Development of kinase inhibitors and activity-based probes

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2 Chemical tools to label kinases

2.1 Introduction

The protein kinase family is one of the largest protein superfamilies in eukaryotic genomes. Due to their central role in cell signaling, physiology and pathophysiology, there is need for biomarker discovery or development of selective kinase agonists or antagonists as therapeutics. To be able to develop more potent kinase inhibitors or to identify and assign function to the whole kinome completely, it is essential to determine the substrates of each individual protein kinase, to define how signaling pathways operate and to determine the (off)targets of kinase inhibitors. For this reason, there is interest in the development and application of chemical biology tools that allow the global analysis of the

protein expression and activity in complex biological samples. Three major strategies used for profiling of small molecule-protein interactions are: affinity-based protein profiling, activity-based protein profiling (ABPP), and photoaffinity labeling. Affinity-based probes (AfBP) are bi-functional small molecules that create a reversible interaction with the target proteins and are linked to either a sorting function or directly to a solid phase. After binding, non-binding proteins are washed away, and the bound proteins are analyzed with an analytical technique (e.g. LC-MS/MS) after elution (Figure 1A).[1,2]

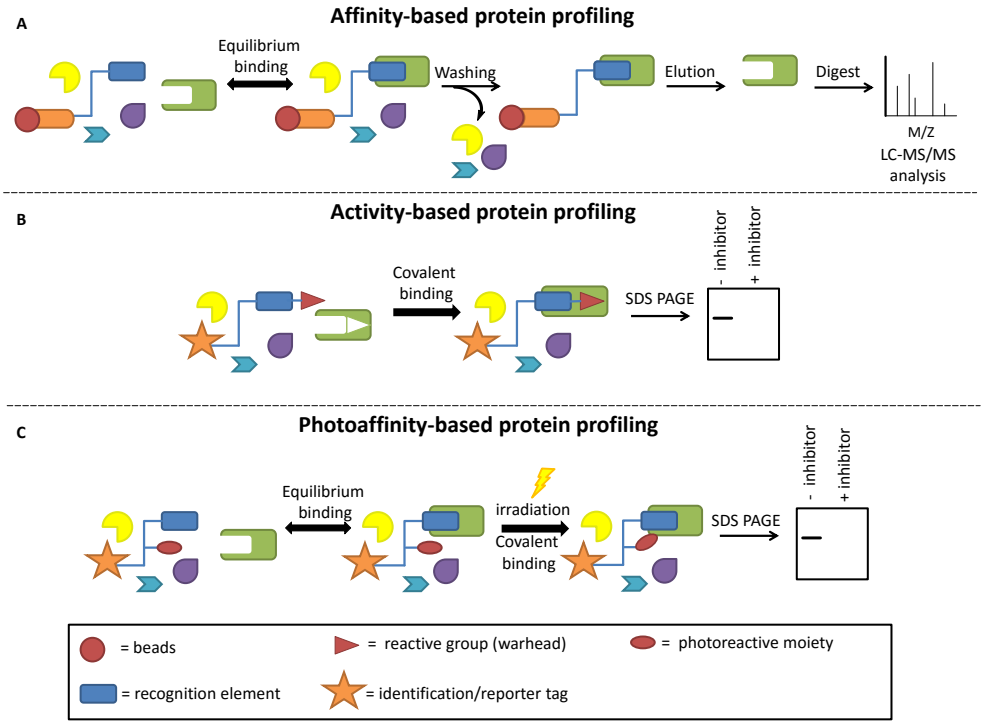


Figure 1. Schematic workflow for affinity pull down (A), activity-based protein profiling (B) and photoaffinity based profiling (C).

In ABPP the probe design consists of three general elements, namely a reactive group (the "warhead"), a recognition site and a chemical tag. The reactive group, which is an electrophilic group, binds and covalently modifies nucleophilic amino acids in the active sites of a single enzyme or a broad range of enzymes from a particular protein class (or classes). Due to the covalent and irreversible nature of this interaction, it enables subsequent sample processing steps employing other parts of the probe such as detection or sorting functions. The structure of the recognition site is in most cases based on the structure of the natural substrate of the target(s) or a known inhibitor (Figure 1B).[3] Finally, photoaffinity probes contain three functional modules: 1) the selectivity function

accomplishes an equilibrium-driven affinity binding to target proteins, 2) the photo-reactivity function binds covalently to the active site of the target protein through photo-activation, and 3) the tag function is necessary for isolation or visualization of the targeted proteins (Figure 1C).[4]

Various techniques are available to visualize the probe-labeled proteins, such as fluorescence scanning of SDS-PAGE gels, fluorescence microscopy, fluorescence assisted cell sorting (FACS), and positron emission tomography (PET). Furthermore, labeling can also be used for enrichment of the sample for subsequent analysis by MS/MS or NMR techniques. For the visualization and/or isolation of the labeled enzymes, numerous reporter tags have been used. Commonly used reporter tags include fluorescent tags (e.g. BODIPY and cyanine dyes (Cy2, Cy3 and Cy5)) and affinity tags such as biotin.[1,3,5,6] Even though the vast majority of chemical proteomics studies have been conducted on lysate samples, it is preferably to probe intracellular proteins in intact cells with membrane-permeable probes. Since only a minority of probes has been reported to be cell permeable and in some cases the bulkiness of the reporter group hampers recognition by the target enzymes, reporter tags in probes are replaced by markedly smaller bio-orthogonal ligation handles to overcome these problems. This strategy uses a two-step labeling method to label proteins in a chemoselective manner. Ligation handles that are frequently used are azide, alkyne and norbornene. Azides and alkynes can be used to perform a copper(I)-catalyzed Huisgen 1,3-dipolar cycloaddition ("click" reaction). Azides can also be used for Staudinger ligation. Norbornenes are used for an inverse-electron demand Diels-Alder reaction. All these ligation methods can be performed in the same biological sample in a chemoselective way (Figure 2).[7,8,9,10]

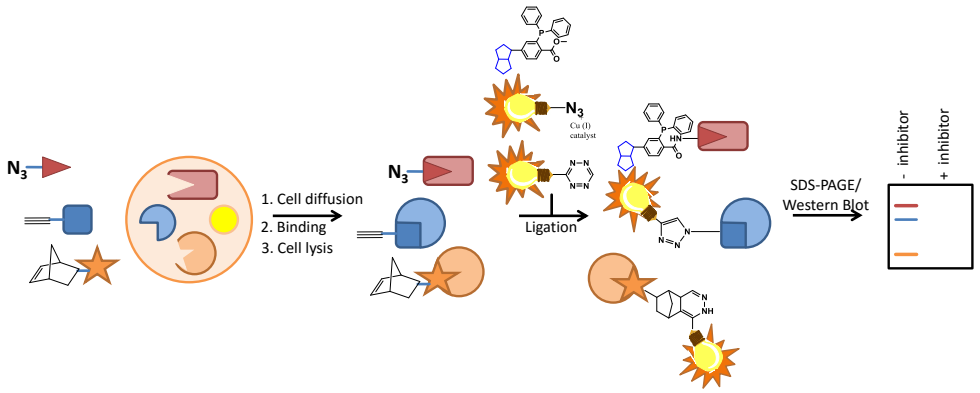


Figure 2. Schematic representation of three different bioorthogonal ligation methods: copper(I)-catalyzed click reaction, Staudinger-Bertozzi ligation and inverse-electron demand Diels-Alder ligation.

Affinity-based protein profiling

A powerful technique for the identification of the intracellular targets of small molecules and substrates is affinity chromatography. This methodology uses affinity-based probes, which contain a molecule of interest that can be immobilized on a solid support, to enrich ligand-binding proteins from cell lysates. This technique is attractive for determining the specificity of protein kinase inhibitors. For these studies, a known protein kinase inhibitor is immobilized on a solid support.[11] A number of notable examples of the application of this methodology are described below.

The first example that uses affinity chromatography to study the intracellular targets of a protein kinase inhibitor was performed with an analog of the CDK-family inhibitor purvalanol B (Figure 3, **1** en **2**).[12] Based on the crystal structure of CDK2-purvalanol, a linker could be attached to the carboxylic acid of the 6-anilino substituent of the inhibitor without interfering with its interaction with the kinase.[13] This site was used to link purvalonal B to an agarose matrix through a polyethylene glycol linker (**2**). As a negative control, an inactive *N*6-methylated purvalanol (**3**) was synthesized and immobilized on agarose (**4**) (Figure 3). The immobilized purvalanol **2** was used to affinity purify interacting proteins from starfish oocytes, diverse mammalian tissues and protozoan parasites. The bound proteins were eluted, resolved by SDS-PAGE and analyzed by Western blotting or microsequencing. As a result, immobilized purvalanol **2** targets next to CDK1, CDK2, CDK5 also ERK1, ERK2, calcium/calmodulin-dependent protein kinase II (CamKII), p70 S6 kinase (S6K) and casein kinase 1 (CK1). The only non-protein kinase identified is the enzyme biliverdin reductase. *In vitro* assays have been performed to validate the interaction of purvalanol B (**1**) with ERK1, ERK2, CamKII, S6K and CK1. It should be noted that soluble purvalanol B (**1**) was less potent for ERK1, ERK2, CamKII, S6K and CK1 than CDK1, CDK2 and CDK5.

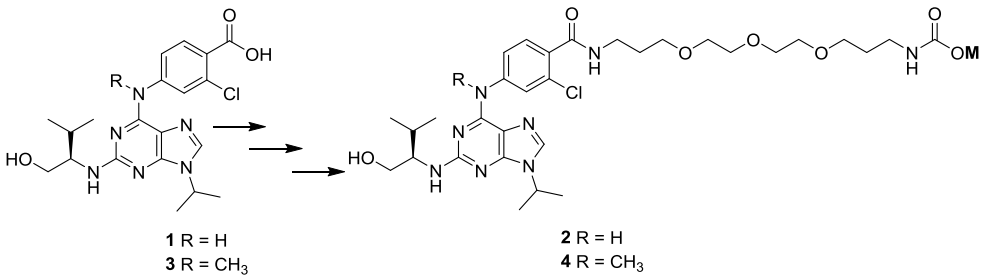


Figure 3. Structures of Purvalanol B (**1**), immobilized purvalanol **2,** the N6-methylated negative control **3** and its immobilized analogue **4**. **M** = agarose matrix.

Following a similar concept for the identification of protein kinase inhibitor targets in order to predict side effects and novel medical uses, Rix *et al.*[14] have immobilized three BCR-ABL tyrosine kinase inhibitors that are used in the current frontline therapy in chronic myeloid leukemia (CML): imatinib (**5**), nilotinib (**6**) and dasatinib (**7**) (Figure 4). These three drugs (**5-6**) were converted into their coupleable analogues **8-10**, respectively, before being immobilized on NHS-activated Sepharose 4 Fast Flow beads via their amino functionalities to yield immobilized imatinib, nilotinib and dasatinib **11-13** (Figure 4). Since the acetylated analogues **14-16**, which are mimicking the immobilized compounds, did not display significantly altered activities, it was assumed that the immobilized drugs would act the same as their soluble analogue. Lysates of K562 and CML primary cells were subjected to affinity chromatography with immobilized drugs (**11-13**), and matrix-bound proteins were identified by liquid chromatography electrospray ionization tandem mass spectrometry (LC-ESI-MS/MS) and/or western blot analysis after being resolved by SDS-PAGE. According to mass spectrometry analysis, imatinib has 11, nilotinib has 14 and dasatinib 38 targets. Among the most prominent targets of dasatinib were the TEC family kinases TEC and BTK, next to BCR-ABL. Since imatinib and nilotinb displayed much more specific target profiles compared with dasatinib, the latter is thus more likely to have a more pronounced impact on multiple biologic processes.

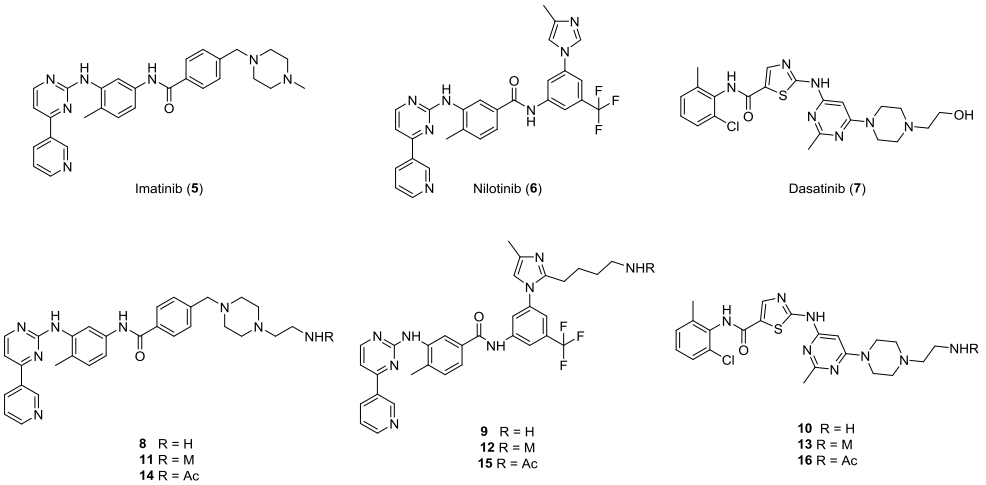


Figure 4. Structures of BCR-ABL inhibitors imatinib (**5**), nilotinib (**6**) and Dasatinib (**7**), their coupleable analogues (**8-10**), their immobilized analogues (**11-13**) (on NHS-activated Sepharose 4 Fast Flow beads) and their acetylated positive controls **14-16**. **M** = sepharose matrix

Other BCR-ABL inhibitors, INNO-406 (**17**)[15] and bosutinib (**18**)[16] were converted into coupleable precursors (**19** and **20**, respectively) prior to immobilization on sepharose beads (**21** and **22**, respectively) and were used to enrich for interacting proteins in human CML K562 cells (Figure 5). INNO-406 is known to inhibit BCR-ABL and LYN efficiently, but

this study additonally identified several novel kinases targets including ZAK, the receptor tyrosine kinases DDR1 and DDR2, and various ephrin receptor kinases (predominantly EPHA2, EPHA5 and EPHA8). Found interaction partners of bosutinib were BCR-ABL, SRC kinases LYN and YES, CSK, BTK, GAK, EPHB4 and PTK2.

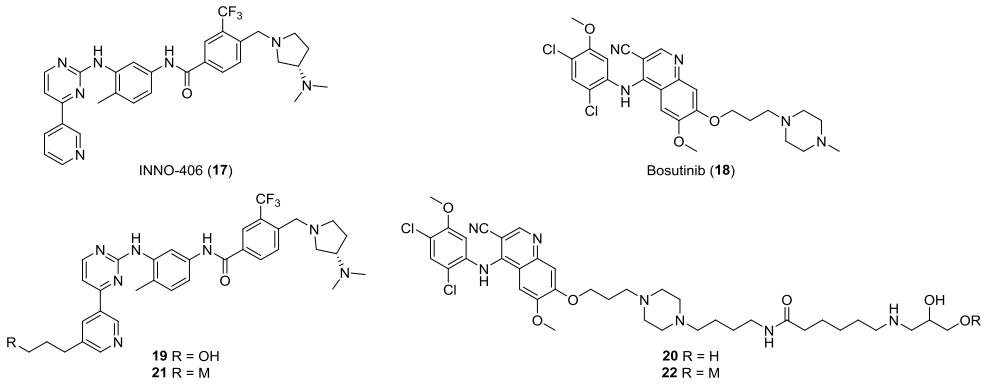


Figure 5. Structures of BCR-ABL inhibitors INNO-406 (**17**) and bosutinib (**18**), their coupleable analogues **19** and **20** and their immobilized analogues **21** and **22** (on NHS-activated Sepharose 4 Fast Flow beads). **M** = sepharose matrix.

Recently, Sewald *et al.* developed an affinity probe in order to understand new links of Janus Kinase (JAK) family members with other signaling pathways, because the JAK signaling pathways play a key role in many cellular processes, and was recently associated with neuronal disorders.[17] The developed probe **24** is an aminopyrimidine derivative based on the known JAK inhibitor **23** (Figure 6), which is known to have high affinity towards all JAK isoforms. To design a probe to address all JAK isoforms, the acetanilide moiety in inhibitor **23** was substituted for an alcohol group and an additional methyl ester group, leading to probe **24**. Probe **24** was immobilized on NHS-activated sepharose beads through covalent linkage using the primary amine group, and pull down experiments were performed using the acute monocytic leukemia cell line MV4-11. The eluted target proteins were digested with trypsin into peptide fragments and analyzed by LC-MS/MS. By this method 133 kinases were identified with probe **24**, and these included the JAK isoforms JAK1, JAK2 and Tyk2. Next to the JAK kinases, a large number of other kinases related to signaling pathways, including MAPK1, MAPK3, MAPK8, MAPK9, MAPK14, MAP4K4, MKNK1 and PI3K, were identified. Due to the broad kinase coverage of probe **24**, it can be applied for the selectivity profiling of kinase inhibitors for the identified 133 kinases.

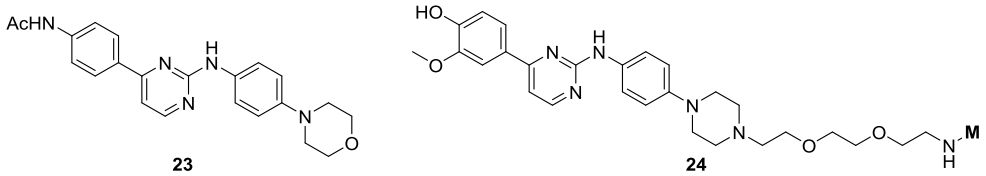


Figure 6. Structures of JAK inhibitor **23** and affinity based probe **24**. **M** = matrix (sepharose bead).

Other studies have also focused on the broadband-isolation of protein kinases combined with quantitative mass spectrometry. Herein, a mixed inhibitor affinity matrix, termed Kinobeads, that carry a collection of >100 ATP-competitive kinase inhibitors including chemical scaffolds, research tool compounds, drug candidates in development, as well as approved drugs, is used to map kinases from complex biological samples and to assess the selectivity profiles of kinase inhibitor drugs. For example, Bantscheff and colleagues applied this methodology to three drugs targeting the oncogenic BCR-ABL kinase, which induces CML.[18] The immobilized compounds were first incubated with lysates of HeLa or K562 CML cells to allow protein binding, which was followed by separation of the beads from the lysate. The bound proteins were eluted, digested with trypsin, and identified by MS. The kinobeads matrix specifically captures roughly 200 protein kinases (two-thirds of the expressed kinome) from any given cell type. This data set works as a control sample (Figure 7a).

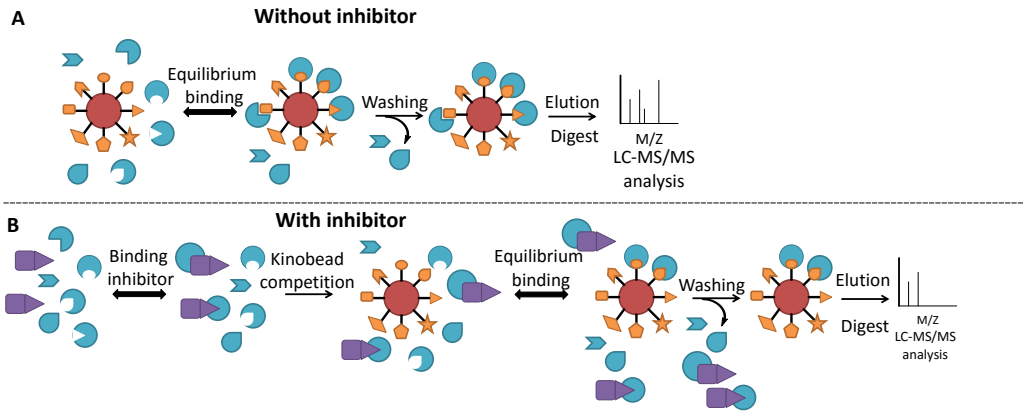


Figure 7. Schematic workflow of proteomic drug target profiling by a kinobead competition assay.

To quantitatively profiling the targets of the three BCR-ABL drugs, namely bosutinib, imatinib and dasatinib, these drugs were added to K562 cell lysates and the lysates were subsequently subjected to kinobeads. When the drug in the lysate has bound to its target and thus has blocked the ATP-binding site, a reduced amount of the free target has been available for capturing by kinobeads, whereas the binding of nontargeted kinases and

other proteins has been unaffected. The kinobead-bound material was subjected for quantification by MS. The selectivity drug profile can be determined by comparing this labeling profile with the control profile (Figure 7b). As a result, for imatinib 13 proteins exhibited >50% binding reduction on kinobeads. These proteins include ABL, BCR-ABL, the ABL family kinase ARG and two novel target candidates DDR1 and NQO2. Dasatinib and bosutinib labeled more targets than imatinib (39 and 53 proteins respectively), including BCR-ABL, ARG and DDR1, BTK, EphB4, focal adhesion kinase (FAK), FER, MER and Syk. Thus, the combination of a mixed-affinity matrix with quantitative MS provides a convenient tool to map a drug's direct and indirect targets in a single set of experiments.

Activity-based protein profiling

Many ABPs have been developed for numerous enzyme classes up to now, including serine hydrolases, cysteine proteases, metallohydrolases, phosphatases, glycosidases, deubiquitylating enzymes, penicillin-binding proteins, palmitoyl transferases, various oxidoreductates and for kinases as well. A characteristic feature of ABPP probes is that they contain a selectivity function capable of covalently reacting with the active site of target enzymes driven by the catalytic mechanism of the enzyme. However, kinases catalyze phosphate transfer from ATP to their substrate by a direct transfer mechanism that does not involve covalent enzyme intermediates. Thus, the kinase active site does not contain conserved nucleophilic residues for catalysis. In these cases, electrophilic ABPs that react with native or engineered noncatalytic nucleophilic residues must be used. Based on sequence comparisons, it is known that almost all protein kinases have at least one conserved lysine residue within their active sites that represents a possible modification site for an appropriate probe. These lysine residues are situated close to β- and γ-phophates of bound ATP, suggesting that the positively charged ε-amino groups of these lysines provide electrostatic interactions with the phosphate backbone. Therefore, it has been reasoned that kinase ABPP probes based on ATP might take advantage of the conserved lysine.[19] In an approach by Patricelli and colleagues, ABPs were used with a biotinylated acyl group positioned at the terminal phosphate of ATP (**25**) or ADP (**26**) to provide broad proteome coverage (Figure 8A).[20] These acyl phosphates have appropriate reactivity with amines and are stable in aqueous solution. Upon addition of the acyl phosphate probes to cell lysates, a stable amide bond with the biotin tag has been formed and an ATP or ADP has been released by a reaction between the active-site lysine(s) and the activated acyl phosphate (Figure 8B). Subsequently, the biotinylated kinases were subjected to proteolytic digestion with trypsin. The labeled peptides were purified on steptavidin-agarose beads prior to analysis by LC-MS/MS to determine the identity of the labeled protein as well as the site of labeling. The acyl phosphate probes were shown to selectively label at least 75% (~ 400) of human kinases in cell lysates. This method is thus

able to profile the functional state of many kinases in native proteomes in a parallel manner.

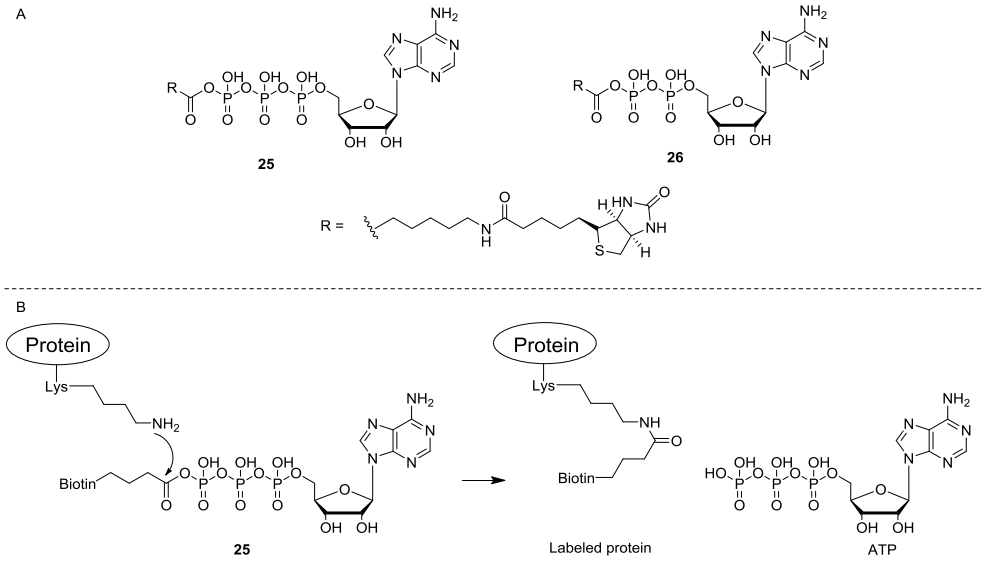


Figure 8. A) Structures of ATP-Biotin probe **25** and ADP-Biotin probe **26**. **B)** Mechanism of labeling of ATP-biotin probe **25** with target lysine.

Another ATP containing ABP has been developed and used for enrichment and identification of kinases and other ATP-binding proteins from complex protein mixtures, namely the isotope-coded ATP ABPP probes (ICAPs) **27** and **28** (Figure 9).[21] The differences between this probe and the ATP probe **25** are that this probe contains a desthiobiotin as the tag instead of biotin and a linker with isotopes. Since chemical isotopic labeling methods like dimethyl labeling are prone to additional experimental errors due to late stage incorporation of the isotopes during sample preparation, the isotopes are already incorporated into the ATP-probe. Two different probes have been developed, one containing a heavy linker (containing six deuterons, **27**) and the other containing a light linker (containing six hydrogens, **28**).

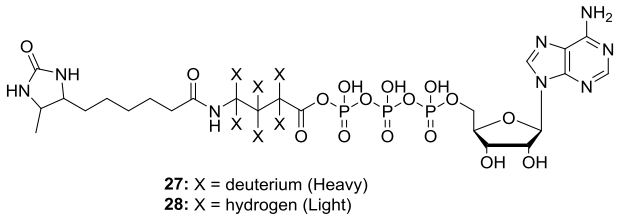


Figure 9. Structures of isotope-coded ATP probes (ICAP).

By using two different ATP probes, two protein labeling profiles of two different samples can be compared. The general procedure with the use of ICAP is as follows: 1) ATP-binding proteins from a sample representing one experimental state are obtained with the isotopically light form ATP probe **28**, whereas the ATP-binding proteins from a sample representing another experimental state are labeled with the isotopically heavy form ATP probe **27**. 2) The two protein samples are mixed and digested with trypsin, and the resulting light/heavy isotope-coded desthiobiotin-labeled proteins are enriched with streptavidin agarose beads. 3) The purified peptides are analyzed by LC-MS/MS. Quantification results can be obtained by comparing the MS peak intensity ratios of the light and heavy forms of desthiobiotin-modified peptide pairs (Figure 10).

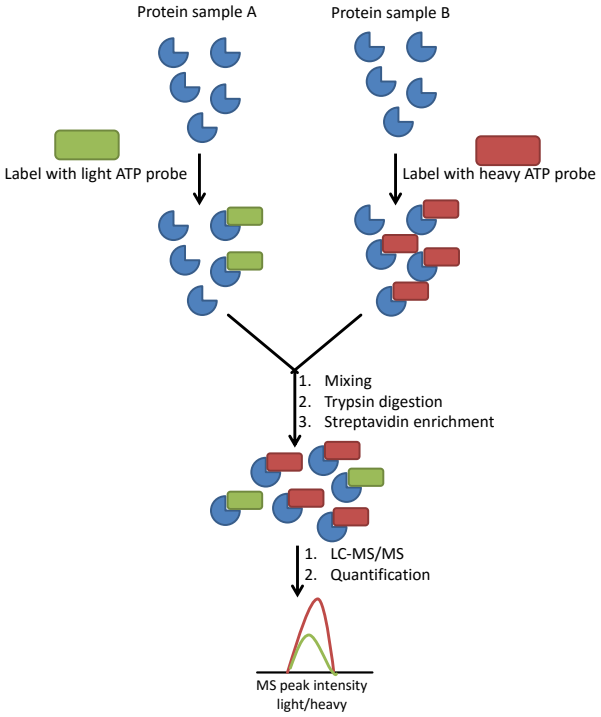


Figure 10. General strategy for quantitative ATP-ABPP using ICAP.

A disadvantage of the ATP-containing probes is that they also label other non-kinase proteins, since ATP is a substrate not only for kinases, but for other ATP-binding proteins as well. Therefore, many studies have focused on the development of kinase-selective ABPP to overcome this problem by using probes based on covalent kinase inhibitors. Wortmannin, which is a metabolite of the fungus *Penicillium funiculosum*, irreversibly inhibits phosphoinositide-3-kinases (PI3Ks) and the PI3K-related kinases (PIKK) families by covalent modification of a lysine in the active site. This lysine is conserved in the different

PI3K and PIKK family members. Therefore, wortmannin is a promising starting point for development of a new ABPP probe. Yee *et al.* have synthesized and characterized three ABPs based on wortmannin. These probes are linked via a water-soluble polyethylene glycol linker to biotin (**29**), or to the fluorescent tags BODIPY (**30**) and TMR (**31**) (Figure 10).[22] This study revealed that all the three wortmannin probes inactivated members of the PI3K and PIKK families in cellular lysates through covalent linkage with the conserved active-site lysine. Biotin-wortmannin (**29**) was used to isolate sensitive kinases from nuclear extracts. After incubation with streptavidin-sepharose, three members of the PIKK family, namely ATM, ATR and DNA PKcs were isolated. The fluorescent wortmannin probes **30** and **31** were used for quantification of kinase activity and determination of expression pattern. Of note, BODIPY-wortmannin is cell-permeable, whereas TMR-wortmannin and biotin-wortmannin are only useful for lysates. Since the activity of some proteins may vary under different cellular conditions and in a manner that can only be detected in the context of a cell, BODIPY-wortmannin is a potential tool for examining the biological activity of wortmannin-sensitive kinases within their native environment.

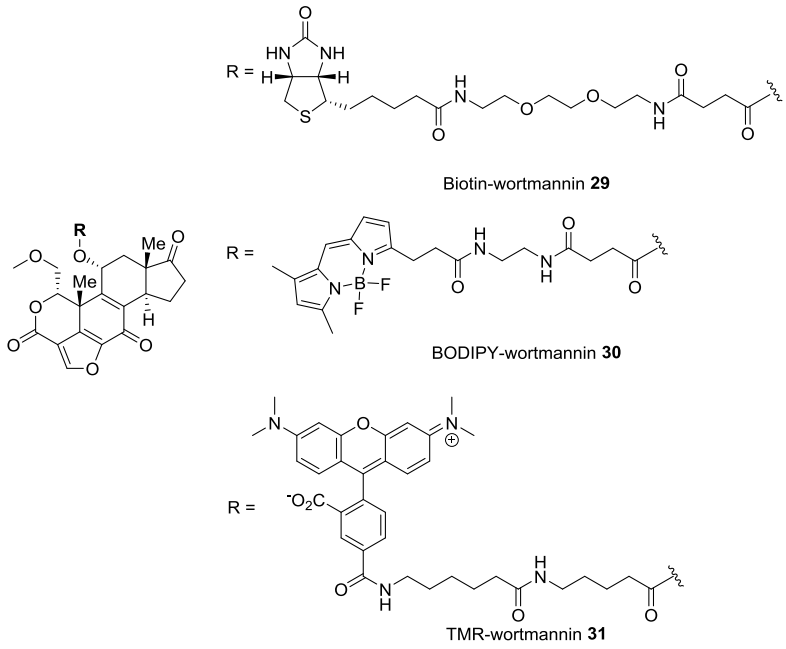


Figure 10. Structures of covalent kinase inhibitor based probes: biotin-wortmannin (**29**), BODIPY-wortmannin (**30**) and TMR-wortmannin (**31**).

Another ATP analogue, namely 5'-fluorosulfonylbenzoyl 5'-adenosine (**32**, FSBA) (Figure 11), which is used to study the mechanism of action of wortmannin, has been used as a general ABP for the protein kinase family.[23] FSBA covalently modifies most protein kinases

by binding irreversibly to the ε-amino group of a conserved ATP pocket lysine. This reagent is structurally similar to ATP, but the phosphoryl groups of ATP are replaced by a fluorosulfonylbenzoyl moiety. To profile protein kinases, biotin-tagged FSBA ABPs have been designed and synthesized.[24] Biotin groups were introduced by modifying the free hydroxyl groups of the ribose ring of FSBA, which resulted in the three different FSBA-biotin probes **33**-**35**, whereof probe **35** carries two biotin tags (Figure 11). Biotin-tagged ABPs allow visualization of labeled proteins after Western blotting and can also be used as handle for protein isolation by streptavidin-coated beads. Ratcliffe *et al.* have proven that FSBA and its biotin-tagged probes can be used to screen and validate ATP competitive kinases inhibitors using LC/MS or Western blot techniques.

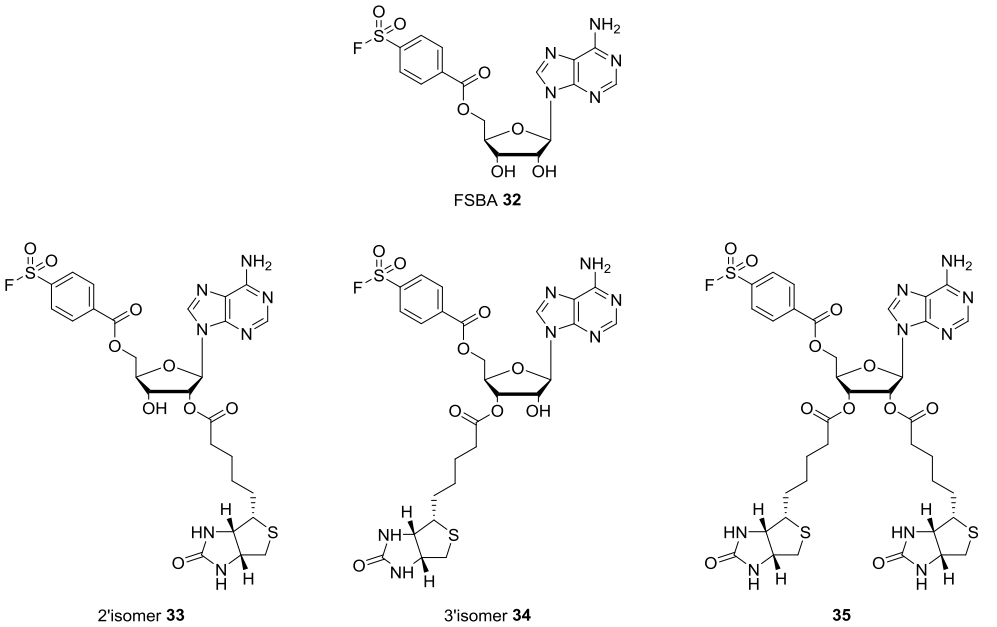
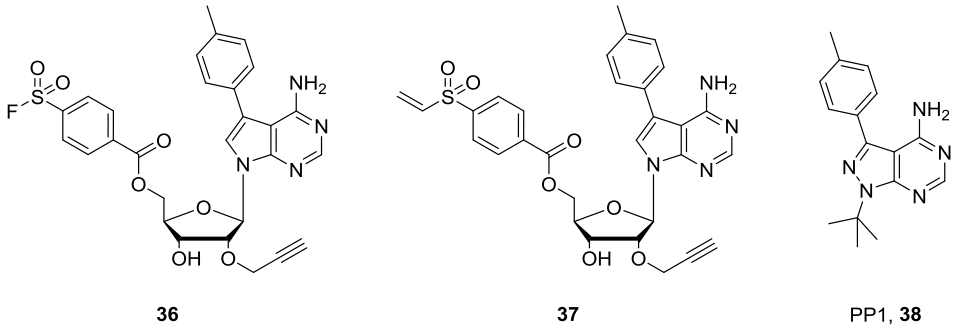


Figure 11. Structures of FSBA (**32**) and its biotin-labeled ABPs **33**-**35**.

Taunton *et al.* have developed irreversible selective Src-family kinase probes by modification of FSBA (**32**).[25] The Src active site contains at least two potentially nucleophilic side chains Lys295 and Cys277. It is known that Lys295 reacts with millimolar concentration of FSBA. The second nucleophile in the Src active site, Cys277, is situated proximal to both Lys295 and the γ-phophate of ATP. Since this cysteine is poorly conserved, it is an attractive target for selective covalent inhibition. Only nine human kinases contain an equivalent cysteine, including Src, Yes, FGFR1-4, LIMK1 and TNK1. To improve the selectivity of FSBA for the Src-family kinases, two hybrid nucleosides (**36** and **37**) which borrowing structural elements from FSBA and the Src-family inhibitor PP1 (**38**)

(Figure 12), have been developed. Since the *p*-tolyl substituent in PP1 exploits a hydrophobic pocket found in all Src-family kinases, this group has been introduced in FSBA. For monitoring of covalent binding to proteins using copper-catalyzed click chemistry, a propargyl ether has been included as bioorthogonal tag. These modifications gave ABPs **36** and **37**. Of note, compound **37** contains a vinylsulfone as the electrophilic trap instead of the fluorosulfonylbenzoyl group. The two probes were compared for their selectivity and their reactivity. Both the probes were able to capture multiple Src-family kinases in intact cells, along with robust competition by kinases inhibitors including the clinical Bcr-Abl inhibitor ponatinib. The fluorsulfonylbenzoyl probe **36** reacts with the conserved lysine and shows little discrimination among related kinases. However, the 5'-vinylsulfonate **37** reacts with the poorly conserved cysteine.



36 **37** PP1, **38**

Figure 12. Structures of two alkyne containing Src-family kinase ABPs based on FSBA (**32**) and PP1 (**38**): 5'-fluorosulfonylbenzoyl probe **36** and 5'-vinylsulfonate **37**.

Other cysteine protein kinases have been targeted by covalent kinase inhibitors as well. The targeted active site cysteines offer a potential selectivity filter, since these residues are not uniformly conserved across the kinome. Thus far, irreversible inhibitors have been developed for some protein kinases, including oncogenic drug targets like the epidermal growth factor receptor (EGFR) and Bruton's tyrosine kinase (BTK), for which the corresponding irreversible inhibitors afatinib and ibrutinib have shown to treat non-small cell lung cancer and chronic lymphocytic leukemia (CLL), respectively. Recently, ABPP combined with quantitative MS was used to perform global and in-depth analysis of proteins targeted by the covalent EGFR inhibitor PF-6271184 (**39**) and BTK inhibitor ibrutinib (**40**) (Figure 13). Both the inhibitors **39** and **40** use Michael receptors (αβ-unsaturated amide) for covalent binding with the nucleophilic active-site cysteines in the ATP-binding pockets. To use these inhibitors as ABPs, an alkyne group was installed as the ligation handle to yield probes **41** and **42** (Figure 13).[26] Rhodamine azide was used as their ligation partner for visualization of targeted enzymes and off targets in A431 and Ramos cells, which possess high levels of the primary inhibitor targets EGFR and BTK, respectively. SDS-PAGE and in-gel fluorescence scanning revealed that the probes **41** and **42** were able

to probe their corresponding on-targets EGFR and BTK, and to detect off-targets. To identify all the targets of these probes, ABPP was combined with MS. Consistent with other studies, EGFR and ERB2 were identified as specific targets for both the probes. However, BTK was primarily labeled by the ibrutinib-probe **41**. Most of the off-targets identified contain the active-site cysteine residue except for MLTK. Non-kinase targets were found as well, including FAM213A and DUS2L for probes **41** and **42**, respectively.

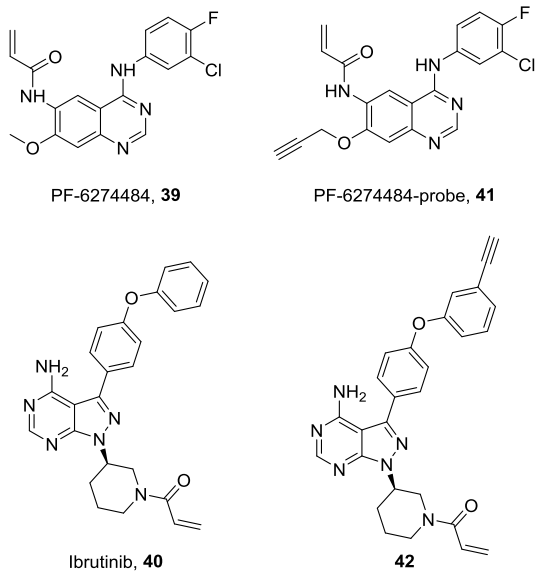


Figure 13. Structures of cysteine kinase inhibitors PF-6274484 (**39**) and ibrutinib (**40**) and their corresponding alkynylated ABPs **41** and **42**.

More ibrutinib-based ABPs have been developed both direct and two-step bioorthogonal probes. Both Honigberg *et al.*[27] and Turetsky *et al*.[28] used a Bodipy-FL containing ibrutinib probe (**43** or **44**, respectively, Figure 14A). Both studies have proven that the direct Bodipy-FL ibrutinib probes were able to label BTK in several BTK-positive tumor cell lines. Recently, Liu *et al.* developed two fluorescent ibrutinib probes (BodipyFL-like **45** and Bodipy-TMR **46**) and three two-step bioorthogonal probes (alkyne **47**, azide **48** and norbornene **49**) to compare the direct and two-step labeling ABPP methodologies in labeling of BTK using Ramos cells and lysates (Figure 14A).[29] The ligation partners of the alkyne **47**, the azide **48** and norbornene **49** were azido Bodipy-FL **50**, alkyne Bodipy-FL **51** and tetrazine Bodipy-FL **52**, respectively (Figure 14B). Cu(I)-catalyzed azide-alkyne [2+3] cycloaddition reactions were performed for the ligation of the bioorthogonal ABPP pairs **47**/**50** and **48**/**51**, and an inverse-electron demand Diels-Alder reaction was performed for the ligation of **49**/**52**. All the five probes (**45** – **49**) were able to label BTK in *in vitro* and *in situ* experiments. Direct imaging using Bodipy-ibrutinib **45** and **46** appears most effective

and with less background labeling in *in situ* experiments than the two-step bioorthogonal analogues. A more detailed discussion will be given in Chapter 5 of this thesis.

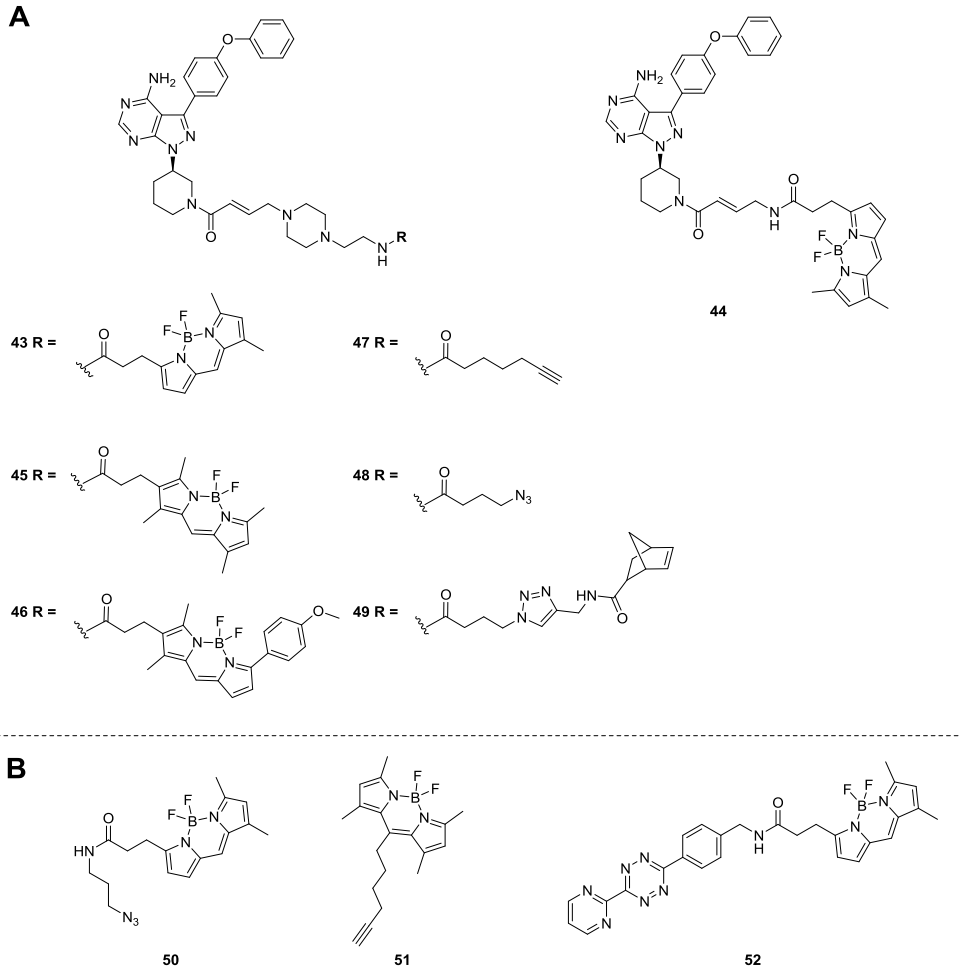


Figure 14. Structures of ibrutinib-based direct and two-step bioorthogonal ABPs and ligation reagents. A) Compounds **43 – 46** are fluorescent probes and compounds **47 – 49** are two-step labeling probes. B) Corresponding ligation reagents **50 – 52** for probes **47 – 49**, respectively.

Photoaffinity labeling

Since not every kinase possesses a conserved and reactive active-site residue for targeting by ABPs, a photochemical group can be used to form a covalent bond between the probe and the kinase. Under UV-exposure conditions, the photoreactive group forms a reactive

intermediate that rapidly reacts and binds to the nearest molecule, which ideally will be the target protein. Probes contain in general three functionalities: an affinity unit (small molecule of interest), a photoreactive moiety and an identification/reporter tag. The affinity unit is responsible for reversible binding to the target kinase, the photoreactive moiety allows permanent attachment to its target and the identification tag is necessary for the detection and isolation of probe-protein complexes. The main photoreactive moieties used for photoaffinity labeling are phenylazides, phenyldiazerines and benzophenones, where under irradiation condition a nitrene, a carbene and a diradical, respectively, are formed as intermediates (Figure 15).[30]

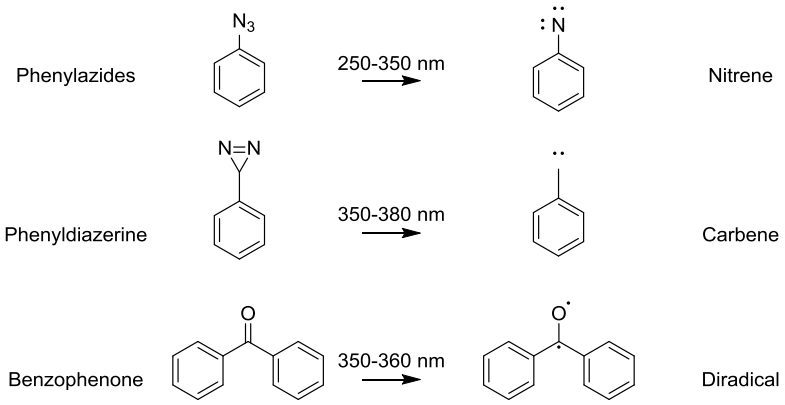


Figure 15. The major photoaffinity groups: phenylazide, phenyldiazerine and benzophenone.

Phenylazides are frequently used as photoaffinity moiety, since they are easily to synthesize and are commercially available. However, damage to biological molecules can be caused by the use of the shorter wavelengths required to excite the phenylazide probes. In addition, the formed nitrene intermediates after expelling molecular nitrogen (N_2) decrease photoaffinity yields compared with carbenes, which might be due to that nitrenes are less reactive than carbenes. Rearrangement of nitrenes can also form benzazirines and dehydroazepines/ketenimines as undesired side products. To prevent the formation of ketenimines, substituted arylazides such as tetrafluorophenylazides have been developed.

Benzophenones are activated by a long wavelength (350-360 nm), which lowers the risk of damaging biomolecules, and thereby a reactive triplet diradical is formed upon UV irradiation. However, the drawbacks of benzophenones are: 1) it is relatively bulky, which can affect the interaction between the affinity pharmocophore and the target protein, 2) the steric hindrance can lead to increased nonspecific labeling and capture, 3) a longer irradiation period is required which can lead to increased nonspecific labeling.

Aryldiazerines are the most commonly used photoaffinity group, since they are highly resistant towards a number of factors such as temperature, nucleophiles, acidic and basic conditions as well as oxidizing and reducing agents. In addition, the photolabile diazerine group absorb most efficiently at a wavelength of 350-380 nm, at which hardly no significant cell damage would occur. Upon irradiation of diazirine, molecular nitrogen is expelled and a singlet carbene is formed. This intermediate is extremely reactive and has a short half life, which leads to fast insertion reactions and less discrimination between reaction sites.
Other functionalities that have been used for photoaffinity labeling include diazo groups, diazocarbonyls, enones, sulfur radicals, halogenated substrates, nitrobenzenes, diazonium salts and alkyl derivatives of azides and diazirines. In the following part, some examples of photoaffinity labeling of kinases using both direct and two-step bioorthogonal probes containing one of the above mentioned photoreactive moieties will be discussed.

Also in the case of photoaffinity labeling, adenine-based probes have been developed. Biotinylated adenine-benzophenone conjugates **53** and **54** (Figure 16) were synthesized to label selectively Lck kinase, which belongs to the Src-family and plays roles in thymocyte differentiation and T-cell activation.[31] These two probes were incubated with six commercially available kinases, PKA, GSK3, CK1, Src, Fyn and Lck. Upon irradiaton at 350 nm, the biotinylated proteins were blotted onto PVDF membrane and visualized after treatment of anti-biotin horseradish peroxidase-conjugated antibody. The *S*-configured probe **53** showed to label Lck only and the *R*-configured probe **54** does not label any of the kinases. This proves that a simple configuration change leads to a drastic change in selectivity and activity of the probe. Based on these findings, five other adenine-benzophenone conjugates were developed to gain insights into the relative importance between target-binding affinity and conformational flexibility of the probe for labeling of LCK kinase. In this study the central Gly residue has been replaced by five other D-amino acids, namely proline (**55**), serine (**56**), piperidine (**57**), glutamic acid (**58**) and γ-aminobutyric acid (GABA, **59**) (Figure 16).[32] This study showed that binding-affinity did not predict photolabeling efficiency of the LCK probes. However, conformational flexibility showed to have correlation with labeling efficiency, since compound **59**, which has the most flexibility around benzophenone, showed to have the highest labeling efficiency. The proline containing probe **55** did not label Lck, because of the conformational constraint on the backbone.

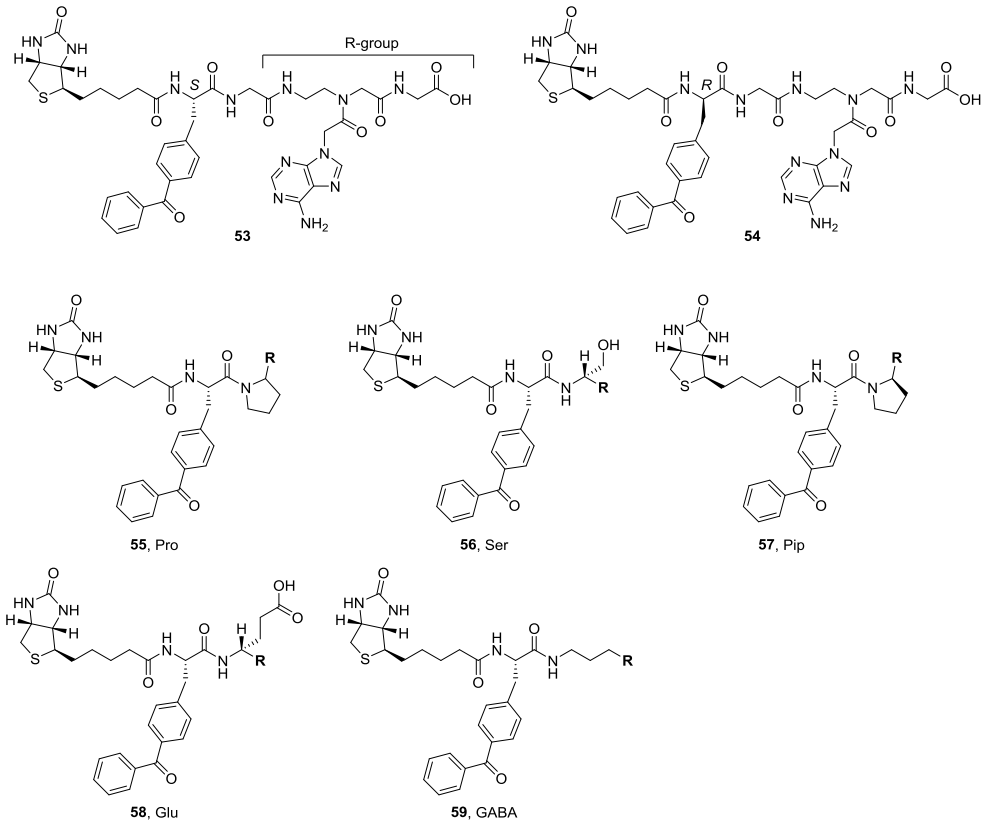


Figure 16. Structure of the biotinylated adenine-benzophenone probes for labeling of LCK **53 – 59**.

The use of known reversible kinase inhibitors as the affinity moiety has been done by many studies. Sewald and co-workers have developed the direct photoaffinity probe **61** by using H-9 (**60**), which is an isoquinolinesulfonamide of the H-series, as the reversible inhibitor (affinity moiety), a benzophenone as the photoreactive group and the isomeric mixture of 5- and 6-carboxyfluorescein as the reporter tag (Figure 17).[33] This study has shown that this isoquinolinesulfonamide benzophenone probe was able to label and visualize four purified proteins, namely hexokinase, creatine kinase, 3PGA phosphokinase and cAMP-dependent protein kinase A. However, a very low binding tendency for the probe has been seen for 3PGA phosphokinase.

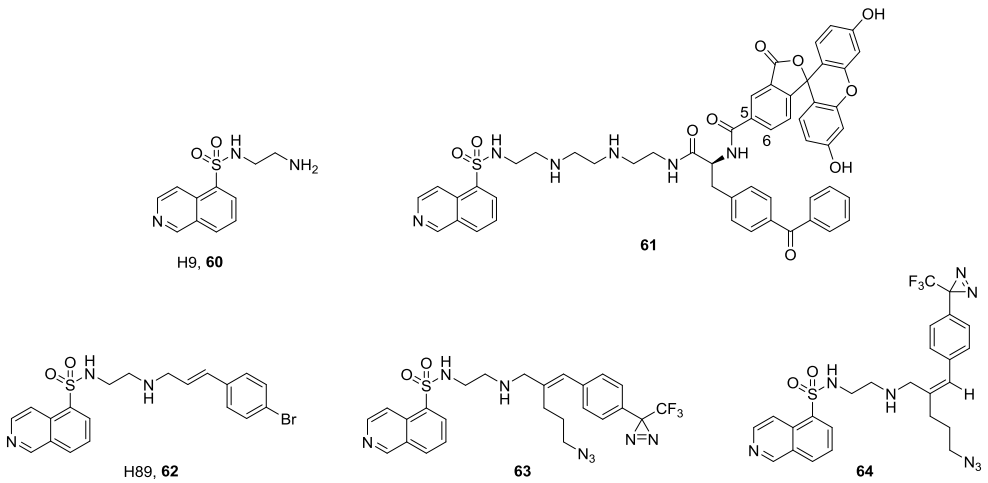


Figure 17. Structures of isoquinolinesulfonamide kinase inhibitors H9 (**60**) and H-89 (**62**) and their corresponding photoaffinity labeling probes **61** and **63** – **64**, respectively.

Another group also has developed photoaffinity labeling probes based on an isoquinolinesulfonamide of the H-series, namely H-89 (**62**, Figure 17), which is known for its inhibition towards PKA and other kinases including AKT1 and AKT2. In this case, the two-step bioorthogonal probes **63** and **64** are developed and contain a trifluoromethylphenyldiazerine, which is a more stable version of the original phenyldiazerine, as the photoaffinity reactive group and an azide group that functions as the ligation handle (Figure 17).[34] Both the probes showed inhibition activity against PKA and AKT1, and for AKT2 and AKT3 in a much lower extent. To prove that these probes were able to be used as photoaffinity labeling probes, both the probes **63** and **64** were incubated with recombinant PKA and AKT1, followed by photo-crosslinking at 350 nm and a final click reaction with an alkyne-modified Cy5 reporter for in-gel analysis. Probe **63** labels both kinases with a higher affinity than probe **64**, which can be explained by the fact that probe **63** is structurally more similar to the lead compound H-89 (**62**) than probe **64**. This study will be discussed in more detail in Chapter 6.

Since most kinase inhibitors target the highly conserved ATP-binding site of kinases, they tend to inhibit multiple cellular targets. To better understand the cellular selectivity of anti-kinase drugs and to anticipate potential side effects of these drugs, known reversible kinase drugs were converted into a photoaffinity labeling probe.[35] An example is the dual Src/Abl inhibitor dasatinib, which is used for the treatment of imatinib-resistant CML. Related method to profile the off-targets of dasatinib has already been performed using immobilized dasatinib on sepharose beads (**7**), which is discussed above. However, disadvantages of this method are: 1) the heavy reliance on a small number of bead-immobilized kinase baits, which affects the accessibility of some target kinases, and leads

to missed targets, 2) due to non-covalent inhibition between the immobilized probe and the kinases, relatively mild wash conditions must be applied, which often leads to accumulation of false positives, as well as exclusion of weaker kinase-drug interaction. Therefore, alkynylated dasatinib-photoreactive labeling probes, which bind covalently to its target have been used to profile the off-targets of dasatinib. Yao and co-workers[36] have designed and synthesized two-step bioorthogonal alkynylated-dasatinib probes, one contains an aliphatic diazirine moiety (**65**) and the other a benzophenone (**66**) as the photoreactive group. For protein profiling and pull-down/target identification, the small terminal alkyne handle was ligated to rhodamine-N_3 (**67**) or biotin-N_3 (**68**), respectively (Figure 18). First, to prove that these probes can be used to profile the targets of dasatinib, docking experiments and *in vitro* kinase assays were performed to determine the binding site and IC_{50} values respectively. As a result, both the probes were able to inhibit c-Src/c-Abl kinase domains in the same range as dasatinib, however only the aliphatic diazirine probe **65** was much more cell-permeable than the benzophenone probe **66**. Therefore, further protein profiling experiments were performed using the cell-permeable diazirine probe **65** only. This aliphatic diazirine probe (**65**) was capable of proteome-wide profiling of potential cellular targets of Dasatinib. Results obtained from different proteomic setups (cell lysates, live cells and immobilized affinity matrix) were compared and it has been found that under similar experimental settings, the photoaffinity probe (**65**) was able to identify more putative kinase targets over immobilized dasatinib affinity matrix. In addition to Abl and Src family tyrosine kinase, six serine/threonine kinases were identified by pull-down/immunoblotting experiments as new targets, namely PCTK3, STK25, eIF-2A, PIM3, PKA Cα and PKN2.

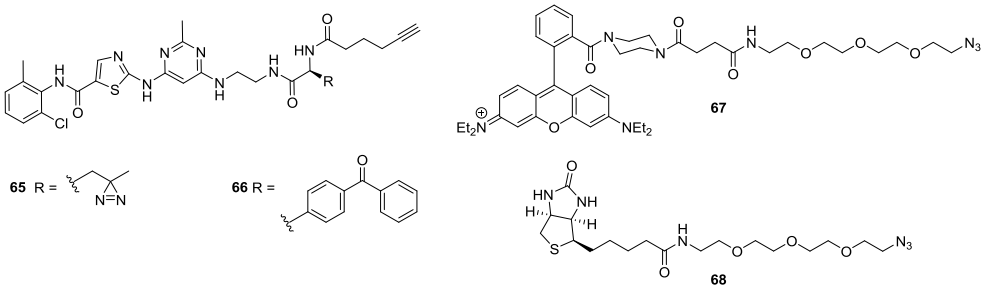
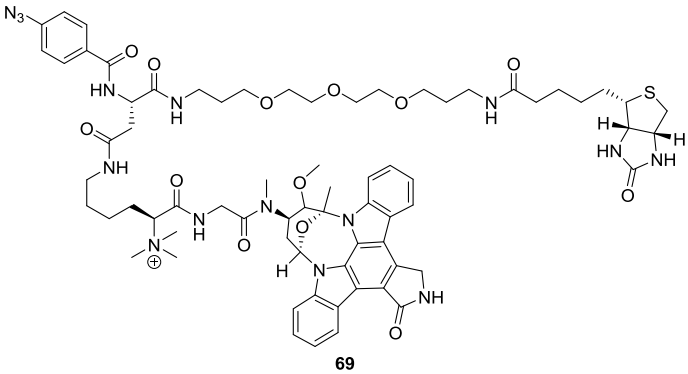


Figure 18. Structures of two-step bioorthogonal phofoaffinity-group containing dasatinib-probes **65** and **66**. Rhodamine-N_3 (**67**) and biotin-N_3 served as the ligation reagents for visualization and pull-down of targets, respectively.

Instead of labeling one particular kinase or kinase family, in some cases large scale labeling of kinases are preferable. For the labeling of kinases in broad-scale, many studies have already done this by using bead affinity-driven techniques as mentioned above. To

overcome the disadvantages of the bead affinity-driven approach, biological samples may be profiled in solution by soluble, multitargeted small molecule probes. To allow labeling of the ATP-binding sites of as much as possible kinases, a few studies has used the reversible ATP mimetic kinase inhibitor staurosporine, which is known for inhibiting at least 253 kinases, as the affinity group.[37, 38] An example is the biotinylated staurosporine-arylazide **69** developed by Fisher *et al.* (Figure 19). The staurosporine probe **69** were incubated with human liver cancer cell line HepG2 whole cell lysates prior to irradiation at 312 nm. Streptavidin coated magnetic beads was used to isolate the covalently cross-linked protein-probe complexes. According to the LC-MS/MS results 101 kinase and 144 non-kinases were labeled. Among these identified kinases, the majority are members of the serine/threonine kinase family.



69

Figure 19. Structure of the biotinylated staurosporine photoaffinity probe, which contains an arylazide as the photoaffinity moiety.

Conclusion

Three methods of chemical profiling are applied for the labeling and identification of kinases. Each method has its own advantages and disadvantages. Thus, dependent on the purpose of the study and under which conditions the experiments need to be performed, one of these methods will suit the best for the experiment and kinase(s) of interest. Even though many studies have already been done up to now, much more experiments need to be done in the future. Not every kinase or kinase subset can be labeled and not every function of the many kinases are determined yet due to the complexity of the kinome and the low abundance of most kinases. Therefore, there is need for the development of more selective and active probes, which in turn can be analyzed using highly sensitive and efficient read-out methods.

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