Probes for Photoaffinity Labeling of Kinases

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Abstract: Protein kinases, one of the largest enzyme superfamilies, regulate many physiological and pathological processes. They are drug targets for multiple human diseases, including various cancer types. Probes for photoaffinity labeling of kinases represent important research tools for the study of members of this enzyme superfamily. In this review, we discuss the design principles of these probes, which are mainly derived from inhibitors targeting the ATP pocket. Overall, insights from crystal structures guide the placement of photoreactive groups and detection tags. This has resulted in a wide variety of probes, of which we provide a comprehensive overview. We also discuss several application areas of these probes, which include identification of targets and off-targets of kinase inhibitors, mapping of their binding sites, development of inhibitor screening assays, imaging of kinases, and identification of protein binding partners.

1. Introduction

The human genome encodes over 500 protein kinases (PKs). This number makes them one of the largest enzyme superfamilies, comprising approximately 1.7% of human genes.^[1] PKs are involved in a wide variety of cellular processes, such as cell growth, division, differentiation, proliferation, apoptosis and metabolism. Apart from their extensively documented role in cancer,^[2] PKs play key roles in inherited genetic,^[3] neurodegenerative,^[4] inflammatory^[5] and infectious diseases.^[6]

Kinases catalyze the transfer of the γ -phosphate group from ATP to the substrate, usually on the hydroxyl group of Tyr, Ser or Thr residues.^[7] The majority of PKs are Ser/Thr kinases (approximately 400), a smaller part of approximately 100 of PKs are Tyr kinases, and some PKs have dual specificity (Ser/Thr and Tyr). Finally, there are ~50 pseudokinases, which share structural features with PKs, but lack catalytic activity.^[8–10] The PK superfamily consists of 7 groups: AGC kinases, Ca²⁺/calmodulin-dependent protein kinases (CaMK), CMGC kinases, tyrosine kinases (TK), the group of casein kinase 1 (CK1), STE kinases, and TK-like (TKL) kinases (Figure 1). The classification of kinases is based on sequence similarity. Proteins that have conserved kinase motifs, but do not belong to any of these 7 groups are referred to as “others”. PKs from the aforementioned 8 groups share a conserved kinase (catalytic) domain and belong to the eukaryotic Protein Kinases (ePKs). Lastly, there are approximately 25 atypical kinases^[11] and around 20 lipid kinases that do not share conserved kinase motifs.^[12]

Figure 1. Evolutionary tree of human kinases with indication of the seven main groups.

Structurally, the approximately 300 residue long conserved kinase domain of ePKs consists of two lobes; the N-terminal lobe (N-lobe) and a larger C-terminal lobe (C-lobe),^[13] which are connected by a flexible segment, also called hinge region. The ATP cleft is formed by the interface of the N- and C-lobe, whereas the substrate binding site is located at the C-lobe, close to the position of the γ -phosphate group. Other important structural features of PKs are the activation loop (A-loop), the Asp-Phe-Gly (DFG) motif, located at the beginning of the A-loop, and the α C-helix (Figure 2A). PKs adopt two extreme conformations: the inactive (autoinhibited) and the active conformation (Figure 2A and B). All these elements coordinate in order to stabilize one conformation over another and facilitate binding and positioning of both the ATP and substrate for successful transfer of the γ -phosphate group.^[14] Even though the inactive conformation differs from kinase to kinase, there are structural similarities between

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some PKs.^[14] A common characteristic is the so-called DFG-out conformation where the Asp is directed outwards of the ATP cleft and the Phe blocks the nucleotide binding.^[14,15] Notably, the DFG-out conformation further induces the formation of an allosteric pocket within the ATP cleft. In the inactive state, the α C-helix is also rotated outwards (α C-helix-out) and away from the ATP cleft.^[13] Contrastingly, the active state conformation of PKs is conserved as certain chemical constraints are required for catalytic activity.^[13] In that state, both the DFG motif and the α C-helix are moved inwards (DFG-in and α C-helix-in, respectively), conformations that are required for catalytic activity of PKs.^[14]

Figure 2. Structure of the kinase domain of ePKs. (A) Crystal structures of the active (left, PDB code: 1M17) and inactive conformation (right, PDB code: 4MJ0) of the kinase domain of EGFR, as a representative example. The kinase domain of ePKs consists of two lobes (N- and C-lobe), at the interface of which the ATP cleft is formed. In the active conformation (left), the α C-helix (red ribbon) and the DFG motif (space filling model with spheres in yellow (carbon), red (oxygen) and blue (nitrogen)) adopt the 'in' conformation, whereas the A-loop (green ribbon) becomes flexible to allow binding of the substrate. In the inactive conformation (right), α C-helix and the DFG motif are moved outwards ('out' conformation). (B) View of the ATP cleft of EGFR in the active state (EGFR bound to gefitinib, PDB code: 2ITY) and in the inactive state (EGFR bound to lapatinib, PDB code: 1XKK). The DFG motif is colored (blue-green-red, respectively) to highlight how the change in the orientation of the G residue influences the formation of the allosteric pocket. Note that the additional benzyl ring of lapatinib occupies the allosteric pocket formed in the inactive states. Also note that the residues in front of the inhibitor structure are not depicted in order to provide a clear view of the ATP cleft.

Dimitris Korovesis obtained his first degree in chemistry from the University of Ioannina and subsequently his MSc degree from University of Leeds. He then started his PhD in a consortium involving King's College London, STFC and Evotec AG, supervised by Prof. Marisa Martin-Fernandez and Dr. Simon Ameer-Beg. There, he developed small-molecule imaging probes for structural studies. In 2018, he joined the lab of Prof.

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Steven Verhelst received a PhD from Leiden University and performed post-doctoral research at Stanford University School of Medicine. In 2008, he became group leader and TUM junior fellow at the Technische Universität München, supported by an Emmy Noether award from the German Research Foundation. Since 2014, he is group leader Chemical Proteomics at the Leibniz Institute for Analytical Sciences ISAS (Dortmund, Germany) and since 2015 associate professor at KU Leuven (Belgium). His lab focuses on the development of covalent chemical probes for enzymes, with a primary focus on proteases.

PKs have been the focus of intense research in drug discovery due to their involvement in various diseases. One of the most commonly used approaches for targeting kinases is using small-molecule kinase inhibitors (KIs). To date, 62 KIs have been granted Food and Drug Administration (FDA) approval with most of them being used as cancer therapeutics.^[16] The majority of KIs inhibit phosphorylation in an ATP competitive manner and have either reversible or irreversible (covalent inhibitors) modes of action, the latter by exploiting a cysteine residue that resides at the entrance of the ATP site in some PKs. KIs can be further classified as type I or type II inhibitors. Type I KIs bind to the active conformation (DFG-in and α C-helix-in) of PKs and type II inhibitors bind to the inactive state (DFG-out and α C-helix-out). Examples of type I KIs include gefitinib (EGFR, reversible), afatinib (EGFR, irreversible) and dasatinib (Abl, reversible). Type II KIs are further substituted, compared with their type I counterparts, in order to occupy the additional allosteric pocket of the inactive kinase conformation (Figure 1B) and include

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inhibitors such as imatinib (Abl, reversible), sorafenib (b-Raf, reversible) and neratinib (HER2, irreversible).^[15,17]

Despite the success of KIs, the development of selective KIs remains one of the major challenges in the kinase drug discovery fields.^[18] The vast majority of KIs bind to the ATP-binding cleft,^[15] which is highly conserved across the kinome. Type II inhibitors are considered more selective than type I, because they exploit the less conserved allosteric pocket.^[19] While this may be true for some KIs, recent studies with type II analogs of dasatinib^[19] and mass spectrometry (MS) based screening of 243 KIs in cell lysates^[20] indicated that this is not a general rule. Covalent KIs, which make use of Michael acceptors, were also thought to have higher selectivity, as these cysteines are non-conserved.^[21] However, chemical proteomics studies by Cravatt and co-workers showed that irreversible EGFR and BTK inhibitors bearing a Michael acceptor, unexpectedly target non-kinases with functional cysteines.^[22,23] The apprehension that KIs also have non-kinase targets of KIs has drawn increasing interest because it may hinder progress in drug development due to undesired adverse effects.^[24]

Covalent chemical probes are important research tools for profiling of enzyme activity, evaluating drug target occupancy and visualizing localization of probe targets in cells and *in vivo* models. In the last decade, covalent probes have been reported for various groups of PKs, most of them based on ATP-competitive KIs. Because the majority of approved drugs for PKs have a reversible mechanism, most probe development has relied on photoaffinity labeling (PAL). This review is dedicated to these PK probes for PAL. We discuss general probe design features, provide an overview of the available photoaffinity probes for different kinase families and illustrate their usage with examples of specific applications.

2. Photoaffinity probes

Covalent probes for PAL are sometimes referred to as affinity-based probes (AfBPs), because they rely on affinity of the scaffold to the target protein(s). They consist of the inhibitor scaffold, a photoreactive group and a detection tag. Obviously, the placement of the last two elements should not interfere with binding to the targets. Below, we will discuss these elements and the design principles of AfBPs for PKs.

2.1. Photoreactive groups

A photoreactive group is a chemical functionality that, upon irradiation with a specific wavelength of light, forms a reactive intermediate that rapidly reacts with functionalities in close proximity. This feature allows transient and/or weak interactions to be trapped, and provides temporal control over the crosslinking reaction. An ideal photoaffinity group is one which produces highly reactive intermediates with the capacity to form stable adducts with many different bond types, but is activated at wavelengths that avoid damage to biomolecules. For example, surplus absorption of UV light by proteins, which absorb at 280 nm (Trp, Tyr and Phe residues) and 200 nm (peptide bonds) can result in degradative chemical transformations.^[25] Furthermore, the photoreactive group should be stable in the dark at a range of pH values, and should not be excessively large that it interferes with protein-ligand binding (see also later).

Various photoreactive groups have been incorporated into AfBPs for PKs. The most commonly used groups are aryl azides, diazirines and benzophenones; these undergo photolysis upon irradiation to form nitrene, carbene, and diradicals, respectively (Figure 3A).^[26] These highly reactive intermediates react with proteins *via* insertion into C-H/X-H bonds (X = N, O, S). Whereas the benzophenone diradical displays a preference for methionine residues,^[27] the singlet carbene produced by irradiation of diazirine has virtually 'indiscriminate' reactivity. Yet, the linear diazo isomer by-product of diazirine photolysis can decompose to the carbocation (see Figure 3A), which reacts exclusively with nucleophilic amino acids. The 3-trifluoromethyl-3-aryldiazirine (TFMD) group often provides enhanced crosslinking efficiencies, due to stabilization of the diazo isomer, disfavoring diazonium formation, and an enhanced stability of the carbene species. Recently, photoaffinity groups with more specific reactivity have been developed (Figure 3B). 2-Aryl-5-carboxytetrazole (ACT) was reported as a novel photoaffinity group by Lin and co-workers.^[28] Upon irradiation at 250 nm, ACT produces a reactive carboxynitrile intermediate which can react with proximal nucleophiles (*e.g.* a Glu carboxylate). However, it should be noted that the shorter, more damaging wavelengths used for activation are non-optimal for application in live cells. The *o*-nitrobenzyl alcohol (*o*-NBA) group is another example of a "residue-selective" photoaffinity group.^[29] *O*-NBA amides undergo photolysis upon UV irradiation to afford aryl-nitroso intermediates. Reaction with proximal lysine residues results in a stable indazolone crosslinked product. Obviously, a suitable nucleophile needs to be present near the ligand-binding site for productive crosslinking to the ACT and *o*-NBA groups, which may prevent covalent reaction with all (off)-targets.

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Figure 3. Photoreactive groups. (A) The most commonly used photoreactive groups of aryl azides (upper), diazirines (middle) and benzophenones (bottom). Note that diazirines can also rearrange to the linear diazo isomer, which can form a diazonium ion upon protonation, and a carbocation following loss of nitrogen. (B) Two recently used photoreactive groups: 2-Aryl-5-carboxytetrazole (ACT, upper) and *o*-nitrobenzyl alcohol (*o*-NBA, bottom).

2.2. Detection tags

In order to detect and isolate probe-labeled proteins, various tags can be appended to the AfBP. For example, fluorescent dyes are used to image the probe-protein adduct, while biotin is a useful affinity handle to enrich labeled proteins prior to tandem MS analysis. Frequently, an alkyne moiety is incorporated into the AfBP and the tag is introduced *via* a copper-catalyzed azide-alkyne cycloaddition (CuAAC) reaction. A common approach is to use a 'minimalist linker' building block containing both aliphatic diazirine and alkyne functionalities.^[30] An advantage of alkyne-functionalized AfBPs is their higher likelihood to retain the cell permeability properties of the parent compounds compared with AfBPs that are directly attached to a fluorophore or a biotin.

2.3. General PK AfBP design principles

The design of a PK AfBP requires three elements: a KI, a photoreactive group and a tag. There are various challenges when placing the photoreactive group and the tag onto the KI scaffold. Bio-activity and target engagement should not be disturbed, covalent modification efficiency should be as high as possible, and the molecular structure should be synthetically accessible. In order to address the first two challenges, most studies take a structure-based design approach: ideally, a co-

crystal structure of the kinase with the KI (or a closely related compound) is available. In absence of this, the KI of interest may be docked *in silico* in order to provide a model of binding. Based on the structure of the KI-PK complex, it is then decided which positions are suitable for functionalization with a photoreactive group and a tag. These are either (1) a solvent exposed group, or (2) a group residing within a hydrophobic pocket (Figure 4). We discuss these two possibilities below.

Figure 4. Design principles of PK AfBPs. Generally, PK AfBPs consists of a KI, a photoreactive group and a tag. The position of incorporation of the photoreactive group and the tag is guided by crystallographic data, *in silico* methods or data on structure-activity relationships. In many cases, the photoreactive group and the tag are incorporated at a solvent-exposed position (upper right). Depending on the application, a shorter or a longer linker are also included. Solvent-exposed substituents on the parent KI may be eliminated in order to reduce the distance between the photoreactive group and the KI core (lower right). Alternatively, the photoreactive group can be placed in a hydrophobic pocket within the ATP cleft, if the KI is amenable to functionalization and if the size of the pocket allows. In this case, the tag is placed on a solvent-exposed position (lower left)

(1) Derivatization of solvent-exposed positions are less likely to interfere with binding to the target PK. The distance between the photoreactive group and the KI core can be varied, depending on the application. Short linkers may achieve higher crosslinking efficiency^[31] and capture the direct PK target (or the off-targets).^[32] A longer distance between the photoreactive group and the KI is required if the purpose is to detect protein interaction partners of the PK of interest.^[33] Derivatization of solvent exposed positions does not place a restriction in the length of the introduced substituent. Therefore, a photoreactive group and the tag are usually introduced within the same substituent. Whereas a longer linker may be preferred if bulky tags such as a biotin or fluorescent dye are used. A short linker will suffice in case of an azide or alkyne 'mini-tag', amenable to click chemistry-mediated introduction of a detection tag at a later stage in the experimental process. Notably, 'minimalist linkers' containing a diazirine and an alkyne group were developed by Yao and coworkers and are now widely used in PAL.^[30]

(2) Derivatization of substituents that bind to hydrophobic pockets of the target PK can also be employed, but the modifications should be small in order to prevent disruption of binding. Hence, azides placed onto an already present aryl group, or the trifluoromethyl diazirine (TFMD) are preferred because these are only a few atoms in size. The benefit of placing the photoreactive group within a pocket of the target PK is the increased likelihood of productive covalent modification with

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surrounding residues. Obviously, the detection tag then needs to be placed at a different, solvent-exposed position.

It is important to note that both above design principles are based on the structure of the target PK. Therefore, it cannot be excluded that interactions with off-targets may be affected. To characterize the full spectrum of off-targets, derivatization at different sites may be necessary.

Figure 5. AfBPs based on pan-kinase inhibitors. (A) AfBP based on the pan-kinase inhibitor H-9.^[34] (B) AfBPs based on the natural product staurosporine.^{[35][36][30][37]}

3. Probes per kinase family

3.1. Pan-kinase AfBPs

The first reported AfBP for a PK was a fluorescent probe based on the isoquinoline sulfonamide-type inhibitor H-9 (1, Figure 5A).^[34] Probe 1 was used to successfully label creatine kinase (among others) when spiked into thylakoid membrane preparations. A number of AfBPs have been developed based on the natural product staurosporine. Staurosporine (Figure 5B) is an ATP-competitive kinase inhibitor known to target more than 250 kinases.^[38] Initially, the aryl azide and biotin-containing AfBP 'Stauro-CC' (2) was developed,^[35] but was only applicable to cell lysate studies. The second generation of staurosporine-based probes (3-4) bear aliphatic diazirine crosslinkers and alkyne handles, which led to the first cell-permeable kinase AfBPs.^{[36][30]} The most recent generation of staurosporin AfBPs contains the tetrazole photoreactive group (5-8); these probes represent the first instance of this reactive group being used as a photocrosslinker.^[37]

3.2. Tyrosine-kinase AfBPs

With over 30 examples, the majority of probes reported for PKs target TKs. Figure 6 lists several examples where one particular inhibitor structure or core scaffold was taken and functionalized with different photoreactive groups, tags or linkers of various lengths. A complete overview of these probes is given in Figure S1.

The largest variety of probes for TKs has been based on dasatinib (Figure 6A). Dasatinib is a dual Bcr-Abl and Src KI that has been granted FDA approval for chronic myeloid and acute lymphoblastic leukaemia. Although all probes use the same solvent exposed site for modification, a wide variety of photoreactive groups (aryl azide, diazirine, benzophenone, ACT) and tags (biotin, alkyne and HaloTag-reactive chlorohexyl) have been incorporated. In section 4, the advantages and limitations of some of these probes are discussed together with their specific application. Imatinib has also been functionalized on a solvent exposed position with various photoreactive groups and tags for detection (Figure 6B). AfBPs based on the quinazoline core (Figure 6C) have been functionalized with photoreactive groups on a solvent-exposed site (22, 23 and 25), but also on a phenyl ring that docks into a hydrophobic pocket of the target kinase (24).

Figure 6: AfBPs based on TK inhibitors. (A) AfBPs based on Dasatinib: **9**,^[39] **10–11**,^[40] **12**,^[41] **13**,^[30,42] **14–16**.^[28] (B) AfBPs based on Imatinib: **17**,^[30] **18–20**,^[43] **21**,^[39] (C) and those with a quinazoline core: **22**,^[30] **23**,^[44] **24**,^[45] and **25**.^[46]

3.3. TKL AfBPs

Tyrosine kinase-like (TKL) kinases are Ser/Thr kinases that share sequence similarity with TKs. AfBPs for TKL kinases are depicted in Figure 7. AfBP **26** is based on a compound developed against the mixed lineage kinase domain-like protein (MLKL), a pseudokinase within the TLK group. Its design was aided by the X-ray crystal structure of the MLKL binder in the nucleotide binding site of the human MLKL pseudokinase domain.^[47] A TFMD group was positioned in place of the terminal phenyl ring, which is buried deep within the binding pocket, to ensure efficient crosslinking to the target, while the terminal alkyne was installed on the aryl sulfonamide moiety which extends into solvent space. Although the binding affinity was slightly affected by the addition of the diazirine substituent, the probe was successfully used to confirm target engagement *in vitro*. Overall, this is a nice illustration of the general design principle 2, as discussed in section 2.3. AfBP **27** (based on the leucine-rich repeat kinase 2 (LRRK2) inhibitor, LRRK2-IN-1) incorporating a 'minimalist linker'

was applied in whole cell proteomic profiling experiments (see section 4.2).

Figure 7: AfBPs based on TKL inhibitors.^{[47][48]}

3.4. AGC AfBPs

PKs A, G and C (PKA, PKG and PKC) are Ser/Thr kinases regulated by secondary messengers such as cyclic AMP (PKA), cyclic GMP (PKG) or lipids (PKC). An overview of all reported

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AfBPs for the AGC family is given in Figure 8. In addition to the 7 photoaffinity inhibitor peptides targeting PKG, shown in Figure 8, a further 8 sequences were synthesized, each containing the Phe^(Tmd) crosslinker at the *N*- or *C*-terminus.^[49] Although it was thought that positioning the Phe^(Tmd) at these locations would prevent detrimental effects on binding, no crosslinking to recombinant PKG was observed. In the end, the Phe^(Tmd) crosslinker turned out to be too far from the protein surface. This example highlights the importance of correctly positioning the photoactivatable amino acid within a peptidic inhibitor.

Figure 8. AfBPs for PKA (28),^[50] PKC (29),^[51] and PKG (30–36).^[49] Bold underlined letter F in the peptide sequence indicates the position of the photoactivatable phenylalanine derivative.

3.5. CMGC AfBPs

Members of the CMGC family are predominantly proline-directed Ser/Thr kinases (*i.e.* they phosphorylate Ser/Thr immediately preceding a proline residue). These include the CDKs (Cyclin-Dependent Kinases), GSKs (Glycogen Synthase Kinase), MAPK (Mitogen-Activated Protein Kinase) and CDK-like kinases.^[52] Figure 9 provides an overview of the available AfBPs for the CMGC group of PKs. Of particular interest is the probe series P1–P12 (37–48; Figure 9A), based on the pan-CDK inhibitor Roscovitine.^[53] Bush and co-workers compared crosslinking efficiencies of AfBPs incorporating a variety of photoaffinity groups, to recombinant CDK2. Interestingly, the TFMD group, usually a preferred photoreactive group because of its efficient formation of covalent bonds, resulted in little to no crosslinking in probes P5–P6. In contrast, alkyl diazirines P7–P8 displayed good crosslinking to all CDKs tested, as did the aryl azides and tetrafluoroaryl azides. Overall, this underlines the effectiveness of a photoreactive group highly depends on your protein of interest. AfBPs have also been developed for other members of the CMGC family, including those based on p38 inhibitors (50–52, Figure 9B),^[30,33] the MEK kinase inhibitor Mirdametinib (53, Figure 9C)^[30] and the JNK inhibitor SP600125 (54, Figure 9C).^[30]

Figure 9. AfBPs for the CMGC kinase family. (A) Photoprobes based on CDK inhibitors Roscovitine (Seliciclib) (37–48)^[53] and Purvalanol B (49).^[30] (B) AfBPs based on p38 inhibitors: phthalazine core containing probes 50 and 51,^[33] and probe 52 derived from the substituted imidazole SB203580.^[30] (C) AfBPs based on the MEK kinase inhibitor Mirdametinib (53)^[30] and the JNK inhibitor SP600125 (54).^[30]

3.6. CaMK AfBPs

For the CaMK group of kinases, only one probe has been reported, based on Dorsomorphin, an inhibitor for AMP-activated protein kinase (AMPK) (Figure 10). CC-1 was one of a set of probes developed by Li *et al.* containing ‘minimalist linkers’, which were used to profile the proteome reactivity of 12 PK inhibitors in HepG2 cell lysates.^[30]

Figure 10. AfBP based on the AMPK inhibitor Dorsomorphin.^[30]

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Figure 11. AfBPs for the (A) Aurora kinases^{[32][30][54]} and (B) for PKM2 (59, 60),^[55] TMPK (61),^[56] PGK1 (62),^[57] IRE1 α (63),^[58] and PI4KB (64, 65).^{[59][60]}

incorporation of photoreactive groups may lead to non-specific protein labeling.^[50] Therefore, the experimental design should include control samples that help sifting out these non-specifically labeled proteins as false positives, such as an AfBP with a closely related structure, but lacking the bioactivity, or a competition of labeling with the parent compound.

Yao and co-workers illustrated the differences in target identification by comparing a dasatinib-based AfBP (10, Figure 6A) with immobilized dasatinib.^[40] Importantly, the authors found that much more targets were found with the AfBP than with the immobilized dasatinib. Whereas only 6 kinase targets were found with the affinity resin, the AfBP led to the identification of 22 (in situ) or 46 kinase targets (in lysates). Probe 10 identified a higher number of total proteins than probe 9 (Figure 6A),^[39] but exhibited lower crosslinking efficiency compared with probe 13 (which is equipped with the minimalist linker; Figure 6A).^[64] Overall, this highlights the effect of the size, type and position of the photoreactive group and the tag on the behaviour of the probe in proteome profiling.

Recently, our group reported the development of an AfBP based on the type II imidazopyrazine KI KIRA6 (kinase inhibitor RNase attenuator 6; 63, Figure 11B). KIRA6 was developed as a KI for inositol-requiring enzyme 1 α (IRE1 α),^[58] a PK that, besides its kinase activity, also possess RNase activity. KIRA6 allosterically inhibits the RNase function of IRE1 α by binding to the ATP-site. While KIRA6 was initially reported to be selective for IRE1 α , subsequent studies indicated that it might not be as selective. PAL experiments in various cancer cell lines showed that the KIRA6-based AfBP labeled several KIRA6-specific protein targets. Interestingly, gel-based MS experiments identified 70 off-targets, none of which were PKs, although more than half were nucleotide-binding proteins. This study further highlights the importance of using KI-based AfBPs for proteome-wide profiling,

3.7. Others

AfBPs for kinases that do not belong to any of the groups described above are given in Figure 11. Many of these have been designed to target Aurora kinases (Figure 11A), whereas the rest target different PKs (Figure 11B).

4. Applications

As illustrated in the previous section, several KI-based AfBPs have been developed for PK targets of different kinase groups. In this section, we discuss some of the most common applications.

4.1. Target identification studies

Proteome-wide profiling of targets and off-targets is an important application area for PK AfBPs, due to the promiscuous behaviour of many KIs. Traditionally, selectivity profiles of KIs are determined with panels of purified PKs.^[61] However, such assays place PKs outside their cellular environment. Moreover, selectivity profiles will be panel-specific.^[62] One of the main proteome-wide alternatives for kinase selectivity screening is the technique of kinobeads, which is a MS-based method applied in cell lysates.^[20] Despite offering advantages over purified proteins, identification of off-targets is restricted to the kinome. Furthermore, this method cannot take place in live cells. These aspects may hinder the identification of off-targets, as previously shown for a lysine-targeted sulfonyl fluoride probe.^[63] Chemical proteomics using KI-based AfBPs overcome the aforementioned limitations and could offer information about the full spectrum of off-targets. The

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as it enables the identification of protein targets in a protein family-independent manner. Lastly, these results may draw the attention to structurally similar KIs, since imidazopyrazine is a common scaffold found in various approved drugs.

The only example of a small molecule AfBP based on a PK agonist instead of a KI, was reported by Cravatt and co-workers.^[51] The authors developed an AfBP derived from ingenol mebutate (IngMeb), a natural product derivative approved for actinic keratosis. While IngMed was known to exert part of its activity through protein kinase C (PKC) activation, it also disrupts mitochondrial structure and function at higher concentrations. Quantitative proteomics in two cancer cell lines and in primary human keratinocytes using AfBP **29** (Figure 8) led to the identification of several off-targets. A high-occupancy target of **29** identified in all three experiments was SLC25A20, a mitochondrial acylcarnitine transporter. The authors showed that IngMed inhibits SLC25A20 in cells, a finding that partially explains the mitochondrial disruption by IngMed.

4.2. Binding site mapping

Beyond target identification, application of AfBPs in proteomics experiments can provide information about the binding site of the probe, which is achieved by tandem-MS identification of the digested peptide that is photocrosslinked to the probe. Such modified peptides not only unambiguously confirm the probe target, but also locate the exact residue where the photoreactive group was inserted.

Piirsoo and co-workers developed an AfBP, based on the KI SU6668 (SUX, Figure S1), to get an insight into the mechanism of inhibition of Unc-51 like kinase 3 (ULK3), a PK that is involved in many cellular processes.^[65] While the authors showed that SU6668 inhibits ULK3, their assay indicated that it may inhibit in a dual competitive and non-competitive manner. In order to determine the exact mode of action, the authors used a diazirine-containing SU6668 derivative (without any tag) on purified ULK3 and the digested protein was subjected to LC-MS/MS. Identification of a single modified residue indicated that the crosslinking occurred at the ATP cleft.

Xiao and co-workers developed a KI AfBP in an attempt to identify the off-target responsible for the antiproliferative activity of a leucine-rich repeat kinase 2 (LRRK2) inhibitor.^[48] LRRK2-IN-1 is an inhibitor developed to selectively target LRRK2 in Parkinson's disease. Despite having shown good selectivity across the kinome in biochemical experiments, LRRK2-IN-1 was shown to exhibit off-target activity in different studies. By performing proteome profiling with AfBP **27** (Figure 7), the authors identified proliferating cell nuclear antigen (PCNA) as the most significant hit in two different cell lines. Surprisingly, PCNA is not a nucleotide-binding protein, but has a binding site for double helical DNA and another binding area for interacting protein partners (the so-called interdomain connector loop, IDCL). LC-MS/MS analysis revealed that LRRK2-IN-1 binds to the IDCL area of PCNA, which results in inhibition of its function.

4.3. Screening

PK-targeting AfBPs can also be used in assays for profiling inhibitors against a certain protein target, in drug selectivity screening or in the identification of conformation-specific inhibitors. These three applications are discussed below.

Bush and co-workers recently developed a MS-based competition assay for profiling KIs for cyclin-dependent kinase (CDK).^[53] Following a series of experiments evaluating AfBPs with various photoreactive groups at different positions (**37-48**; Figure 9A), the authors found that probe **48** was the most efficient. The authors used this probe in a MS-based competition assay for screening CDK inhibitors (Figure 12A). The ability of the competitors to displace the probe was monitored by intact protein MS. 20 CDK inhibitors were screened and their derived IC₅₀ values were in a good agreement with those obtained in a commercial functional assay. Although the throughput is still limited, this may be improved by higher throughput analytical instrumentation. Moreover, it has the potential to be used in live cells.

Traditionally, target identification by proteomics is followed by biochemical confirmation of the identified targets by Western blot. However, this is a low throughput and expensive process. Yao and co-workers combined AfBP-based proteome-wide profiling with microarrays for rapid and miniaturized confirmation of identified targets.^[31] The principle of their assay involves live cell treatment with AfBPs, click chemistry-mediated introduction of a biotin, immobilization of probe-modified proteins on microarray slides and finally detection with antibodies against different PKs to simultaneously identify and validate targets of the AfBPs (Figure 12B). They exemplified the applicability of the assay by using 6 KI-based and 3 non-kinase AfBPs in combination with antibodies for the expected on-targets. The results were consistent with traditional pull-down and Western blot analysis but with higher throughput and with lower cost. However, it should be noted that the number of protein targets is restricted to the number of used antibodies.

Figure 12. AfBPs applied in screening methods. (A) AfBP binding leads to a mass shift in intact protein MS, as exemplified by CDKs.^[53] By treatment of the kinase with a competitor, this signal of the shifted mass is decreased in intensity. Dose-response curves of this displacement is comparable with IC₅₀ values in kinetic assays. (B) Workflow for microarray-based detection of AfBP targets from in situ experiments. After PAL in live cells, covalently tagged protein targets are captured on microarray slides and subjected to detection with antibodies against various targets.

A novel gel-based assay for profiling of PK by AfBPs was developed by Maly and co-workers.^[66] The authors used the dasatinib-based AfBP **12** (Figure 6A), because it binds over 40 PKs with the similar affinity. Essential to their assay was the use of a hexylchloride moiety on the probe, which selectively reacts with HaloTag protein. This leads to a substantial increase in the molecular weight of the probe-modified PK compared with unmodified PK, which allows to determine the modification efficiency by a ratiometric gel shift assay. Because AfBP **12** binds both active and inactive PK conformation, the authors were able to use the novel assay in combination with mutant Src-family

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kinases that were biased towards a specific conformation. As a result, ATP-competitive inhibitors were found with an over 50-fold preference for the inactive conformation, which was further confirmed by X-ray crystallography.

4.4. Imaging

Fluorescent microscopy-mediated imaging of the covalent AfBP-PK complexes would allow monitoring of probe uptake and subcellular localization. It can be performed by attaching fluorophores directly to a PK AfBP or by using bioorthogonal chemistry to incorporate it at a later stage in the experiment. However, it is a less explored detection technique. Only few examples can be found in literature, and below we discuss two studies reported by Yao and co-workers.

Cell division is regulated by various PKs, such as CDKs and Aurora kinases. Su *et al.*^[32] functionalized the selective Aurora kinase A (AKA) inhibitor MLN8237 with a minimalist linker, yielding AfBP **56** (Figure 11A). The probe was successful in detecting AKA in all five mitotic cell phases by fluorescence microscopy. Lastly, the authors were also able to image two kinases in parallel by using CDK1-targeting probe **49** with an alkyne tag (Figure 9) in the same experiment as a non-covalent *trans*-cyclooctene (TCO) functionalized MLN8237. Because of the mutual compatibility of CuAAC and the TCO-tetrazine ligation, two different fluorophores could be introduced. AKA and CDK1 both showed nuclear localization and reasonable co-localization during all five stages of cell division, whereas AKA levels were virtually undetectable in the G1/S phase.

Li *et al.*^[37] reported the development of novel turn-on AfBPs for imaging kinase activities in cells. The probes consist of the pan-KI staurosporine, a tetrazole as a photoreactive group and a dye (**5-7**, Figure 5B). The turn-on feature relies on the quenching effect of the tetrazole on the dye. Upon irradiation-induced formation of a nitrile-imine reactive intermediate and reaction with a nucleophile, the fluorescence of the dyes is substantially affected (Figure 13A). Notably, the increase is more pronounced if the nucleophile is a carboxylic acid than with a water molecule. Such a turn-on property allows application of these AfBPs in live cell imaging as non-bound probe will display lower fluorescence background signal, which is a benefit over AfBPs with bioorthogonal alkyne tags. Assessment of the tetrazole probes **5-7** in live cells showed weak signal before tetrazole activation, but an increase upon UV irradiation. The intensity was unaffected by subsequent washes, indicated that the AfBPs were covalently bound to proteins.

Figure 13. AfBP applications in imaging and PPI identification. (A) Tetrazole-containing photoreactive group forms a reactive nitrile-imine, which leads to an

increase in fluorescence of the dye upon reaction with a carboxylic acid on the protein target but much less after reaction with water. (B) AfBPs with long spacer arms can be used to capture protein interaction partners with detection by, e.g. gel-based or MS-based methods.

4.5. Identification of protein partners

Identification of interaction partners of a protein of interest can provide information regarding its regulation by protein-protein interactions (PPIs). This is often achieved by classical co-immunoprecipitation (co-IP). However, protein complexes that are held together by weak and transient interactions may not be identified. The use of suitable AfBPs can facilitate the identification of PPI partners by covalent modification, provided that the photoreactive group is placed on a sufficiently long linker (Figure 13B).

Maly and co-workers developed AfBPs **50** and **51** (Figure 9), which they called label transfer reagents (LTRs), to capture interaction partners of p38.^[33] The authors equipped p38 inhibitors with a flexible linker, at the end of which a benzophenone and an alkyne handle were placed. Despite the relative long linkers, the LTRs retained similar activity and selectivity as their parent compounds. They covalently modified p38, but more importantly, they labeled p38 interaction partners only in the presence of p38. Importantly, the interaction partners comprised not only substrates, but also activators and repressors that bind at other sites as substrates. It was also demonstrated that the LTRs were compatible with co-IP workflows.

A similar concept was adopted by Pezacki and co-workers in order to investigate PPIs of phosphatidylinositol 4-kinase beta (PI4KB) and how hepatitis C virus (HCV) infection affects those.^[60] They employed AfBPs **64** and **65** (Figure 11B), which incorporate a PI4KB inhibitor, linkers of various lengths, a benzophenone and an alkyne function. The probes were successful in labeling proteins in complex proteomes, as well as in identifying known PI4KB interacting partners by LC-MS/MS, including ACBD3, which recruits PI4KB to the Golgi membrane. Labeling of ACBD3 was dependent on PI4KB expression levels, demonstrating that capturing of interaction partners was through interaction with PI4KB, rather than through direct probe binding. Interestingly, during HCV infection, labeling of ACBD3, but not of other interaction partners, reduced dramatically, suggesting that the interaction between ACBD3 and PI4KB is specifically disrupted.

5. Conclusions

In conclusion, probes for photoaffinity labeling are powerful research tools for the study of PKs. We have provided a comprehensive overview of the currently reported AfBPs for PK and distilled some general design principles from these.

Although it is difficult to compare AfBPs designed for different targets, several studies have utilized a common core structure and functionalized this with various photoreactive groups or at different positions.

Generally, the aryl TFMD photoreactive group has superior labeling efficiency compared with alkyl diazirine, because it more efficiently forms a carbene intermediate, stabilizes the carbene moiety and disfavors diazonium ion formation by stabilizing the linear diazo isomer.^[67] However, this does not mean that incorporation of the aryl TFMD group always leads to better AfBPs.

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For example, Bush and co-workers found that roscovitine-based AfBPs with an aryl TFMD exhibited the lowest labeling efficiency compared with 4 other photoreactive groups.^[53] This might have been due to the fact that such a moiety is rigid and not suitable for solvent-exposed functionalization. Yao and co-workers found that a tetrazole-staurosporine AfBP was more effective in labeling proteins in cells than a diazirine derivative, although tetrazoles exhibit preference towards carboxylic acids and diazirines are non-selective.^[37]

Besides the type of photoreactive group, placement of this group onto the KI scaffold is also crucial. An illustrative example was provided by a study of Lee and co-workers, who developed AfBPs based on a scaffold targeting IGF-1R and Src kinases (OPALs, see Figure S1). Of the three different positions that were derivatized with a photocrosslinker, one failed to efficiently label proteins.^[68]

Above examples illustrate that in the design of AfBPs – even if based on available crystal structures of the target PK with the chosen KI scaffold – one should aim for more than one probe structure to be synthesized and evaluated.

The presence of a bulky tag may have a positive or negative effect of labeling efficiency. Maly and co-workers found that a biotin tag on a p38-targeting AfBP was unable to label its target PK, whereas the same probe with a less bulky alkyne tag did lead to labeling.^[33] In contrast, a study from the same group found that a dasatinib-based AfBP with a hexylchloride moiety displayed higher crosslinking efficiency if the bulky HaloTag was introduced prior to irradiation.^[66] While in most cases the presence of a bulkier tag results in reduced crosslinking efficiency, reaction with the HaloTag may have contributed in reducing the flexibility of the long linker used, which in turn may have brought the photoreactive group closer to the protein surface.

Besides the design of AfBPs for PKs, we have discussed various application areas for these probes. AfBPs have been used as tools to characterize the off-target landscape of KIs by chemical proteomics, understand their mode of action by mapping their binding site, to facilitate inhibitor screening, to visualize PKs by fluorescent microscopy and to interrogate their interactome.

Arguably, one of the most prominent applications of kinase AfBPs is understanding the proteome-wide selectivity of KIs. There are several techniques for selectivity profiling, some of which require derivatization of the small molecule, while others do not. The technique of Kinobeads,^[20] for example, does not require modification of the KI of interest, but it cannot be applied in live cells and it is protein family-specific. Thermal protein profiling (TPP)^[69] is another technique where chemical modification of the small molecule is not required. More importantly, it can also be applied in live cells and target identification is not restricted to the kinome. A disadvantage of TPP is the requirement for costly isotopic labeling and long LC-MS/MS instrument times. In addition, target identification does not result from direct observation of an interaction, but is inferred from a higher protein melting temperature. Furthermore, identification of membrane^[70] or low-abundant proteins remains challenging. In contrast to above methods, the development of clickable or biotinylated probes requires often challenging chemical synthesis. However, their utilization overcomes some of the aforementioned limitations. Noteworthy is a study by Taunton and co-workers, who developed a clickable sulfonyl fluoride probe that led to the identification of PKs that were not found detected by kinobeads.^[63]

While probes with reactive electrophiles are advantageous, target identification is limited to proteins with proximal nucleophilic residues. The use of photoreactive groups on probes potentially expands the target identification space to any protein. Although this feature may also lead to non-specific binding,^[50] the use of appropriate control samples renders AfBPs very powerful tools that identify protein targets in a non-biased way and can take place in live cells.

Future technological advancements may be made to overcome several of the current limitations of kinase AfBPs. For instance, cleavable linkers, which have previously been applied in target identification of many chemical probes,^[71] may improve the identification of off-targets by reducing false-positive hits. Additionally, some linkers – after cleavage – lead to positively charged amino groups which may increase ionization of probe-modified peptides and help identification of the modification site. Application of PK AfBPs in animals is currently problematic, because the required UV-light is harmful to organisms and the short wavelength prevents tissue penetration. Some recent reports of two-photon activation of aryl azide derivatives may overcome such limitations.^[72]

PKs have received major interest from the pharmaceutical industry as targets for anti-cancer therapy, leading to FDA approval of dozens of KIs for treatment of patients suffering from different types of cancer. However, it has become clear that kinases also play important roles in other human diseases, such as neurodegenerative diseases,^[4] which will be a major future challenge in view of the worldwide ageing population. We expect that the currently available AfBPs for PKs and further development of these will substantially contribute to molecular understanding of the role of kinases in these diseases.

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Entry for the Table of Contents

Light up the kinome. Probes for photoaffinity labeling of kinases are powerful research tools for detection of (off)-targets of kinase inhibitors, image kinases within the cell or identify binding partners. In this review, we provide a comprehensive overview of the currently available kinase photoaffinity probes and discuss how such probes are designed. We illustrate the applicability with various examples.

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