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Target identification of natural products and bioactive compounds using affinity-based probes

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HIGHLIGHT

Target identification of natural products and bioactive compounds using affinity-based probes

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Advances in isolation, synthesis and screening strategies have made many bioactive substances available. However, in most cases their putative biological targets remain unknown. Herein, we highlight recent advances in target identification of natural products and bioactive compounds by using affinity-based probes. Aided with photoaffinity labelling, this strategy can capture potential cellular targets (on and off) of a natural product or bioactive compound in live cells directly, even when the compound-target interaction is reversible with moderate affinity. The knowledge of these targets may help uncover molecular pathways and new therapeutics of currently untreatable diseases. In this highlight, we will introduce the development of various photoactivatable groups, their synthesis and applications in target identification of natural products and bioactive compounds, with a focus on work done in recent years and from our laboratory. We will further discuss the strengths and weaknesses of each group and the outlooks for this novel proteome-wide profiling strategy.

1 Introduction

The identification of new drug targets is of great importance in the biomedical research, but has been comparatively slow, with only less than 0.15% of genes in the human genome currently being used as drug targets.[1] Despite the technological advances in isolation, synthesis and screening strategies that make many bioactive substances available, in most cases their putative biological targets remain unknown.[2,3] Typically, these substances are discovered from phenotypic screening, which assesses multiple cellular targets.[1,2,4] Thus, the ultimate goal of target identification is to study all binding targets that account for the biologically interesting phenotype, and off-targets that might be relevant to toxicity and side effects.[5] This knowledge could help uncover new therapeutic targets and molecular pathways of currently untreatable diseases.[2] In recent years, there has been extensive reviews on target identification of small bioactive molecules by using different types of strategies[6-12]. In this highlight, we will discuss recent advances in the target identification of natural products and bioactive compounds under *in situ* settings (that is, in live-cell environments), by using affinity-based probes, with a focus on work done from our laboratory.

1.1 Activity-based protein profiling and photoaffinity labelling

An ideal strategy of target identification captures drug-target interactions directly *in situ*, while allowing for subsequent proteome-wide target deconvolution which is normally done *in vitro*.[7] One such strategy is the activity-based protein profiling (ABPP), where the enzyme of interest is identified on the basis of its activity/state rather than its expression level.[13] For example, we previously reported an activity-based probe (ABP, **1**) based on Orlistat (a US Food and Drug Administration (FDA)-approved lipase inhibitor with potential antitumor activities), and successfully used it to identify unknown cellular targets (Fig. 1).[14] But in the case where the enzyme activity does not rely on a nucleophilic residue in its active state or the compound is a non-covalent binder, an affinity-based probe (A*f*BP) can be applied instead.[5,8,15] By taking advantage of both ABPP and photo-affinity labelling (PAL), natural products and bioactive compounds can be converted to A*f*BPs via the addition of a photoactivatable group and a tag (reporter or analytical handle). With such a strategy, even if the A*f*BP is made from a reversible binder with moderate affinities to its molecular targets, the photoactivatable group can create a covalent bond between the probe and targets (Fig. 1). Hence moderate probe-target interactions can be analyzed by subsequent *in vitro* biochemical methods that often require harsh denaturing conditions.[6] On the other hand, various tags have been used for distinct analytical purposes as shown in Figure 1: an isotope or fluorophore can make the probe-target complex visible by using in-cell imaging, or in-gel fluorescence scanning after the sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) separation; a biotin can be used with avidin beads for affinity purification and enrichment of captured targets for subsequent Western blotting and/or mass spectrometry (MS) analysis; an analytical handle, typically a terminal alkyne, can be ligated to either fluorophore-azide or biotin-azide *via* the copper (I)-catalyzed azide-alkyne cyclo-

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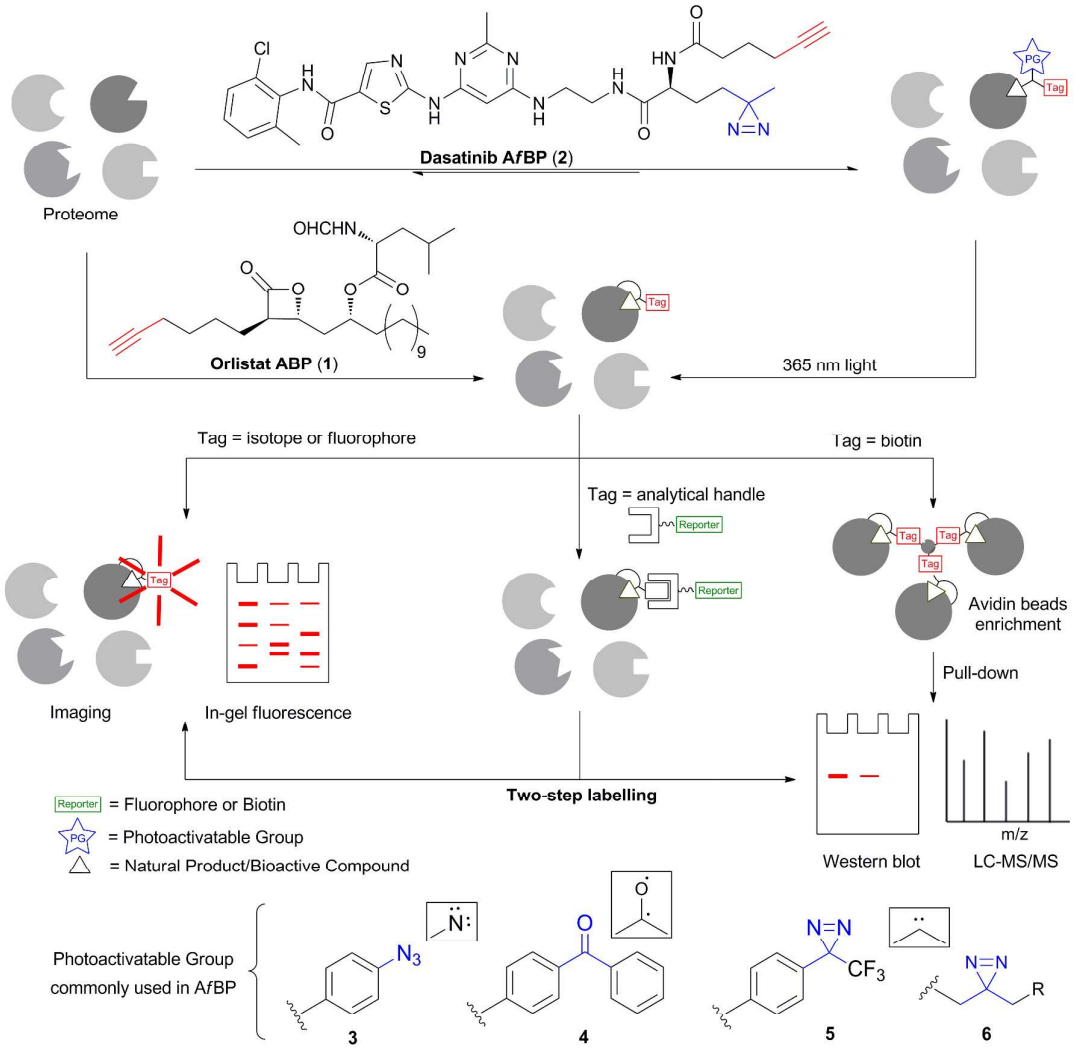

Figure 1 General workflow (one-step and two-step) of proteome-wide target identification by ABPs and A*f*BPs, using Orlistat and Dasatinib as examples.

addition for visualization or enrichment of labelling events as stated above. This two-step labelling strategy can make an A*f*BP more cell-permeable with less perturbation to the native binding. Our laboratory has applied this strategy to convert Dasatinib (a FDA-approved cancer drug targeting Src/Abl) into an A*f*BP (**2**), which was subsequently used to identify a number of previously unknown cellular targets (Fig. 1).[16] This was the first time that large-scale proteome-wide profiling of kinase-drug interactions was successfully demosntrated directly in the native cellular environments.

2 Photoactivatable groups

There are three types of photoactivatable groups commonly used in A*f*BP: aryl azides (**3**), benzophenones (**4**), as well as diazirines, which are further classified into aryl diazirines (**5**) and aliphatic diazirines (**6**). Each of these groups generates different reactive species after photolysis (Fig. 1, in boxes).

2.1 Aryl azides

2.1.1 Development of aryl azides. The use of aryl azide in PAL was first reported in 1969 when 4-azido-2-nitrophenyl was chosen as the nitrene source.[17] Although its major absorption peak is at about 260 nm, it has a minor peak at 460 nm that is clear of the protein absorption wavelength (280 nm) and thus is suitable for photo-crosslinking. The properties of aryl azides can be fine-tuned with diverse substituents on the benzene ring. A successful example is tetrafluorophenyl azide, whose photo-labelling efficiency was improved by reducing the ring expansion rearrangement and red-shifting the excitation wavelengths to 320-350 nm.[8,18]

2.1.2 Synthesis of aryl azides. Traditionally, aryl azides (**3**) are prepared from the corresponding anilines (**7**) in two steps: anilines are first diazotized with sodium nitrite under strong acidic conditions (**8**), and then treated with sodium azide at low temperatures to afford **3** (Fig. 2A).[17] In recent years, a one-step synthetic method of aryl azides was developed by employing Cu(II)-catalysed diazo transfer from triflyl azide (TfN_3, Tf = CF_3SO_2) to anilines.[19] Later, a new azide source, imidazole-1-sulfonyl azide hydrochloride was reported with

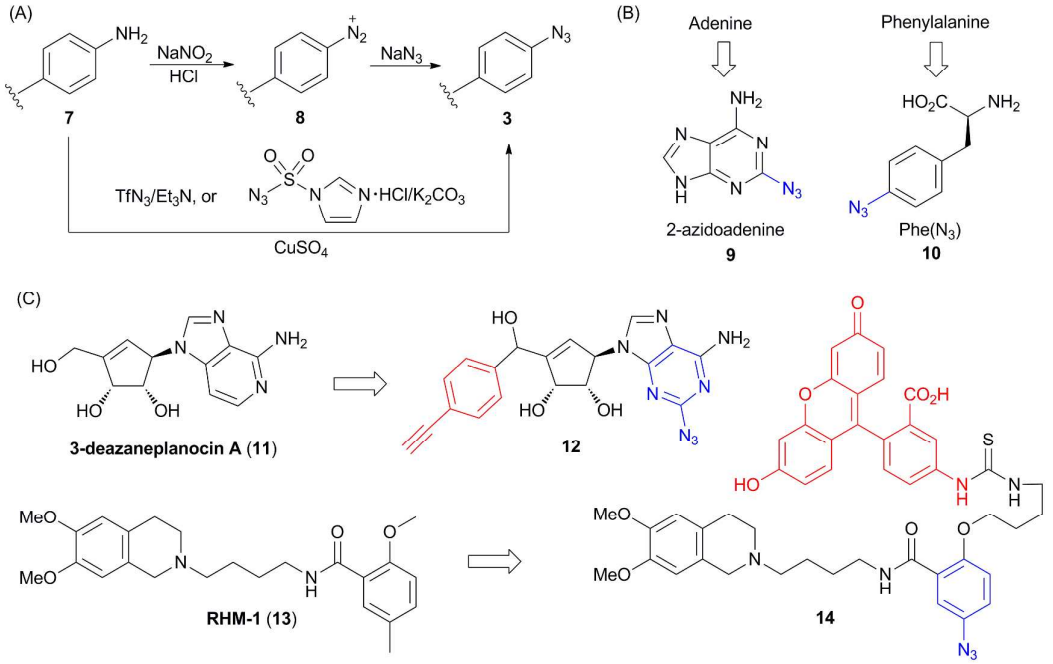

Figure 2 Synthesis and applications of aryl azides. (A) Synthetic scheme of aryl azides from anilines by one- or two-step methods; (B) Structures of azide modified adenosine and phenylalanine; (C) Structures of 3-deazaneplanocin A, RHM-1 and the corresponding A*f*BPs.

improved stability.[20] In practice, a variety of aryl azides have become commercially available in recent years, and could be readily incorporated into a desired compound through simple aromatic substitution or amide coupling.

2.1.3 Applications of aryl azides. The short activation wavelength and the low crosslinking yields raised from side reactions have limited the application of aryl azides.[8,18,21] However, they are easy to prepare and incorporate in A*f*BP. They are also relatively small, which is particularly useful in modifying aromatic moieties commonly found in bioactive substances, e.g. purines (**9**)[22] and phenylalanine (**10**)[23] shown in Fig. 2B.

3-deazaneplanocin A (**11**) is a global histone methylation inhibitor, whose molecular mechanism of action and cellular off-targets were not fully understood. Thus, an A*f*BP (**12**) was developed from **11** by replacing its adenine-like moiety with the photoactivatable 2-azidoadenine and also installing an aryl alkyne at a site far from the protein-binding interface (Fig. 2C). **12** was subsequently used to study the cellular uptake and localization of **11**. And a number of unknown putative targets were identified through large-scale *in situ* pull-down (i.e. biotin affinity purification) coupled with liquid chromatography–tandem mass spectrometry (LC-MS/MS) analysis.[22]

The sigma-2 receptor is a useful biomarker of the tumour cell proliferation, though its structure and sequence remained unknown. In order to identify potential protein complexes involved in the binding to sigma-2 receptor and investigate its roles in cell proliferation, an A*f*BP (**14**), based on a sigma-2-selective ligand RHM-1 (**13**), was developed with the strategic addition of an aryl azide for photo-labelling and a fluorescein for visualization (Fig. 3C). Subsequently, rat liver membrane homogenates were incubated with (**14**), and then separated by SDS-PAGE. A band at around 24 kDa was detected and subsequently identified to be the progesterone receptor membrane component 1 (PGRMC1). Further studies suggested that the putative sigma-2 receptor binding site was within the PGRMC1 protein complex.[24]

2.2 Benzophenones

2.2.1 Development of benzophenones. Since the first report in 1973, benzophenones have become a popular tool in PAL.[25] They are activated by wavelengths of 320-360 nm that are less harmful to the aromatic residues of nucleic acids and proteins. Furthermore, the reactive triplet state formed is essentially inert to water, making benzophenones suitable for biological applications.

2.2.2 Synthesis of benzophenones. Although they can be readily prepared by Friedel-Crafts acylation of arenes with benzoyl chloride, various benzophenone building blocks are commercially available, and are often purchased and used directly. Typically they contain functional groups such as carboxylic acids, amines or halides that are linked to targeted molecule *via* amide coupling or nucleophilic substitution.

2.2.3 Applications of benzophenones. In spite of their bulkiness which may interfere with native binding of the parental compound, benzophenones are widely used in A*f*BP because of its good labelling potential, long excitation wavelengths and ease of introduction. Benzophenones react with C-H bonds that produce relatively stable carbon radicals, such as amino acid α-carbons, benzylic carbon and tertiary carbons.[8,21] However, their inertness to being quenched by water and long irradiation time may cause severe non-specific

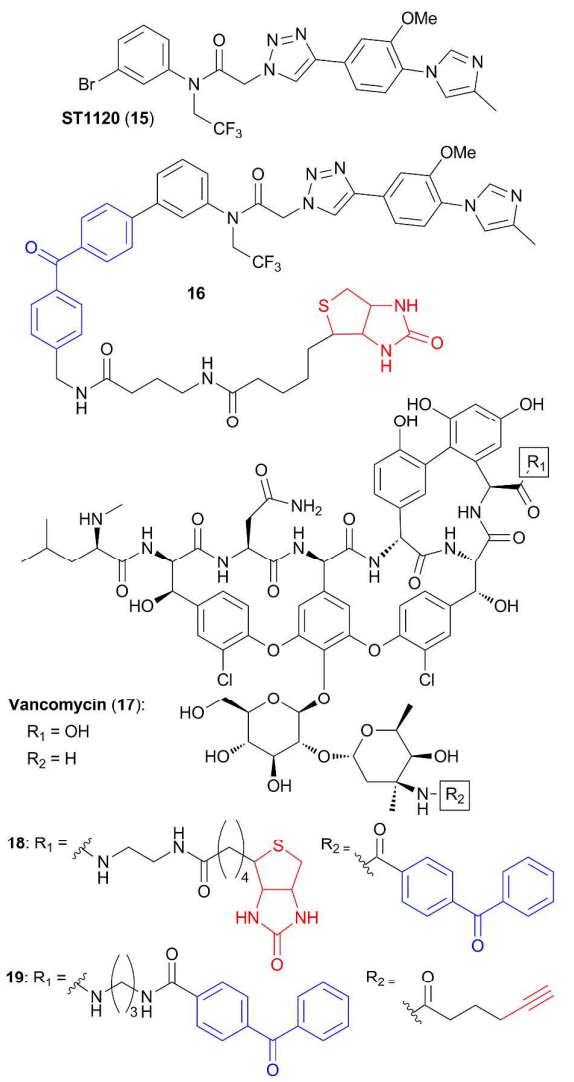

Figure 3 Structures of ST1120, vancomycin and the corresponding A*f*BPs.

background labelling. It should also be noted that the presence of hydroxyl group in the labelling product may also be problematic for MS analysis, as such products are prone to dehydration (e.g. when cross-linked to glycine[26]).

γ-Secretase is an intramembrane protease responsible for the production of amyloid-β (Aβ) peptides, which might play an important role in the pathogenesis of Alzheimer's disease. It is known that phenylimidazole-type modulators can reduce the production of pathogenic long Aβ peptides while elevating the levels of short Aβ peptides. However, the precise mode of binding and the molecular mechanism remained unclear. Thus, a phenylimidazole-type modulator ST1120 (**15**) was converted to an A*f*BP (**16**) by coupling with benzophenone and biotin (Fig. 3). **16** showed similar biological activities as**15**, and it was subsequently used in photo-labelling and pull-down to identify two potential protein targets, Aph-1aL and the *N*-terminal fragment of a catalytic subunit (presenilin 1) in γ-Secretase. In the following alanine screening, three residues were found to be essential for the photo-labelling. This finding was further investigated with systematic mutational studies, which suggested that **15** bound to a previously unknown extracellular pocket within the *N*-terminal fragment of presenilin. This binding induced a conformational change in γ-secretase, resulting in modulation of its activity for Aβ production.[27]

Vancomycin (**17**) is an important glycopeptide antibiotic first isolated in 1953, but its inducible drug resistance has since emerged in the late 1980s. While its activation mechanism of drug resistance was still under much debate, it was reported that such resistance required the expression of multiple genes including receptor histidine kinase VanS. Hence, an A*f*BP (**18**) was prepared by installing a benzophenone at the amino group of the sugar moiety in vancomycin, and a biotin at the *C*-terminus (Fig. 3). These modifications did not influence the activities of vancomycin. By employing **18** in photo-labelling, followed by MS analysis, it was revealed that a direct binding existed between **17** and VanS, which correlated with the formation of resistance and was required for the expression of other genes relevant to resistance.[28] In an extended study of protein targets of **17** in living bacterial cells, another A*f*BP (**19**) was designed with a benzophenone at the *C*-terminus and a terminal alkyne at the sugar moiety. Two previously unknown targets, the major autolytic protein Atl and the nutrition transporter ABC, were identified from *in situ* photo-labelling and subsequent quantitative MS analysis, which further unravelled the mode of vancomycin binding and inhibition.[29]

2.3 Aryl diazirines

2.3.1 Development of aryl diazirines. 3*H*-aryl diazirines is the first aryl diazirine applied in PAL, as reported in 1973 with an absorption at 340-380 nm.[30] Nevertheless, its photolysis would generate the linear diazoisomer as a major product (50-70%) and a carbene as a minor product. Instead of the desired carbene X-H (X = carbon or heteroatom) bond insertion, diazo compounds are known to be sensitive toward protonation and subsequent nucleophilic attack. Therefore, 3-trifluoromethyl-3-aryl diazirines were designed with less diazoisomer (30%) formed after irradiation. This diazoisomer is stable towards acids owing to the strong electron-withdrawing effect of the trifluoromethyl group. As a result, more efficient carbene X-H bond insertion could be achieved.[31]

2.3.2 Synthesis of aryl diazirines. As shown in Fig. 4A, the preparation of aryl diazirines (**5**) commonly starts from aryl bromide (**20**), which could be first converted to trifluoromethyl ketone (**21**) by the Grignard reaction with trifluoroacetic acid, or lithiation and reaction with various trifluoroacetyl reagents. Next, (**21**) is condensed with hydroxylamine to afford (**22**), whose OH group is then activated into a good leaving group (**23**) by using either *p*-tosyl chloride (TsCl) or mesyl chloride (MsCl). The corresponding diaziridine (**24**) is next obtained by treating (**23**) with liquid ammonia (usually under pressure). Finally, oxidation of (**24**) by freshly prepared silver oxide or iodine with triethylamine can give aryl diazirines.[31,32] Very recently, a direct conversion of the tosyl oximes (**23**) to aryl diazirines (**5**) was reported using liquid NH_3 either at 80 °C or with lithium amide.[33]

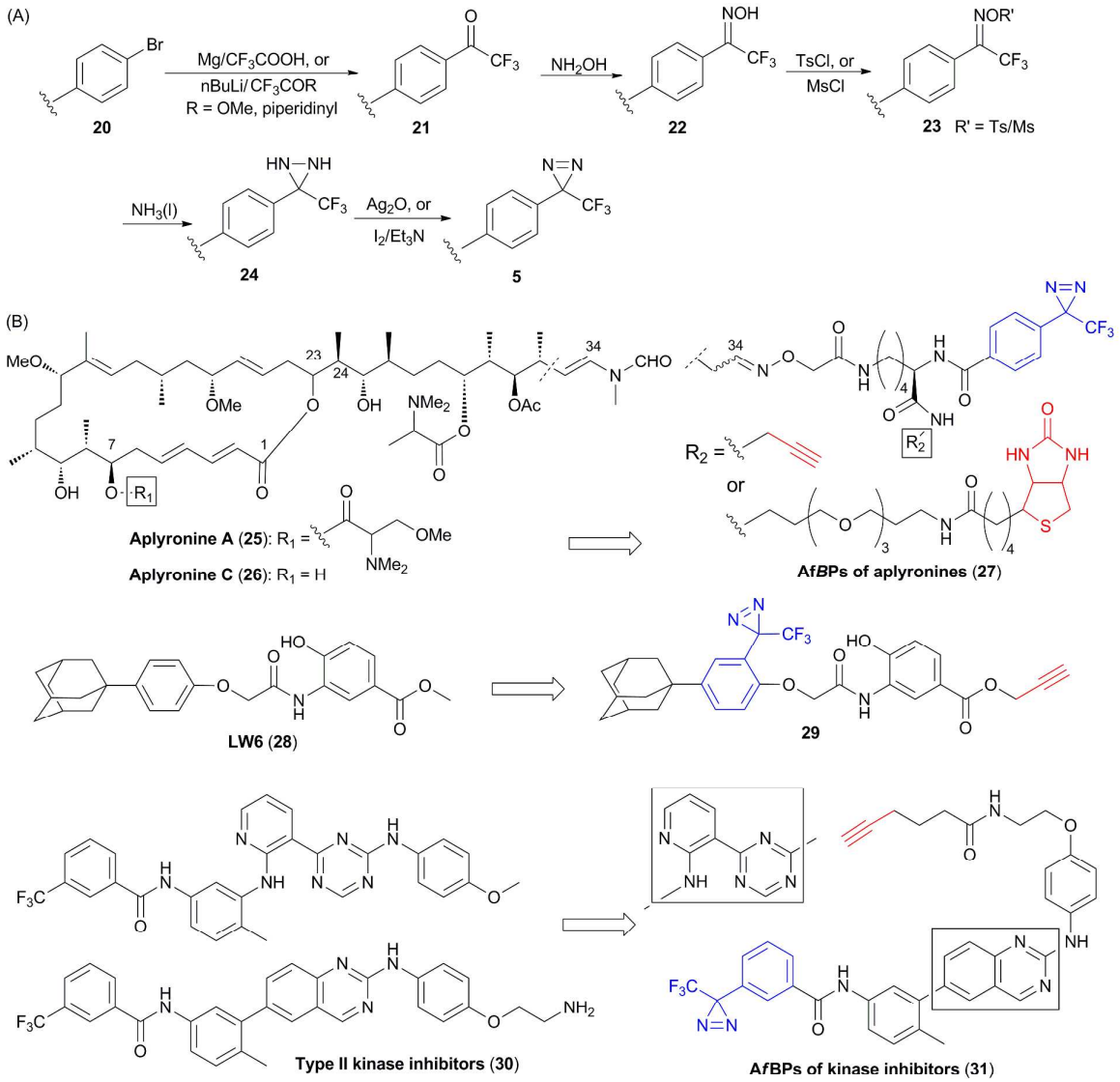

Figure 4 Synthesis and applications of aryl diazirines. (A) Common synthetic scheme of aryl diazirines from aryl bromides; (B) Structures of aplyronine A/C, LW6, two type II kinase inhibitors and the corresponding A*f*BPs.

2.3.3 Applications of aryl diazirines. While it requires some efforts to synthesize aryl diazirines, they are activated at 365 nm which causes minimal damages to biological systems. In addition, the reactive carbenes formed are readily quenched by water, thus reducing non-specific protein labelling. Aryl diazirines have good labelling yields, and the smaller size can further minimize interference to the native target binding. They are also stable towards many common reaction conditions, including strong acidic/basic environment and oxidizing/reducing reagents. In general, aryl diazirines favour insertion into O-H bonds over C-H bonds.[8,21] However, when it is interposed into N-H bonds, the product will easily expel a HF molecule, giving an enamine, which is subject to further hydrolysis with concomitant loss of the captured proteins.[34]

Aplyronine A (**25**) is a antitumor macrolide that exhibits intriguing potency against actin polymerization. X-ray analysis of the actin–aplyronine A complex showed that the C24-C34 tail is important for target binding, while the C7 ester moiety projects toward the solvent-accessible surface. Interestingly, aplyronine C (**26**), an analogue of **25** with absence of the C7 ester, showed similar actin-depolymerizing activity, while possessing significantly lower cytotoxicity. To investigate the precise antitumor mechanism of these compounds, several A*f*BPs (**27**) were prepared from aplyronine A/C (Fig. 4B). Acidic hydrolysis of aplyronines could afford the C34 aldehyde. This intermediate could be condensed with alkoxyamine, which was linked with 3-trifluoromethyl-3-aryl diazirine and a alkyne/biotin, for subsequent in-gel fluorescence scanning or pull-down/Western blotting visualization. With the help of **27**, two actin-related proteins, Arp2 and Arp3, were identified as the binding proteins along with actin from tumour cell lysate.[35] However, a follow-up study using live cells revealed that a heterotrimeric complex was formed among **25**, actin and tubulin, whereas **26** could not induce such protein-protein

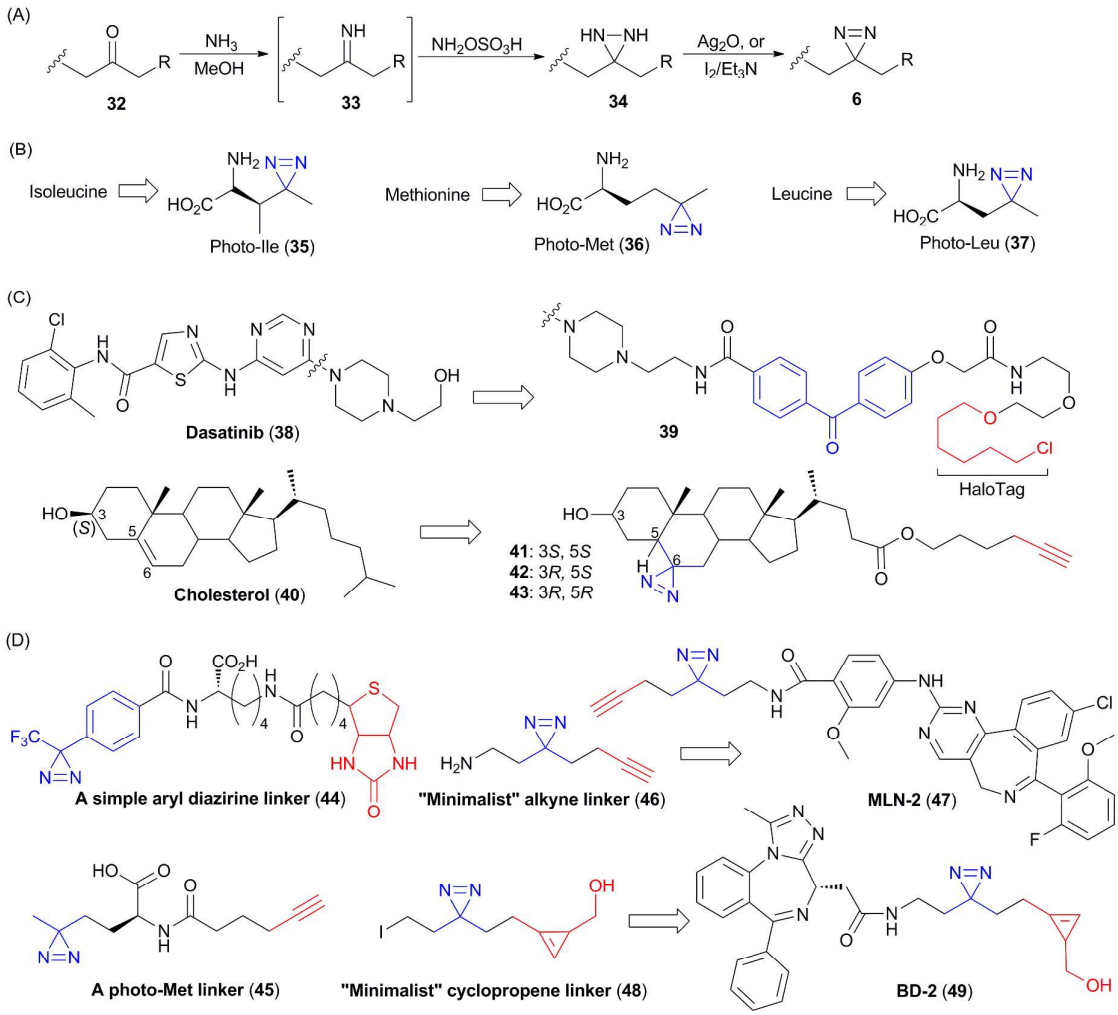

Figure 5 Synthesis and applications of aliphatic diazirines. (A) Common synthetic scheme of aliphatic diazirines from ketones; (B) Structures of diazirine modified isoleucine, methionine and leucine; (C) Structures of Dasatinib, cholesterol and the corresponding A*f*BPs; (D) Different generations of diazirine linkers, and the "minimalist" linker deriving A*f*BPs **MLN-2** and **BD-2**.

interaction. Thus, **25** represents a novel pharmacophore that can inhibit two distinct cytoplasmic proteins to exert highly potent antitumor activities.[36]

Hypoxia-inducible factors (HIFs) are transcription factors that regulate tumour angiogenesis and metastasis in response to decreases in oxygen, or hypoxia. HIF-1α, a subunit of HIF-1, is overexpressed in many human cancers and hence is an important therapeutic target. The accumulation of HIF-1α in the cancer cells can be inhibited by LW6 (**28**), an analogue of aryloxyacetylamino benzoic acid through the induction of the degradation of HIF-1α. To identify its molecular target, a trifluoromethyl diazirine and a terminal alkyne were added to **28**, affording an A*f*BP (**29**, Fig. 4B). From *in situ* photo-labelling and in-gel fluorescence scanning, malate dehydrogenase 2 (MDH2) was identified as the potential cellular target of **28**. Further studies indicated that **28** inhibited the MDH2 activity by suppressing mitochondrial respiration, which eventually reduced the accumulation of HIF-1α.[37]

Kinases are critical drug targets as they are involved in the regulation of virtually every signal transduction process in cells. Apart from the highly conserved ATP-binding site, there are also distinct types of inactive conformations throughout the mammalian kinome. One such conformation is the 180° rotation of Asp-Phe-Gly motif (DFG-out), which is specifically recognized by the type II inhibitors. In order to perform the conformation-selective kinase profiling, two type II kinase inhibitors (**30**) with different scaffolds were selected and modified into the corresponding A*f*BPs (**31**) by addition of a trifluoromethyl diazirine and a terminal alkyne (Fig. 4B). **31** was then used in proteome-wide profiling and quantitative MS analysis, and a diverse set of putative kinase targets was identified, including several protein kinases that have not been previously characterized to adopt the DFG-out conformation.[38]

2.4 Aliphatic diazirines

2.4.1 Development of aliphatic diazirines. Although their properties are almost identical to those of aryl diazirines, the use of aliphatic diazirines has remained limited mainly due to two reasons: they tend to generate less carbene after photolysis, and the subsequent crosslinking process is

competed by an intramolecular rearrangement reaction (which generates an alkene instead). Nevertheless, aliphatic diazirines have gained increasing attention in recent years due to their extremely compact sizes, which is highly useful in the study of protein-protein interactions in living cells.[39]

2.4.2 Synthesis of aliphatic diazirines. Unlike the synthesis of aryl diazirines, the ketone intermediate (**32**) of aliphatic diazirines (**6**) is first converted to an imine (**33**) with methanolic ammonia or liquid ammonia under high pressure, whereafter a suitable aminating reagent (i.e. hydroxylamine-O-sulfonic acid) is added, in one pot, to obtain the corresponding diaziridine (**34**). The compound is then oxidized by a freshly prepared silver oxide or iodine with triethylamine to provide the aliphatic diazirine (**6**, Fig. 5A).[40,41]

2.4.3 Applications of aliphatic diazirines. The moderate labelling efficiencies that limit the use of aliphatic diazirines can be compensated by their comparatively small sizes, which make them highly attractive in cases where the drug-target interactions are sensitive to the size of modification introduced into the probes. For example, aliphatic diazirines were used to modify several natural amino acids, including isoleucine (**35**)[39], methionine (**36**)[41] and leucine (**37**)[42], in an effort to directly monitor residue-target interactions (Fig. 5B). Aliphatic diazirines display a broad range of photo-crosslinking reactivity with various side chains of amino acids, with the most efficient being aspartate, glutamate, and tyrosine, followed by glutamine, serine, cysteine, and histidine.[43]

Dasatinib (**38**) is a promising drug that has been approved by FDA for chronic myelogenous leukemia. Because of the highly conserved ATP-binding pocket amongst most kinases, **38** is expected to inhibit multiple cellular targets just like many other well-known protein kinase inhibitors. In the study of cellular selectivity and potential on- and off-targets of **38**, two A*f*BPs were initially developed by our group with a linker containing both a photoactivatable group (aliphatic diazirine or benzophenone) and a terminal alkyne handle. Since the benzophenone A*f*BP showed poor cell permeability, only **2** was utilized in the following proteome-wide profiling study, and a number of previously unknown cellular targets was identified through pull-down/LCMS. Six serine/threonine kinases (PCTK3, STK25, eIF-2A, PIM3, PKA C-α, PKN2) were further validated by preliminary pull-down/Western blotting analysis.[16] In another study, **38** was chemically modified with a long benzophenone-containing aliphatic linker, to obtain a different kind of A*f*BP (**39**), which was subsequently used for the profiling of kinase active sites and led to the identification of conformation-specific inhibitors (Fig. 5C).[44]

Cholesterol (**40**) is a type of lipid molecule, which is essential to cell membranes as well as the biosynthesis of several signalling molecules. In order to fully investigate the cholesterol-binding proteins in mammalian cells, three diastereomeric A*f*BPs (**41**-**43**) were prepared by introducing a diazirine at the C6 position of cholesterol and a terminal alkyne at its alkyl side chain (Fig. 5C). These changes were shown to have minimal disturbance to the cholesterol binding and its biophysical properties. Additionally, the stereocenters at C3 and C5 positions were manipulated to provide different diastereomeric features. With these probes, in combination with the stable-isotope labelling by amino acids in cell culture (SILAC) followed by quantitative MS experiments, over 250 cholesterol-binding proteins were successfully identified.[45]

2.4.4 Development of "minimalist" diazirine linkers. The design of A*f*BP is critical for successful target identification. Generally, two-step labelling can increase the cell permeability of the probe and reduce perturbation to the native target binding (Fig. 1). It is also beneficial for live-cell labelling, whose importance has been illustrated in the studies of Aplyronine A (**25**). In practice, a photoactivatable group is placed in proximity to certain residues of the intended cellular targets for efficient crosslinking, whereas an analytical handle is placed at the solvent-accessible surface for the convenience of subsequent reactions. These positions are typically estimated by known co-crystal structures of the parental compound and a target (if any), to ensure minimal influence on the interactions between targets and the compound once the needed chemical modifications are introduced.

A more convenient way for deriving an A*f*BP is to use a linker containing both a photoactivatable group and an analytical handle. As show in Fig. 5D, initially the design of our linker was based on lysine that joined both parts (**44**)[15], which was later simplified with the use of photo-Met (**45**)[16]. In order to further minimize the interference on target binding, we have reported a set of "minimalist" linkers in which both aliphatic diazirine and a terminal alkyne were concurrently incorporated (**46**). These linkers were applied to modify 12 reported kinase inhibitors, which were subsequently utilized in large-scale cell-based proteome profiling in an attempt to identify potential on- and off targets. Indeed, compared to our previously reported bulkier linkers[16], these "minimalist" linkers demonstrated better protein labeling and cellular imaging properties.[46] One such linker has recently been used in the preparation of an A*f*BP (**47**) for simultaneous imaging and target profiling of MLN8237, an Aurora kinase A inhibitor.[47] Recently, we have also developed new "minimalist" linkers (one of which is shown as **48**), by making use of the rapid, copper-free, tetrazine-cyclopropene ligation reaction. The potential application of these second-generation minimalist linkers has recently been demonstrated successfully in a case study where a small molecule protein-protein-interaction inhibitor, GW841819X, was converted into an A*f*BP (**49**), which exhibited the dual capability of both live-cell imaging and *in situ* proteome profiling of bromodomains.[48] The most obvious improvement in these cyclopropene-containing A*f*BPs is their compatibility with live-cell imaging experiments.

3 Conclusion and Outlook

The use of A*f*BP in target identification of natural products or bioactive compounds has been described herein with various photoactivatable groups. As discussed above, each group has its strengths and weaknesses, and should be chosen on a case-by-case basis. In spite of the successful examples reported, it remains a challenge of A*f*BP to reduce non-specific photo-labelling and to improve accuracy in the proteome-wide

identification of molecular targets through MS analysis or live-cell imaging, or both. These topics have been addressed to some extent with the development of novel analytical approaches, such as SILAC and chemical labels in quantitative proteomics, and with the use of suitable photoactivatable groups and reporters (e.g. two-photo dyes).[49,50] Therefore, we expect that the strategy of A*f*BP will continue to have a strong impact on retrospective identification of potential on- and off-targets and the study of novel molecular pathways in medicinal chemistry and chemical biology.

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