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Developments of bioorthogonal handle-containing photo-crosslinkers for photoaffinity labeling

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Photoaffinity labeling (PAL) has been widely applied in various research areas such as medicinal chemistry, chemical biology and structural biology, owing to its capability of investigating non-covalent ligand–protein interactions under native environments and elucidating protein structures, functions etc. One important application of this technique is to use affinity-based proteome profiling (AfBP) coupled with bioimaging for profiling drug–target interactions *in situ*. In order to accurately report drug–target interactions *via* these approaches, several considerations as follows need to be made: (1) maximally retaining bioactivities of photo-probes upon functionalization with a photoreactive group and a reporter tag from a parental compound; (2) performing proteome profiling and imaging *in situ* simultaneously, to monitor drug–target interactions in different manners; and (3) developing excellent photo-crosslinkers capable of photo-crosslinking and fluorescence turn-on at the same time. With these considerations in mind, we have developed three versions of “minimalist” bioorthogonal handle-containing photo-crosslinkers (L3–L6) during the years and successfully applied them in all kinds of small bioactive molecules for protein labeling and cellular imaging studies. In this mini-review, the features and functions of these linkers are specifically highlighted and summarized.

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Introduction of photoaffinity labeling

Photoaffinity labeling (PAL), first introduced by Frank Westheimer in the early 1960s, is the use of photoaffinity labels that can covalently attach to bound targets upon specific-wavelength light irradiation.¹ During the past few decades, it has been successfully applied in medicinal chemistry, chemi-

cal biology and structural biology,^{2,3} areas for identification of cellular targets of bioactive compounds, characterization of ligand binding sites,⁴ elucidation of the structure and function of biological molecules, isolation and identification of unknown enzymes or receptors *etc.*⁵ During the course of these important applications, this technique has been greatly evolved over the past few decades.⁶ In this mini-review, we focused on the important applications of PAL in relation to the studies of drug–target interactions by affinity-based proteome profiling (AfBP) and bioimaging approaches.^{7–10}

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A typical photoaffinity probe (PAP) is composed of three important functionalities, namely a “warhead” (a target-specific ligand), a photoreactive group and a reporter tag (as shown in Fig. 1A).¹¹ The warhead is usually a pharmacophore unit that is responsible for reversible binding to target proteins. Photoreactive groups can generate highly reactive species that rapidly crosslink with bound proteins in photolysis. Amongst the three frequently used photo-crosslinkers, benzophenones,¹² arylazides¹³ and diazirines,¹⁴ the diazirine group is the most popular one due to its small size, chemical stability and superior crosslinking reactivity. Importantly, the excitation wavelength for diazirine-derived photoprobes is in the range of 350–380 nm, which can reduce damage to the biological system.^{15,16} The advantages and disadvantages of these photo-crosslinkers are well-known and have been delineated in prior photoaffinity labeling reviews.⁴ Other photoreactive groups include pyrone, pyrimidone,¹⁷ and tetrazole,¹⁰ (Fig. 1B) which have seldom been applied in PAL so far. As these moieties exist in many natural and synthetic biologically active compounds, they are capable of facilitating corresponding PAL studies.⁴ In addition, the unique crosslinking mechanisms of these photo-crosslinkers give them the potential to reduce nonspecific labeling, which is a key issue encountered in photoaffinity labeling.

Three types of reporter tags, fluorophore, radioisotope and biotin, are normally used in PAL for target analysis. However, direct introduction of these reporter tags into photoprobes generally leads to significantly reduced bioactivities and poor cell-permeability, which is therefore not suitable for live-cell studies to monitor genuine ligand–protein interactions.¹⁸ To alleviate these issues, bioorthogonal handles are now adopted to replace the reporter tags, forming a tandem photoaffinity labeling–bioorthogonal conjugation strategy.⁵ Azide and alkyne are the most popular bioorthogonal handles due to their superior stability and bioorthogonality, and reporter tags can be introduced into probe-labeled proteins *via* copper-catalyzed azide–alkyne cycloaddition (CuAAC) reac-

tion.¹⁹ However, they fall short of being ideal due to the need of using toxic copper which is not compatible with live cells. As the inverse electron demand Diels–Alder (IEDDA) reaction between tetrazine and cyclopropene/*trans*-cyclooctene can occur under catalyst-free conditions,²⁰ *trans*-cyclooctene/cyclopropene are therefore suitable bioorthogonal handles for proteome profiling and imaging *in situ*.^{21–23} The principles of introducing bioorthogonal handles and photoreactive groups are based on the structure and activity relationship (SAR) of parental compounds and the crystal structures of ligand–protein complexes to avoid compromising the bioactivities.

One important application of PAL is to use affinity-based proteome profiling (A/BP) combined with bioimaging to identify cellular on/off-targets of bioactive molecules.^{24,25} The general workflow of this approach for *in situ* studies is as follows: photoprobes are firstly incubated with live cells and then irradiated with specific-wavelength light to initiate crosslinking, and samples are treated with ligation reagents for conjugation with reporter tags. Lastly, labeled proteins can be analyzed by LC-MS/MS and microscopy upon downstream processes. Notably, nonspecific labeling usually occurs due to the intrinsic properties of photo-crosslinkers;² competition experiments, where samples are treated with excess unmodified parental compounds and photoprobes together, are usually carried out concurrently to eliminate the background.^{7–9} The specificity and background inventory of different photo-crosslinkers have been comprehensively illustrated in several recent photoaffinity labeling studies,^{26–28} which can help distinguish between background labeling and specific cross-linking.

Development of “minimalist” bioorthogonal handle-containing photo-crosslinkers applied in A/BP and bioimaging

As mentioned earlier, two key components, a photoreactive group and a bioorthogonal handle, need to be embedded in original molecules to form the corresponding photoprobes. They are usually installed in two separate sites,^{29–32} which would lead to complexities in synthesis and compromised bioactivities. This thus calls for “minimalist” photo-crosslinkers containing a photoreactive group and a bioorthogonal handle to alleviate these issues. In addition, the minimalist linker-containing probes would offer opportunities for simultaneous A/BP and bioimaging to accurately determine drug–target interactions. With these considerations in mind, we developed three versions of minimalist bioorthogonal handle-containing photo-crosslinkers which possess the following properties, respectively: (1) capable of facilitating the construction of photoaffinity probes that retain most bioactivities of parental compounds; (2) realizing simultaneous proteome profiling and imaging *in situ*; and (3) enabling protein labeling and real-time imaging at the same time; the linkers were successfully applied in all kinds of

Fig. 1 (A) Overall process of the PAL strategy. (B) Reported photo-crosslinkers and bioorthogonal handles.

small bioactive molecules for proteome profiling and cellular imaging studies (Fig. 2A and B).

Terminal alkyne-containing diazirine/benzophenone photo-crosslinkers (L1/2)

In 2012, Shi *et al.* reported two terminal alkyne-containing photo-crosslinkers (L1/2),⁷ where benzophenone and diazirine were used as photo-crosslinkers (Fig. 2A). They were incorporated into a hydrophilic moiety of dasatinib to produce their corresponding photoprobes termed DA-1/2 for *in situ* off-target identification (Fig. 2B). In order to assess the effect of linker units on the probes, molecular docking experiments, *in vitro* kinase assay, antiproliferation assay *etc.* were carried out and the results proved that both probes prettily retained the bioactivities of their parental compound. Next, cellular imaging and labeling experiments in different experimental settings demonstrated that DA-2 was a good mimic of dasatinib and could be further used to report dasatinib-kinase interactions in live cells. Finally, large-scale pull-down/LC-MS experiments were performed and successfully identified 84 proteins by subjecting DA-2 to both cellular lysates

and live cells of K562 and HepG2 cancer cell lines. By comparison, only six kinases were identified under identical conditions with the immobilized dasatinib matrix. The identified protein hits included known targets such as MAPKAPK2, RSK2, p38 α , PRKDC, STK6, CDK2, and PKC- β and a number of unknown targets including PCTK3, STK25, eIF-2A, PIM3, PKA C- α , and PKN2, which were further validated by pull-down/immunoblotting experiments with corresponding antibodies and kinase inhibition assays. These results thus confirmed that clickable probes like DA-2 are better than resin-immobilized probes³³ and biotin-tagged probes³⁴ to profile the intended protein targets in live-cell environments by pull-down/LC-MS experiments.

Another example of using a similar linker unit of L2 to profile the cellular targets of bioactive compounds was also reported by Shi *et al.* in 2011.³⁵ The linker unit was introduced into staurosporine, a pan-kinase inhibitor, to produce the clickable probe STS-1 (Fig. 2B). Similarly, different validation experiments, molecular docking, inhibition assays *in vitro* and *in vivo*, labeling profile and cellular imaging, were carried out and proved that STS-1 was indeed suitable for proteome profiling of cellular targets of staurosporine.

Fig. 2 (A) Recently developed bioorthogonal handle-containing photo-crosslinkers L1-L6 for A β BP and cellular imaging studies. (B) Structures of the photoprobes containing different photo-crosslinkers (L1-L6).

Subsequent large scale pull-down/LC-MS identified a total of 43 kinases, which is comparable to a biotin-containing probe under similar conditions.³⁶ Amongst these protein hits, several unknown targets such as PRPS6KA6, PFKM, CKB, PRPS2, and ADK were further validated by *in situ* pull-down/immunoblotting, which provided valuable information for studying potential off-targets of staurosporine. In summary, the linker unit (L2) improved the accuracy of the affinity-based proteome profiling (A/BP) approach for target identification of small molecules based on these successful applications.

Minimalist terminal alkyne-containing diazirine photo-crosslinker (L3)

Given that the size of L2 is relatively large, which could potentially affect the binding with target proteins and the synthesis of L2-derived probes is complex, we thus developed a minimalist photo-crosslinker (L3), where both the photo-reactive group and bioorthogonal handle were incorporated into a small aliphatic chain (Fig. 2A and 3A).⁸ It was functionalized with carboxyl, amino and iodo groups so that it could be conveniently conjugated to all kinds of biologically active molecules through acylation and alkylation reactions. The synthesis of the linker unit starting using readily available ethyl acetoacetate gave a hydroxyl-functionalized linker over 5 steps. Subsequent conversion of $-OH$ to $-NH_2$, $-COOH$ and $-I$ was achieved under standard conditions. They were subsequently incorporated into 12 well-known kinase inhibitors to form their corresponding probes by replacing the water-soluble structural motifs (Fig. 1B) through amine/acid coupling and alkylation reactions.

With these probes in hand, standard *in vitro* kinase inhibition assays and cell-based anti-proliferation assays were first performed and confirmed that the biological activities of the probes were not significantly compromised, indicating that the minimalist linkers had indeed reduced the interference

of the linker unit in probes' binding with the target protein under different conditions. To assess whether these minimalist linkers performed better than the previous version of L2, we made a comparison between STS-2/DA-3 and the well-established probes, STS-1/DA-2, through biological evaluation and proteomics studies. Two sets of probes were first compared by *in vitro* kinase inhibition assays and cell-based XTT assays, and the results appeared that STS-2/DA-3 were stronger than their counterparts, STS-1/DA-2. Next, labeling in bacterial proteomes and cellular imaging experiments demonstrated that STS-2/DA-3 were more efficient than STS-1/DA-2 to label their target protein in complex cellular proteomes (Fig. 3B and C).⁸ Lastly, endogenous proteome labeling and PD/LC-MS/MS target identification with STS-1/2 in both lysates and live HepG2 cells proved that STS-2 consistently identified more kinases *in vitro* and *in situ* than STS-1 (Fig. 3D).⁸ Importantly, the number of identified protein hits was significantly higher than that from a previous study using a different photo-crosslinking approach.³⁶ These results proved that the minimalist linkers could exhibit the desired functionalities by making the L3-derived probes excellently retain the bioactivities and efficiently capture target proteins.

Next, to confirm the ability of the 12 probes to label their known kinase targets, we performed proteome labeling followed by PD/western blotting (WB). The results turned out to be that 10 out of the 12 probes were shown to successfully label their known kinase targets both *in vitro* and *in situ*. Obvious difference between *in situ* and *in vitro* labeling profiles can be observed, which indicated the different protein targets under the two experimental settings. Finally, a "cocktail" approach was applied in the proteome profiling in rat kidney tissues by standard proteome labeling/PD/LC-MS/MS; a total of 94 kinases were effectively identified which include several known kinase protein such as c-Src, PKAC- α , AMPK α 1/2, JNK2/3, CDK5, P38, VEGFR, MEK, LRRK2 *etc.*, and a number

Fig. 3 (A) Workflow of the first-generation minimalist photo-crosslinkers applied in A/BP and cellular imaging. (B) In-gel fluorescence imaging of PKA (left) and c-Src (right) overexpressed bacterial proteomes labeled by STS-1/2, DA-2/3 and NP. (C) Live cell imaging of HepG2 cells with STS-1/2. (D) Analysis of the number of kinases enriched by STS-1/2 upon proteome labeling/PD/LC-MS/MS using HepG2 cells.⁸

of unknown protein hits including Grk1, CKB, PRPS2, MRKCB, Nek1, Nek7, ILK, Gsk3a, TLK2, TAOK3, WNK1, and WNK4. Three of these newly identified targets, Grk1, CKB and PRPS2, were further validated by PD/WB experiments. This evidence proved that the minimalist linker (L3) performed better than previous methods, and can be applied in different bioactive compounds for target identification.

Minimalist cyclopropene-containing diazirine photo-crosslinker (L4/5)

Although L3 enables its corresponding photoprobes to improve their synthetic accessibility and proteome profiling capability, it requires a Cu(i) catalyst in CuAAC, which is not suitable for both simultaneous photo-crosslinking and reporter-tagging *in situ*. Herein, we developed the second-generation minimalist linkers (L4/5, Fig. 2A and 4A) containing a novel cyclopropene moiety and an alkyl diazirine within the same molecule,⁹ aiming to perform proteome profiling and imaging *in situ* simultaneously through copper-free tetrazine–cyclopropene ligation reaction.

It is a great challenge to incorporate diazirine and cyclopropene moieties into a small linker due to their instability. Noted that cyclopropene is synthetically accessible from its terminal alkyne precursor, we chose to synthesize L4 by treating L3 with ethyl diazoacetate and a catalytic amount of Rh₂(OAc)₄. Further reduction of the ester group of cyclopropene to afford the corresponding hydroxymethyl-containing linker (L5) resulted in faster ligation reaction with tetrazine ($k_2 = 5.03 \pm 0.34 \text{ M}^{-1} \text{ s}^{-1}$). The stability of the cyclopropene moiety (L5) was tested by NMR and LC-MS in the presence of biological nucleophiles (L-cysteine) for different periods of time, which proved that it was stable enough for

in situ applications. Moreover, L5 showed an obvious turn-on effect upon ligation with different tetrazine-containing fluorescent dyes within 5 min. To assess the performance of L4/5, they were conjugated with a protein–protein-interaction (PPI) inhibitor of BET bromodomains to produce the probes BD-1/2. The L3-containing probe (BD-3) was also made for comparison (Fig. 2B). Firstly, an isothermal titration calorimetry (ITC) experiment was carried out and showed that the probes could bind to recombinant BRD-4 protein with similar affinity to that of (+)-JQ1; secondly, concentration- and time-dependent labeling experiments showed that BD-2 was better than BD-1/3 in both labeling efficiency and ligation speed (Fig. 4B).⁹ Live cell imaging experiments demonstrated that BD-2 was able to monitor drug uptake and subcellular distribution (Fig. 4C).⁹ Finally, large scale proteome profiling was carried out with BD-2/3 in live HepG2 cells; 420 and 326 protein candidates for BD-2 and BD-3, respectively, were successfully identified and around 41% of the proteins are the same (Fig. 4D).⁹ The differences could be account for either variability of the instrument or linker difference. Two likely genuine off-targets of (+)-JQ1, DDB1 and RAD23B, were further validated by pull-down/WB. Taken together, these results proved that L5-containing photoprobes are capable of simultaneous proteome profiling and imaging *in situ*, and such dual-purpose probes could increase the reliability of protein hits identified from pull-down/LC-MS/MS experiments.

Minimalist alkyne-containing tetrazole photo-crosslinker (L6)

The linker unit (L5) enabled its corresponding photoprobes for simultaneous proteome profiling and imaging *in situ* to accurately report drug–target interactions. Due to the background of using the tetrazine reporter, this method still has

Fig. 4 (A) Workflow of the second-generation minimalist photo-crosslinker applied in A/BP and cellular imaging studies. (B) Concentration- and time-dependent labeling of BD-1/2/3 with recombinant BRD-4. (C) Live-cell imaging of BD-2/NP-2. (D) The number of proteins enriched by BD-2/3 upon *in situ* labeling/PD/LC-MS/MS with live HepG2 cells.⁹

Fig. 5 (A) Workflow of the third-generation minimalist photo-crosslinker applied in protein labeling and imaging studies. (B) Labeling profiles of BSA by NP-1/2 with or without UV irradiation. (C) Concentration-dependent and UV irradiation-time-dependent labeling of STS-2/3 with recombinant PKA. (D) Labeling of a PKA-spiked HepG2 lysate by STS-2/3 with or without 10 $\times$ STS. (E) Proteome reactivity profiles of live HepG2 cells treated with STS-2/3 with or without excess STS. (F) Images of live HepG2 cells treated with STS-2/3.¹⁰

space for further improvement. Photo-crosslinkers that possess the properties of both photo-crosslinking and fluorescence turn-on would be complementary to this approach. In 2015, we unexpectedly found that tetrazole, a moiety, can display fluorescence turn-on properties based on previous studies³⁷ and can behave as a general photo-crosslinker owing to its high reactivity with biological nucleophiles such as amino, acidic and thiol groups upon UV irradiation.¹⁰ The terminal alkyne was introduced into tetrazole to form the third generation minimalist linker, L6 (Fig. 2A and 5A), which was then introduced into staurosporine to assess the photo-crosslinking efficiency. Labeling with different proteomes and imaging studies proved that L6 derived probes were comparable to diazirine-containing probes in labeling target protein (Fig. 5B–F).¹⁰ Next, a library that was composed of 14 fluorogenic probes by introducing tetrazole into Bodipy and acedan dyes was constructed, most of them turned out to be good protein biosensors. By further introduction of these photo-activatable, fluorogenic compounds into staurosporine, the resulting probes were capable of no-wash imaging of kinases in live HepG2 cells. These results indicated that L6-containing photoprobes can efficiently label target proteins, and the corresponding fluorogenic probes were capable of photoinduced, no-wash imaging *in situ*. These functions would enable more accurate determination of drug–target interactions. In addition, our findings that tetrazole can be a general photo-crosslinker with excellent crosslinking efficiency were further validated by several independent works from different labs.^{38,39}

Conclusion

Bioorthogonal handle-containing photo-crosslinkers (L2–6) facilitate the synthesis of photoprobes with improved proteome profiling and imaging applications, which would increase the confidence level of protein hits in target identification of small bioactive molecules. Gratifyingly, these linkers, especially L3, have already been applied in PAL by many labs since they were published,^{40,41} and we expect that they would find broad applications in various research areas. Encouraged by these outcomes, we are trying to develop other kinds of linkers which would further improve the accuracy of target identification through proteome profiling and bioimaging approaches.

Conflict of interest

The authors declare no competing interest.

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