The use of click chemistry in the emerging field of catalomics†

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Of the thousands of known chemical reactions, a handful of reactions, called "click" reactions, stand out with features such as good chemoselectivity, good solvent compatibilities, modularity, minimum synthetic demands, bioorthogonality and high yields. Among them, the Cu(I)-catalyzed 1,3-dipolar cycloaddition reaction between azides and terminal alkynes has emerged as a powerful tool in chemical biology and proteomics. This perspective surveys the significant contributions of click chemistry in catalomics (a sub-area in chemical proteomics), with special emphasis on activity-based protein profiling (ABPP), posttranslational modifications (PTMs) and enzyme inhibitor developments.

Introduction

Living organisms carry out a myriad of complex biological functions using highly intricate, yet tightly balanced and self-controlled, networks of biological molecules including nucleic acids, proteins, lipids, carbohydrates and metabolites. A better understanding of the life process therefore requires powerful ways to investigate these molecules and their interactions in various contexts, either using isolated and purified biomolecules or those present in their native cellular environment. Towards this end, chemistry, traditionally being the science of synthesis and structural manipulations of molecules, has gradually undertaken the more challenging task of biology-oriented synthesis.[1] As the generation of molecules/molecular assemblies possessing well-defined biological functions remains an extremely challenging task, immediate refinements in conventional synthetic tactics are necessary. New and more efficient chemical reactions and methodologies, which may override the laborious protection/deprotection and purification steps in conventional total synthesis, could revolutionize the next-generation chemical and biological research.[2] A set of chemical reactions, known as bioorthogonal reactions, that are orthogonal to most functional groups in biological systems, have so far shown promising applications in biological research.[3] Of these reactions, a handful have been defined by Barry Sharpless as click reactions.[4] The Cu(I)-catalyzed version of the Huisgen 1,3-dipolar cycloaddition reaction[5] between azides and terminal alkynes, discovered independently by Meldal *et al.*[6] and Sharpless *et al.*,[7] is the best-known click reaction so far, and has recently emerged to become one of the most powerful tools in chemical biology and proteomic applications.[8]

With the advances from genome-sequencing projects, the functional annotation and characterization of newly discovered proteins have become increasingly important. We are particularly interested in the understanding of the context-dependant catalytic roles and interaction networks of protein-based biocatalysts, or enzymes. As key regulators of virtually every biological process, even minor imbalances in the activity of certain enzymes might lead to severe pathological conditions. Detailed understandings of how an enzyme works at its molecular and cellular level will

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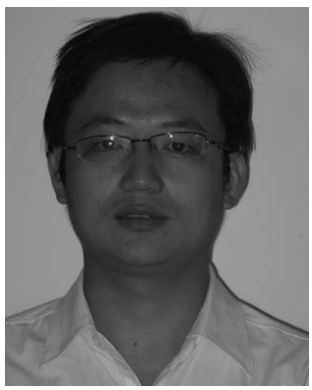

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have direct relevance to drug discovery. To meet the goal of large-scale studies of a significant number of enzymes present in any particular organism, high-throughput amenable chemistry and enzyme-screening tools are needed. The different combinations of chemical/biological and technological advances directed towards elucidating the catalytic functions of enzymes *en masse* have been recently grouped in a unified field termed Catalomics (Fig. 1).[9] Herein, we review the important roles of click chemistry (CC) and its recent developments in this emerging field, with special emphasis on activity-based protein profiling (ABPP), post-translational modification (PTM) and enzyme inhibitor discovery.

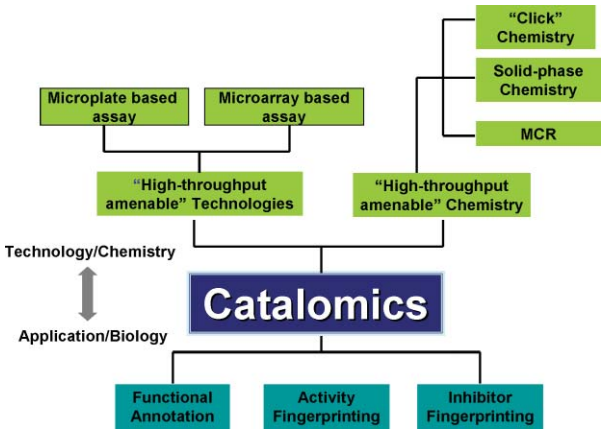

Fig. 1 Overview of Catalomics. For a detailed review on the subject, see ref. 9.

CC in activity-based protein profiling (ABPP)

Activity-Based Protein Profiling (ABPP) has been emerging as a powerful chemical proteomic method towards the analysis of functional states of proteins in a complex proteome.[10] The strategy, pioneered by Cravatt and co-workers, uses active site-directed, mechanism-based probes, which report the functional integrity of the active site in enzymes. The general designs of activity-based probes (ABPs) contain three parts (Fig. 2): (1) active site-targeting reactive group (*e.g.* warhead or **WH**) that directs the probe to the active site and causes covalent reaction with the protein; (2) a reporter unit, which is typically a fluorophore or biotin for the direct readout of the protein's activity, and (3) a linker/spacer, which minimizes the possible disruption from the reporter tag in the protein reactivity/recognition of the reactive unit, or in some cases serves as an additional recognition element. The early-stage developments of ABPs solely relied on known electrophilic

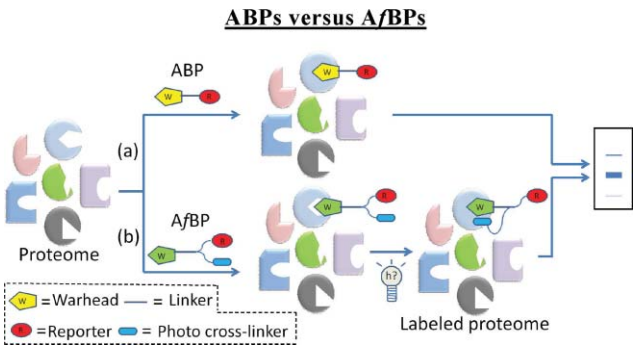

Fig. 2 Schematic of ABPP showing two different approaches using either ABPs (activity-based probes) or A*f*BPs (affinity-based probes). (a) In this approach, a crude proteome is treated with the activity-based probe which bears a reactive group or warhead (**W**, shown in yellow) and the reporter unit (**R**, shown in red). The probe covalently reacts with the active site of the target protein and the labeling is visualized by in-gel fluorescence scanning following separation of proteins on SDS-PAGE. (b) In this approach, the crude proteome is treated with an A*f*BP whose warhead (**W**, shown in green) first recognizes the target protein's active site *via* non-covalent interactions. Subsequently upon UV irradiation, a reactive intermediate generated from the photo-reactive unit (depicted as blue oval) covalently reacts with suitable residues near the active site. The labeled protein is separated by SDS-PAGE and visualized by in-gel fluorescent scanning.

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Shao Q. Yao joined NUS in 2001. He is currently the Dean's Chair Professor, and Associate Professor at the Departments of Chemistry and Biological Sciences at NUS. His current research interests are in Catalomics—the large-scale studies of enzymes at the organism level using chemical and biological tools. He has received numerous research awards, including the 2002 Young Investigator Award funded by the Biomedical Research Council (Singapore), the 2005 Young Researcher Award (NUS), the 2006 Mr and Mrs Sun Chan Memorial Award (International), the 2007 Outstanding Scientist Award at the Faculty of Science of NUS, and the 2008 ASAIHL-Scopus Young Scientist Award (International).

irreversible inhibitor scaffolds capable of forming stable covalent bonds with nucleophilic residues near the active site of the target proteins. This approach, although highly desirable, encounters significant challenges from proteins lacking such active site-targeting, covalently-binding scaffolds (*i.e.* suicide inhibitors). Alternatively, affinity-based probes (A*f*BPs),[11] based on well-documented photo-affinity reagents,[12] have been developed recently by several research groups.[13] These probes are normally made of conjugates of tight-binding reversible inhibitor scaffolds of target proteins and a photoreactive molecule (Fig. 2). When properly designed, they can also label target enzymes in an activity-dependent manner, therefore offering a powerful alternative strategy to the ABP approach.

In many cases, the A*f*BPs are used to profile enzymes normally inaccessible by ABPs. For example, the labs of Cravatt and Yao independently reported the first A*f*BPs for metalloproteases.[13c,14] Cravatt's lab designed a photoreactive probe equipped with a succinyl hydroxamate as the zinc-binding group, benzophenone as the photo cross-linker and a rhodamine dye as the reporter (**1**, Fig. 3). The metalloprotease probes from Yao *et al.* were equipped with a similar succinyl hydroxamate as the zinc-binding group, an aryl diazirine as the photo-reactive group and a Cy3 dye as the reporter unit (**2**, Fig. 3). By using a set of probes with varied P_1' amino acids, the authors demonstrated the utility of the probes to generate unique activity-based fingerprints of various metalloproteases.

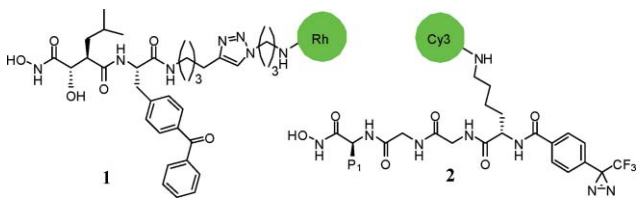

Fig. 3 Affiniy-based probes (A*f*BPs) against metalloproteases reported by the laboratories of Cravatt and Yao.

CC-facilitated synthesis of ABPs and A*f*BPs

In recent years, click chemistry has been adopted extensively in the field of ABPP, which has significantly expanded its utilities and potential applications. Some early designs of ABPs employed CC as a convenient reaction for introducing the linker unit (still in use). This is justified by both the high specificity of the reaction that does not require functional group protection/deprotection, and also the unique structural and hydrogen bonding features of the triazole ring, formed as the product of azide/alkyne ligation, which may facilitate certain protein-binding interactions.

Cravatt and co-workers utilized CC as a convenient linking method for synthesizing both rhodamine-, and rhodamine and biotin-tagged (trifunctional) probes from the natural product (–)-FR182877 (**3**, Fig. 4).[15] With the probes, the researchers observed selective labeling of carboxyesterase-1 (CE-1) from mouse heart proteomes. In a similar approach, Thompson and co-workers utilized CC to link a rhodamine tag to covalent inhibitors of protein arginine deiminase 4 (PAD4) based on fluoro and

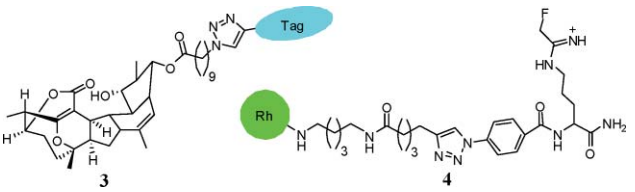

Fig. 4 CC-based ABPs. **3**: ABP of CE-1 derived from the natural product (–)-FR182877. **4**: ABP that targets the active form of PAD4.

chloroacetamidine moieties.[16] PAD4 is an enzyme implicated in rheumatoid arthritis, and the ABPs thus generated, in particular the fluoroacetimidine-based probe (**4**, Fig. 4), were shown to specifically target the calcium-bound, and hence the active form of the enzyme *in vitro*.

Probe libraries

The daunting task of functional profiling of an ever-expanding list of structurally and functionally poorly understood proteins more or less necessitates the use of probe libraries, rather than single probes. The library approach may facilitate isoform-selective labeling of well-characterized protein families as well. However, even designing a single multifunctional probe using chemical manipulations of structurally complex small molecules is often met with synthetic difficulties. Therefore, the very first consideration in designing a probe library is the possibility of conveniently utilizing chemoselective reactions that minimize synthetic challenges. For this purpose, CC-based combinatorial approaches for probe synthesis have been adopted, which essentially take advantage of the highly modular, chemoselective, and solvent-compatible nature of this reaction. Yao and co-workers employed CC to generate a library of hydroxamate-containing small molecule probes against matrix metalloproteases (MMPs) (**5**, Fig. 5).[13f] With these second-generation metalloprotease probes, the authors generated activity-based fingerprints of different MMPs and subsequently showed the feasibility of the method in discriminating highly homologous enzymes.

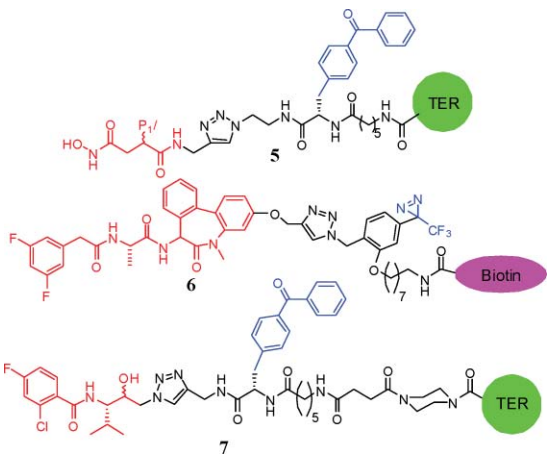

Fig. 5 CC-based A*f*BPs. General structure of metalloprotease probes (**5**). Representative chemical structure of dibenzoazepine inhibitor incorporated, biotinylated photo-reactive probes for γ-secretase protein (**6**). Chemical structure of a selective probe (**7**) for the detection of all four plasmepsins in the malarial parasite *Plasmodium falciparum*.

The laboratories of Fuwa and Tomita recently reported a CC-facilitated divergent synthesis of photoreactive probes of the enzyme γ-secretase (an important aspartic protease related to Alzheimer's disease) based on dipeptidic inhibitors.[17] A small library (5 members) of azides containing biotinylated photoreactive molecules was clicked to alkyne-incorporated benzodiazepine/dibenzoazepine inhibitor scaffolds (2 members) to generate a 10-member probe library (**6** as representative, Fig. 5). Subsequent photo-affinity labeling experiments using the probe library provided direct evidence that both signal peptide peptidase (SPP) and N-terminal fragments of the presenilin-1 subunit within the γ-secretase complex act as molecular targets of the two types of inhibitor scaffolds.

Yao and co-workers recently combined small molecule microarray (SMM) technology with CC to facilitate the high-throughput discovery of A*f*BPs against γ-secretase.[13g] The researchers first developed a solid-phase method for combinatorial synthesis of hydroxyethylene-based inhibitors of aspartic proteases (hydroxyethylenes are well-known transition state analogues of aspartic proteases). The 198-member inhibitor library consisted of two sub-libraries; a 123-member N-terminal library with varied aromatic and aliphatic moieties which could potentially interact with the S_1 and S_2 residues of the protease, and a 75-member C-terminal library, which could potentially interact with the S_1, S_1' and S_2' residues of the protease. The small molecule inhibitor microarray, upon screening against fluorescently labeled membrane fraction of cell lysate from γ-30 cell line (a γ-secretase overexpressing CHOK1 mammalian cell line) revealed distinctive inhibitor binding patterns with a very clear preference for the C-terminal sub-library by the protease. From the microarray result, the two strongest binders and a weak binder (as a negative control) were conveniently converted to A*f*BPs by click conjugation of the azido derivatives of these three inhibitors with a TER-benzophenone-alkyne reporter. The A*f*BPs thus generated were subsequently treated with the γ-30 cell lysate, and shown to selectively label the presenilin-N-terminal fragment (PS-NTF) of γ-secretase.

Elegant chemical proteomic strategies have recently been developed to study the pathogenic activities of the malaria parasite *Plasmodium falciparum*.[18] Along with cysteine proteases, the parasite growth requires aspartic proteases such as plasmepsins (PMs) for metabolizing human hemoglobin.[19] Yao and co-workers recently reported a CC-facilitated chemical strategy for the functional profiling of all four known plasmepsins (*i.e.* PM-I, PM-II, PM-IV and HAP) in the intraerythocytic malaria parasite.[13a] The researchers first developed a set of A*f*BPs, which were assembled *via* clicking hydoxyethyl warhead-containing azido precursors bearing varied P_1 and P_2 groups with a TER-benzophenone-alkyne reporter. The probes, upon UV-mediated labeling of the parasite proteome (obtained at different stages of the parasite development), showed highly specific labeling of all four known PMs. One of the various probes, compound **7** (Fig. 5), consistently gave the strongest labeling signal and thus was used for further studies. The researchers subsequently performed *in situ* inhibitor screening against the parasite proteome with a 152-member hydroxyethyl inhibitor library, synthesized *via* click assembly of the same azido precursors (of the probes) with 19 different aromatic alkynes, and identified a cell-permeable compound, **51** (Fig. 17), which showed good inhibition against all four PMs and parasite growth in infected red blood cells (RBCs) with minimum cytotoxicity (against mammalian cells).

Label-free clickable ABPs and A*f*BPs

The earlier generations of ABPs, due to the presence of their reporter tags, suffer obvious limitations both from the synthetic point of view and in their labeling performance. Reporter-labeled probes are difficult to deal with synthetically due to the poor solubility of most fluorophores and biotin. An even more serious drawback is their poor cell permeability in most cases, which limits their application to profiling only recombinant proteins or crude proteome extracts. Moreover, the bulky reporter unit in the probe in many cases disrupts the proper binding interaction between the reactive unit and the protein's active site, rendering the probe inactive.

To override these problems, the labs of Cravatt and Overkleeft independently reported the first "label-free" versions of ABPs, where a two-step protein-profiling strategy making use of a bioorthogonal labeling of the probe-treated proteome was employed (Fig. 6a).[20] Overkleeft and co-workers used an azide-functionalized vinyl sulfone probe to target proteasomes in cell lysates as well as in live cells.[20b] After the probe treatment, the lysates were subjected to Staudinger ligation with a biotinylated triarylphosphine reagent and the labeling was visualized by immunoblotting. The Cravatt lab, on the other hand, utilized CC for this purpose.[20a] In their original work, Cravatt and co-workers treated cell lysates from COS7 cells overexpressing glutathione S-transferases (GSTO 1-1) with an azide-derivatized phenyl sulfonate (PS) probe, **8** (Fig. 6b). Upon click conjugation with a rhodamine alkyne, the probe-treated sample was analyzed by SDS-PAGE and in-gel fluorescence scanning. In addition to the desired target GSTO 1-1, endogenously expressed levels of aldehyde dehydrogenase (ALDH-1) and enoyl CoA hydrolase (ECH-1) were detected. Furthermore, the probe was found to

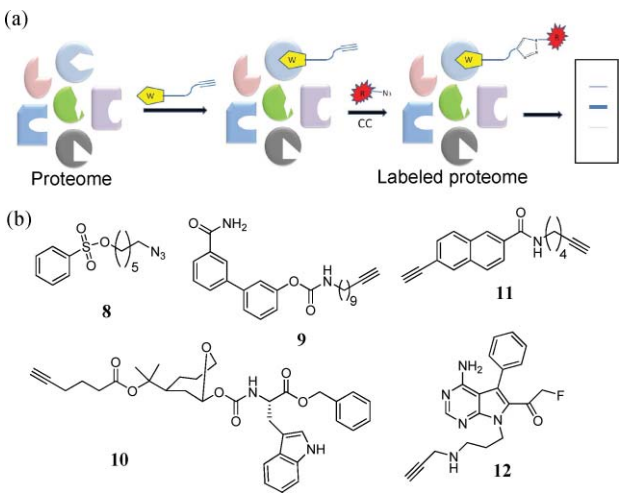

Fig. 6 (a) Schematic of ABPP using clickable activity-based probes. The proteome is treated with the clickable probe whose warhead **W** (yellow) covalently reacts with the active site of the target protein. The reporter **R** (shown in red) is then incorporated into the probe-protein complex using CC, which enables subsequent visualization of the labeled protein with in-gel fluorescent scanning. (b) Chemical structures of clickable ABPs. **8**: Phenylsulfonate-based clickable probe for GSTO 1-1. **9**: Probe JP104 that targets FAAH. **10**: A natural product-based probe, MJE3, for phosphoglycerate mutase 1. **11**: A representative example from the probe library of cytochrome P450s. **12**: RSK kinase probe fmk-pa.

detect GSTO 1-1 in MDA-MB-435 cells (a human breast cancer cell line) as well as in live animals. A high background labeling problem observed in this study was subsequently eliminated by switching the azide-alkyne reactive partners between the probe and the reporter.[21]

With the initial success, the clickable ABPP strategy has been quickly adopted for other classes of proteins. Alexander and Cravatt utilized an N-alkynyl derivative of a known tight binding inhibitor against fatty acid amide hydrolases (FAAHs), named JP104 (**9**, Fig. 6b), for the comprehensive evaluation of the *in vivo* proteome reactivity of these enzymes.[22] The FAAHs are a special class of serine hydrolases, belonging to the amidase signature family, which regulate several signaling lipids. With the probe, the researchers observed selective labeling of the animal brain FAAH, whereas multiple off-targets were observed in the liver and kidney fractions. In an effort to determine protein targets of bioactive natural products, Cravatt and co-workers performed cell-based screening with a small-molecule clickable probe library bearing different natural product scaffolds.[23] The researchers identified one probe, MJE3, (**10**, Fig. 6b), which possesses a 1-oxa-spiro[2,5]octane scaffold, showing significant inhibition of breast cancer cell proliferation. The probe was found to covalently label a glycolytic enzyme called phosphoglycerate mutase 1 (PGAM1) in cancer cells, pointing to the possibility of considering PGAM1 as a cancer therapeutic target.

Cravatt and co-workers recently reported a set of clickable probes for the enzymes Cytochrome P450s (**11** as a representative, Fig. 6b).[24] The Cytochrome P450s are a group of important membrane-bound monooxygenases that catalyze the metabolism of a large number of endogenous signaling molecules and drugs.[25] The researchers observed that their probes were able to detect both inhibition and activation of cytochrome P450s by different small molecules.

Clickable ABPP methods have been extended to various signaling enzymes including protein kinases (PKs). Taunton and co-workers employed a clickable probe, called fmk-pa (**12**, Fig. 6b), to study the autoactivation mechanisms of the RSK kinase.[26] The probe design was based on a selective irreversible inhibitor, called fmk, for the C-terminal kinase domain (CTD) of RSK1 and RSK2 kinases.[27] Initially, a biotinylated fmk was used to selectively detect the CTD of RSKs 1 and 2 in HEK293 cell lysates but the probe could not be used in intact cells due to its poor cell permeability. The group subsequently designed the clickable probe, *i.e.* **12**, which, upon treatment with RSK-expressing cells followed by CC with an azide-conjugated fluorescent dye, was found to label the CTDs of endogenous RSKs 1 and 2 with very high selectivity and sensitivity.

Similar to ABPs, clickable versions of A*f*BPs have also been developed (Fig. 7a). The first design of a clickable A*f*BP was from the Pieters group.[28] Based on the known binding interaction of β-galactoside-containing motifs to galectins (a medically relevant subfamily of lectins), the group designed a clickable photo-affinity probe (**13**, Fig. 7b), which was shown to bind purified galectins. The same group subsequently reported a divalent version of the original probe (**14**, Fig. 7b), which possesses improved detection limits and enables specific labeling of endogenous galectin 3 in human colon carcinoma lysate.[29] The same group also reported peptide-based clickable probes capable of detecting spiked galectin 3 in bacterial cell lysates (**15** as a representative, Fig. 7b).[30] Sieber

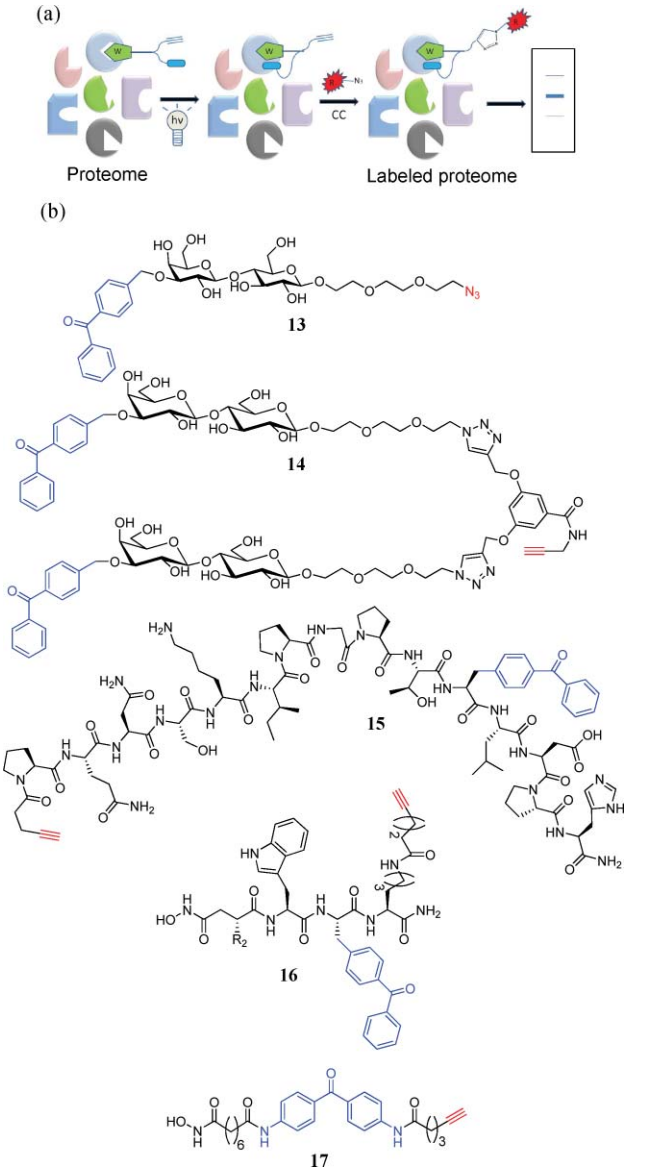

Fig. 7 (a) Schematic of A*f*BPP using clickable photo-reactive probes. The warhead, **W** (shown in green), first occupies the target protein's active site. Subsequent UV irradiation leads to covalent cross-linking of the probe with the target protein. The reporter is then incorporated into the probe–protein complex using CC and the labeled proteins are visualized by in-gel fluorescent scanning. (b) Chemical structures of various clickable A*f*BPs. **13**: Monovalent probe for galectins. **14**: Multivalent probe for galectins. **15**: A representative example of peptide-based clickable probe for galectin 3. **16**: A representative example from the clickable MMP probe library with R_2 representing different amino acids. **17**: Selective HDAC probe, SAHA-BPyne.

et al. adopted the same strategy for targeting metalloproteases using hydroxamate-containing peptide-based probes.[31] A small library of probes (**16** as a representative, Fig. 7b) was synthesized using CC, and used as cocktails in administration to mouse tissues and human cancer cell lines. The researchers identified more than 20 metalloproteases as potential targets of the probes. Further profiling of these enzymes with the probes showed significantly elevated activities of some of these proteins in certain invasive

tumor cells, suggesting the possible involvement of these proteins in cancer pathogenesis. They also observed that the clickable probes have greater sensitivity in labeling exogenous matrix metalloproteases when compared with their rhodamine-tagged counterparts. In a separate study of parasitology of *Chlamydia trachomatis* bacteria using the same set of probes, it was found that certain metalloprotease inhibitors could suppress the chlamydial growth by targeting the bacterial peptide deformylase, pointing to the potential development of therapeutic agents for the treatment of chlamydial disease.[32]

In another recent development, Cravatt's laboratory reported a very sensitive clickable photoreactive probe, SAHA-BPyne, **17** (Fig. 7b), for detection of histone deacetylases (HDACs).[13b] The probe design was based on a known general reversible inhibitor of HDAC called suberoylanilide hydroxamic acid (SAHA). The SAHA-BPyne was found to detect endogenous class-1 and class-2 HDACs as well as some of the HDAC-interacting proteins in the close proximity of the probe in highly complex whole cell proteomes and live cells. The same group subsequently reported the synthesis of a set of SAHA-BPyne analogues. While some analogs were found to have greater HDAC inhibition potency, the original SAHA-BPyne turned out to be the best probe in terms of labeling performance.[33]

Click chemistry in the study of posttranslational modifications (PTMs)

Posttranslational modifications (PTMs) incorporate a wide range of diverse chemical moieties such as phosphate, sugars, lipids, alkyl, acyl and sulfates into proteins. These protein-modifying chemical processes are carried out by different classes of enzymes. One of the key motivations in catalomics is to systematically characterize these very important enzyme–substrate interactions.[9] Of the various PTMs, protein glycosylation controls important cellular mechanisms related to cell–cell communication. It plays important roles in immune responses, inflammation and cancer metastasis. Although about 50% of eukaryotic proteins are estimated to be glycosylated,[34] the study of glycosylation in the cellular environment is traditionally considered highly challenging, due to the presence of several diverse classes of sugars in the body as well as the lack of selective *in vivo* glycan-labeling and visualization strategies.

To tackle this difficult problem of visualizing glycosylation at the systems level, elegant labeling strategies based on certain bioorthogonal reactions such as Staudinger ligation,[35] ketone–hydrazide,[36] ketone–aminooxy[37] and Michael addition,[38] with chemically modify sugars, have been developed. Among these methods, the Staudinger ligation, which uses the exquisite bioorthogonality of the azide functional group, has been extensively exploited.[3] Staudinger ligation, although highly biocompatible, has an intrinsic drawback of slow reaction rate, and the phosphine (the reactive partner of the azide) might undergo oxidation catalyzed by air or metabolic enzymes present in the living system. The solubility property of phosphine in aqueous media is also problematic and more importantly the reaction is typically not sensitive enough for the detection of low-abundance species in biological systems. The discovery of Cu(I)-catalyzed click reaction between azides and terminal alkynes, which proceeds more than 20 times faster than Staudinger ligation has fuelled the glycan-labeling efforts in recent years.

The basis of metabolic incorporation using unnatural sugar analogues relies on the finding that certain metabolic pathways were shown to tolerate minimally modified sugar precursors (Fig. 8). This was originally reported by Reutter and co-workers, who successfully demonstrated the incorporation of chemically synthesized N-propanoyl-D-mannosamine into membrane and serum glycoproteins.[39] Bertozzi and co-workers adopted this approach with a ketone-modified N-acetyl-mannosamine.[37] They showed the unnatural sugar was efficiently taken up by mammalian biosynthetic pathways and incorporated into sialic acid which was eventually incorporated into glycoproteins. The unnatural sugar-bearing glycoconjugates on the cell surface were subsequently labeled by a biotin-derivatized hyrazide. Bertozzi's group further refined this strategy by utilizing the azide-modified N-acetyl-mannosamine, and selective labeling of the azide-modified cell-surface glycoconjugates were observed with a modified Staudinger ligation using a biotinylated triarylphosphine.[40]

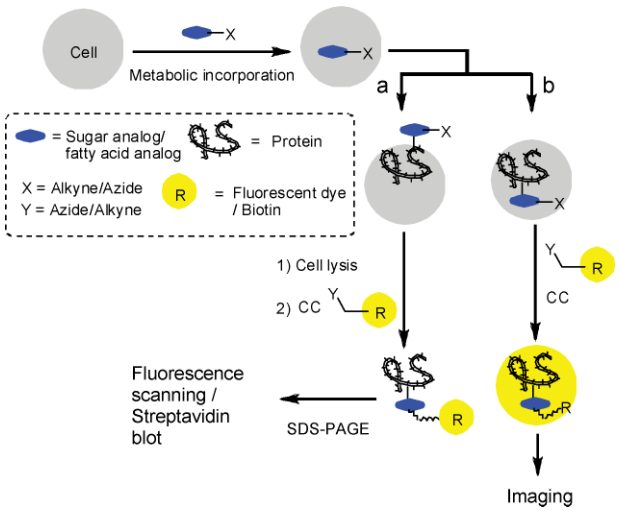

Fig. 8 Schematic of metabolic incorporation of sugars or fatty-acid analogues followed by labeling using CC. (a) Cell-surface incorporation (b) Intracellular incorporation.

With the development of CC as a superior bioorthogonal labeling strategy, Bertozzi's group recently reported a metabolic labeling strategy to probe protein fucosylation.[41] Compound **18**, a fucose analogue with an azide at position 6 (Fig. 9) was incorporated into cell surface glycans utilizing the fucose salvage biosynthetic pathway. The cell lysates were then subjected to CC with a biotin–alkyne, which then selectively "fished out" azide-labeled glycans. In the same year, Wong and co-workers reported a click-activated fluorescent probe for fucosylated glycoconjugates.[42] The researchers used the same fucose analogue with an azide at position 6 to incorporate the unnatural fucose into glycoproteins. Subsequent visualization of the glycoconjugates was achieved by treating the cells, under click conditions, with a latent fluorophore, 4-ethynyl-*N*-ethyl-1,8-naphthalimide, which becomes fluorescent upon conversion of the alkyne to triazole. Such fluorogenic click reactions represent another useful application of CC.[43] The fluorogenic click-based detection has the advantage that only the click product remains intensely fluorescent, whereas the unreacted

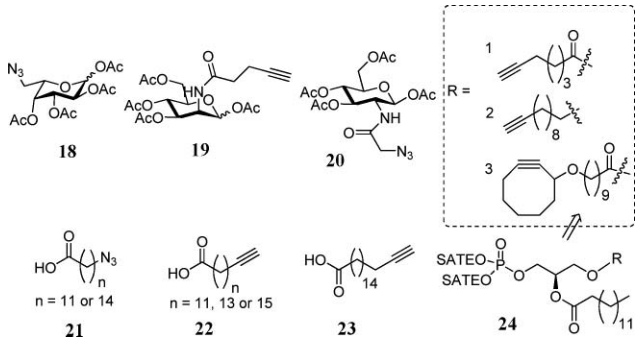

Fig. 9 Chemical structures of some of the metabolically incorporated molecules probed by CC. **18**: Fucose-analogue with azide at position 6 used for identifying fucosylated proteins. **19**: Alkynyl N-acetyl mannosamine that was shown to get incorporated into biosynthesis of sialic acid. **20**: Azide-derivatized peracetylated N-acetylglucosamine used in the metabolic labeling of O-GlcNAc modification of proteins. **21** and **22**: Fatty-acid chemical probes used for metabolic labeling of fatty-acylated proteins in mammalian cells. **23**: 17-octadecynoic acid used as a clickable probe for *in situ* labeling and identification of several s-palmitoylated proteins in human cells. **24**: Metabolically incorporated alkyne-modified phosphatidic acid analogues used for visualizing lipid bilayers.

reagents remain undetected with very minimum background noise, enabling the sensitive visualization of intracellular localization of fucosylated glycoproteins with fluorescence microscopy. Wong's group further extended this strategy for probing sialic acid-containing glycoproteins, which are overexpressed in many tumor cells. The researchers synthesized an alkyne-derivatized *N*-acetyl mannosamine (**19**, Fig. 9), which was shown to be incorporated into the sialic acid biosynthetic pathway, and the alkyne-bearing sialyl glycans were subsequently detected both on the cell surface and in fixed-cell intracellular environments using click conjugation with a biotin–azide or a fluorogenic coumarin azide.[44]

A more detailed investigation of sialyl glycans *via* metabolic labeling and CC with biotin–azide, followed by enrichment of labeled proteins with streptavidin beads and site-selective proteolysis with Peptide N-Glycosidase F, revealed the sites of sialic acid modifications of glycoproteins from cellular lysates of prostate cancer (PC3) cells.[45] Wu, Bertozzi and co-workers recently utilized the alkynyl *N*-acetyl mannosamine, originally developed by Wong's group, to perform *in vivo* labeling experiments with live animals, demonstrating the potential of the combined metabolic labeling and click conjugation at systems-level experiments.[46] Lemoine and co-workers synthesized azide and alkyne-derivatized peracetylated *N*-acetylglucosamine (**20**, Fig. 9) for the metabolic labeling of O-linked β-*N*-acetylglucosamine (*O*-GlcNAc) modification of proteins,[47] another important glycosylation event that plays regulatory roles in the activity of many nuclear and cytoplasmic proteins. The researchers observed that only the azido analogue was accepted by the mammalian cells (MCF-7). Upon click conjugation with biotin–alkyne, conjugated proteins in the cell lysate were enriched by streptavidin beads and identified by nano-HPLC-tandem-MS. A total of 32 *O*-GlcNAc incorporated proteins were identified, of which 14 had not been reported previously.

Azide-modified sugar analogues and CC have also been used for the investigation of glycosylated proteins without metabolic incorporation as well. For instance, using CC as a superior labeling method, Hsieh-Wilson and co-workers demonstrated the labeling of several glycosylated proteins (with O-linked β-*N*-acetylglucosamine) from mammalian cell lysates using an UDP-azidogalactose substrate and an engineered β-1,4-galactosyltransferase enzyme (Y289T GalT).[48]

In recent years, the combined approach of metabolic labeling and CC has been adopted by various research groups in the study of other types of PTMs which are otherwise difficult to investigate. Protein fatty-acylation is an important event known to regulate a large array of cellular processes, ranging from trafficking and localization of proteins to cell growth and differentiation.[49] It is traditionally detected by autoradiography following metabolic incorporation of radiolabeled fatty-acid analogues.[50] An acyl–biotin exchange strategy has been developed as a useful non-radioactive approach towards identifying S-palmitoylated proteins.[51] Ploegh and co-workers recently reported a Staudinger ligation-based detection of both N-myristoylated and S-palmitoylated proteins in mammalian cells using metabolically incorporated azido analogues of fatty-acids and a biotinylated triaryl phosphine reagent.[52] After this report, the Staudinger ligation has been successfully employed by various research groups for the detection of different fatty-acylated proteins.[53] More recently, Hang and co-workers reported the metabolic labeling of fatty-acylated proteins in mammalian cells using azide- or alkyne-modified fatty acid chemical probes (**21** and **22**, Fig. 9), followed by CC with a fluorophore-containing alkyne or azide, respectively.[54] Comparative analysis of the unnatural fatty acid-modified cell lysates with Staudinger ligation and click ligation revealed significant improvement in the detection with the click strategy, which allowed the researchers to efficiently detect endogenously expressed levels of fatty-acylated proteins using in-gel fluorescence scanning, fluorescence microscopy and flow cytometry. Cravatt and co-workers recently utilized the commercially available compound 17-octadecynoic acid (**23**, Fig. 9) as a click-based probe for *in situ* labeling and identification of several previously unknown s-palmitoylated proteins in human cells.[55]

Schultz and Neef recently reported the metabolic incorporation of alkyne-modified phosphatidic acids into mammalian cells.[56] The terminal alkyne-modified phospholipids (**24**, Fig. 9) were visualized using CC with an azidocoumarin dye, whereas the cyclooctyne-incorporated phospholipids enabled visualization of lipid bilayer dynamics using the strain-promoted cycloaddition.

One of the inherent drawbacks of the Cu(I)-catalyzed CC that limits its application for visualizing dynamic processes in living systems is the toxicity of the copper catalyst. In order to overcome this limitation, Bertozzi's group developed a metal catalyst-free, strain-promoted cycloaddition reaction between a cyclooctyne and azides (Fig. 10a; compound **25** in Fig. 10b).[57] The intrinsic bond angle stain of the cyclooctyne and the corresponding relative destabilization of the ground state compared to the transition state make it highly vulnerable to react with azides that otherwise require activation *via* a Cu(I) catalyst. With a cyclooctyne bearing a biotin, the researchers demonstrated cell surface labeling of metabolically incorporated azide tags on glycoproteins of CHO cells. Live cell labeling of Jurkat cells with metabolically incorporated azido sugars showed that the strategy could be applied without affecting the cell viability. Since the original report, other cyclooctyne analogs have been developed (Fig. 10, **26–32**).[58–62] For example, Bertozzi's group recently improved their original design

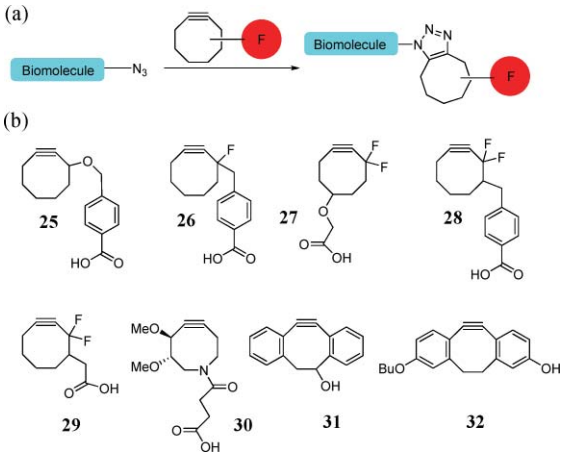

Fig. 10 Copper-free CC using cyclic alkynes. (a) Azide-incorporated biomolecules undergo strain-promoted, [2 + 3] cycloaddition reaction with cyclooctynes leading to efficient "copper-free" biomolecular labeling. (b) Examples of different cyclic alkynes. **25**: ref. 57; **26**: ref. 59; **27**, **28**, **29**: ref. 58; **30**: ref. 60; **31**: ref. 61; **32**: ref. 62

of the cyclic alkyne with mono and difluorinated cyclooctynes (**26-29**) where the electron-withdrawing fluorine moiety enhances the rate of cycloaddition with azides.[58] With these new substituted cyclooctynes, the authors were able to successfully study the dynamics of glycan trafficking in live cells and identified a group of sialoglycoconjugates with fast internalization kinetics.[58–60]

Boons and co-workers recently developed a new reagent, dibenzocyclooctyne-ol (**31**), for the copper-free CC.[61] This compound was reported to have the advantage of nontoxicity and fast reaction kinetics, which enabled the researchers to visualize metabolically labeled glycoconjugates in living cells. Boons, Popik and co-workers subsequently reported a photo-triggering strategy for the *in situ* generation of the otherwise masked alkyne (dibenzocyclooctyne) functionality (**32**) from the corresponding cyclopropenone.[62] This photo-unmasking strategy may be useful for temporally and spatially controlled labeling experiments in living organisms. Thus, the strain-promoted cycloaddition is providing chemical biologists with unprecedented opportunities for dynamic imaging of highly complex biological events in living systems.

Click chemistry in the developments of enzyme inhibitors

Conventional drug development efforts typically rely on medicinal chemistry both at the very early stage of *lead finding* and at the next stage of *lead optimization*. High-throughput synthetic methods could potentially expedite these processes. Many enzymes are known to possess multiple binding pockets, yet conventional inhibitor developments generally focus more on only the active site. However, in many cases the secondary/allosteric binding sites confer selectivity as well as potency. Within this context, click chemistry, due to its highly modular and efficient reaction nature, has been identified as one of the most practical methods towards fragment-based inhibitor assembly where N + M combinations of inhibitor fragments lead to the generation of N × M potential bidentate inhibitors (Fig. 11). Because of the efficiency and water-compatible nature of click reaction, in most cases, the assembled

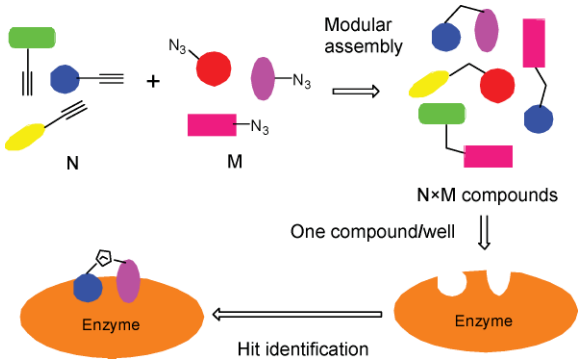

Fig. 11 CC facilitates a highly modular and rapid assembly of potential bidentate inhibitors (N × M compounds) from N number of alkynes and M number of azides. This high-throughput inhibitor assembly can be performed in a 96- or 384-well plate (one compound per well) and the inhibitors in each well can be directly screened with the enzyme (*in situ* screening) without the need to purify the compounds.

products could be directly screened for inhibition without the need of any purification.[63]

Protein tyrosine phosphatases (PTPs)

Protein tyrosine phosphatases (PTPs) are signaling enzymes responsible for the dephosphorylation reaction of tyrosine-phosphorylated proteins. The discovery of an allosteric site in PTP1B by Zhang's group[64] prompted Yao and co-workers to evaluate the possibility of designing bidentate inhibitors of this important enzyme. The researchers used CC to construct a 66-member inhibitor library derived from five different alkynes (which were designed from known PTP core-binding scaffolds) and 13 different azides (which are potential allosteric-site binders).[65] Subsequent enzymatic screening of the library with different PTPs and non-PTP proteins revealed compound **33**, a selective and moderately potent cell-permeable inhibitor of PTP1B (Fig. 12).

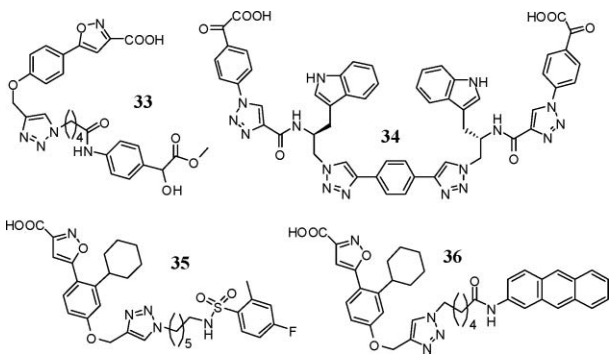

Fig. 12 Chemical structures of PTP inhibitors generated *via* CC. **33**: A selective PTP1B inhibitor (IC_{50} = 4.7 μM against PTP1B). **34**: A potent inhibitor identified from two-stage, click-based library of PTPs (IC_{50} = 0.55 μM against Yersinia PTP and IC_{50} = 0.71 μM against TC-PTP). **35**: Compound F1S-6C-W11, a potent inhibitor of MptpB (K_i = 150 nM). **36**: Compound H1-6C-W11, a potent inhibitor of MptpB (K_i = 170 nM).

In a similar approach, Xie and Seto employed CC to generate sequential libraries of PTP inhibitors.[66] In the first stage of the library synthesis, the researchers clicked an azide-functionalized α-ketocarboxylic acid (a known phosphate mimic) with 56 different

alkynes. The hit identified from this first library (which was 9 times more potent than the parent warhead) was further modified to incorporate an azide functionality and used for the second step click conjugation with the library of 56 alkynes. Subsequent inhibitor screening revealed a potent inhibitor, **34**, with IC_{50} values of 550 nM against *Yersinia* PTP and 710 nM against TC-PTP (Fig. 12).

Yao and co-workers recently reported a solid-phase reaction strategy for high-throughput synthesis of azide libraries.[67] With this new and more efficient way to synthesize azide libraries, large "click" inhibitor libraries are more easily accessible. As a proof of concept, the researchers first synthesized a 325-member PTP inhibitor library by clicking the azide library with an alkyne-modified isoxazole warhead. The potential of such a novel combination of high-throughput amenable chemistries has been further demonstrated in a recent report from the same lab, where the researchers synthesized a ~3500-member click library of potential bidentate inhibitors against PTPs using the azide library and several different alkyne warheads.[68] Subsequent high-throughput inhibition assays led to the discovery of highly potent and selective inhibitors of *Mycobacterium tuberculosis* tyrosine phosphatase (MptpB), some of which represent the most potent MptpB inhibitors identified to date (Fig. 12, compounds **35** and **36**).

Protein kinases (PKs)

Protein kinases catalyze the phosphorylation of serine, threonine, tyrosine and histidine residues in proteins. Aberrant kinase expression and activities have been implicated in many diseases including several cancers.[69] Hence, a significant portion of drug discovery efforts have protein kinases as primary targets. Yao and co-workers recently employed CC to generate inhibitors of Abelson (Abl) tyrosine kinase.[70] The researchers first generated a 344-member generic kinase inhibitor library by clicking an ADP–alkyne (a promiscuous ATP-site binding scaffold) with a 344-member library of azides. Subsequent inhibition assays revealed a clear preference for short-chain azide scaffolds by the Abl kinase. This led to the subsequent design and synthesis of a 90-member click-inhibitor library directed towards Abl kinase using short-chain azides and two different alkyne warheads derived from the core structure of the Abl kinase selective drug Imatinib, **37** (an FDA-approved drug for the treatment of chronic myelogenous leukemia or CML). From the inhibitor screening, the researchers identified 11 hits (compound **38**, the most potent hit identified in the screening is shown in Fig. 13) with moderate potency and 10 to 20 folds in selectivity for Abl compared to the **41** closely related kinase c-Src. In a similar approach Grøtli and co-workers employed CC to generate *Plasmodium falciparum* protein kinase 7 (PfPK7) inhibitors.[71] The researchers used alkyne/azide-derivatized purine analogues to click to different aromatic azides/alkynes and subsequent inhibition assays of the click-products revealed two moderately potent inhibitors, **39** and **40** of PfPK7 (Fig. 13).

Fucosyltransferases

Fucosyltransferases catalyze the transfer of an L-fucose moiety from GDP–fucose to proteins, lipids or carbohydrates. Fucosylation is the final step in the biosynthesis of important carbohydrates such as sialyl Lewis^x and sialyl Lewis^a. Cell-surface glycoproteins and glycolipids bearing these carbohydrate epitopes are responsible for vital cell–cell recognition events such as immune responses, embryogenesis, fertilization, lymphocyte trafficking and cancer metastasis.[72] Potent and selective inhibitors of these enzymes would prevent the biosynthesis of the fucosylated products and may provide a viable way to treat associated diseases. Wong and co-workers, using a GDP-alkyne warhead and 85-member azide library, generated a "click" inhibitor library targeting fucosyltransferases.[73] The enzyme inhibition assays revealed nanomolar inhibitors of which one compound (**41**, Fig. 14) was identified to be the most potent and selective inhibitor of human α-1,3- fucosyltransferase VI.

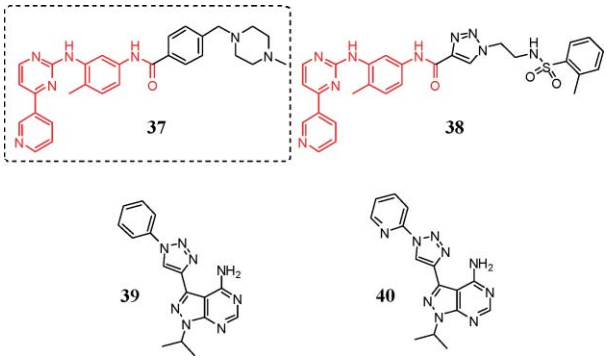

Fig. 13 Chemical structures of protein kinase inhibitors. **37**: Imatinib (a selective and potent inhibitor of Abl, IC_{50} = 800 nM). **38**: A potent and selective click-inhibitor (W1-SA20) of Abl kinase identified from the click-inhibitor library (IC_{50} = 700 nM). The Imatinib core structure is highlighted in red. **39** and **40**: Click-inhibitors of PfPK7 with IC_{50} values of 20 and 10 μM respectively.

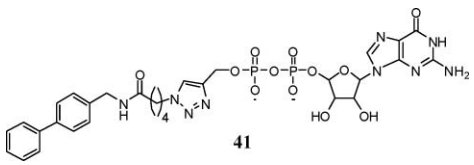

Fig. 14 Chemical structure of the most potent inhibitor against human α-1,3- fucosyltransferase VI (K_i = 62 nM).

Metalloproteases (MPs)

Metalloproteases (MPs) are proteolytic enzymes whose catalytic mechanisms involve the use of metal ions. Most MPs are Zn^{2+}-dependent enzymes. Of the different MPs, matrix metalloproteases (MMPs) are known to play key roles in the degradation of extracellular matrix and are tightly regulated by a number of endogenous inhibitors. Even minute imbalance between their activation and inhibition mechanisms could lead to several pathological states including cancer.[74] Structure–activity-relationship (SAR) studies with peptide-based right-hand side MMP inhibitors have revealed that the P_1' and P_4' positions of the inhibitors are the most critical sites responsible for potency and selectivity.[75] Based on these findings, Yao and co-workers designed the first "click" MMP inhibitor library.[76] The authors first synthesized two libraries; an 8-member alkyne library with a succinyl hydroxamate (a potent

Zn-binding group widely used in MP inhibitor designs) warhead and varied substituents for the P_1' position, and a 12-member azide library with varied alkyl chain lengths for the P_4' position. The two fragment libraries were then clicked together, in a modular way, to generate the inhibitor library, which upon subsequent screening revealed **42** as a selective and potent inhibitor of MMP-7 over other metalloproteases tested (Fig. 13a).

Based on the finding that certain rhodanine-based molecules act as selective nanomolar inhibitors of Anthrax Lethal Factor (ALF),[77] Yao and co-workers recently reported a small molecule MMP "click" inhibitor library equipped with rhodanine as the zinc-binding scaffold.[78] The inhibition assays revealed moderately potent inhibitors against MMP-7 and MMP-13 over other MMPs (**43**, Fig. 15).

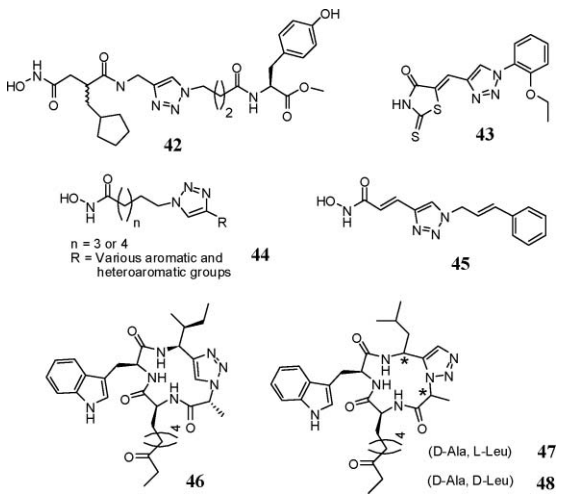

Fig. 15 Metalloprotease inhibitors. **42**: MMP inhibitor generated using the click-library approach with IC_{50} = 6.5 μM against MMP-7. **43**: A small-molecule based MMP inhibitor generated using CC with IC_{50} = 24 μM against MMP-7 and 36 μM against MMP-13. **44**: SAHA-like HDAC inhibitors. **45**: HDAC-1 inhibitor generated using CC with IC_{50} = 104 nM. **46**, **47** and **48**: Triazole analogues of a naturally occurring cyclic tetrapeptide inhibitor of HDACs with IC_{50} values of HDAC1 inhibition as 25, 7 and 75 nM, respectively.

Inhibitors of histone deacetylases (HDACs) have recently been identified as effective chemotherapy agents. Oyelere and co-workers recently synthesized a small library of SAHA-like hydroxamates where the amide bond in SAHA was replaced with a triazole ring prepared *via* CC.[79] Both the linker chain length and the aromatic ring were varied. Inhibition assays revealed several HDAC inhibitors (**44**, Fig. 15) with improved potency compared to SAHA. In a similar approach, Wang and co-workers reported the synthesis of a 14-member library of HDAC inhibitors using CC.[80] Inhibition assays revealed one compound **45** (Fig. 15), with improved inhibition of HDAC1 as compared to SAHA. The compound also exhibited potent antiproliferative activity against several cancer cell lines.

Ghadiri and co-workers recently reported the synthesis of cyclic pseudotetrapeptides containing 1,4 or 1,5-disubstituted 1,2,3-triazoles analogues to a naturally occurring cyclic tetrapeptide inhibitor of HDACs called apicidin.[81] As the 1,4 and 1,5-substituted triazoles act as surrogates for *trans* and *cis* amide bonds respectively, the resultant molecules (**46**, **47** and **48** Fig. 15) provide useful for identifying the most bioactive conformation (*cis-trans-trans-trans*) of the original cyclic-tetrapeptide inhibitor of HDAC.

Caspases

Caspases are a class of cysteine proteases which specifically cleave after the aspartic acid residue of protein substrates. As they are the enzymes responsible for apoptotic mode of cell death, inhibitors of caspases may have the potential to be developed into therapeutic agents for both acute cellular degenerative diseases as well as chronic neurodegenerative diseases.[82] Reversible (*e.g.* aldehyde) and irreversible (*e.g.* vinyl sulfones) inhibitors based on electrophilic reactive units targeting the catalytic cysteine residue of this class of proteins have been developed.[83] Most peptide-based caspase inhibitors contain a tetrapeptide sequence, P_4–P_3–P_2–Asp, coupled to a suitable warhead where the Asp at P_1 position and the residue at P_4 position play critical roles in enzyme/substrate recognition. Yao and co-workers developed an efficient strategy for high-throughput synthesis of caspase inhibitors containing aldehyde and vinyl sulfone warheads.[84] A total of 198 caspase inhibitors were assembled by using CC and subsequent enzymatic screening with caspase-3 and -7 led to the identification of a moderately potent reversible inhibitor, **49**, and an irreversible vinyl slufone-based inhibitor, **50**, for caspases 3 and 7 (Fig. 16), respectively.

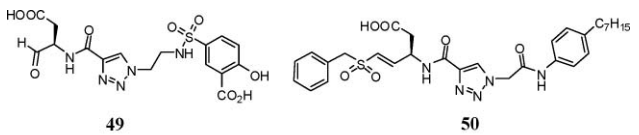

Fig. 16 Caspase inhibitors. **49**: Aldehyde-based reversible inhibitor with IC_{50} values of 4.67 and 7.70 μM against caspases 3 and 7, respectively. **50**: Vinyl sulfone-based irreversible inhibitor with IC_{50} value of 5 μM against caspase 7.

Aspartic proteases

Aspartic proteases, although the smallest protease class with only about 15 members encoded by the human genome, have proven to be a rich source of drug targets for the pharmaceutical industry. For instance, human immunodeficiency virus-1 (HIV-1) protease is one of the main targets in the treatment of AIDS;[85] plasmepsins in malaria,[86] β and γ-secretase in Alzheimer disease,[87] cathepsin D in breast cancer metastasis[88] and rennin in hypertension,[89] all are aspartic proteases.

Elegant strategies for the synthesis of inhibitor libraries of γ-secretase and plasmepsins facilitated by CC have been developed and are described in previous sections of this review along with discussions on A*f*BPs.[13g,17,19] In particular, Yao and co-workers recently reported a click assembly of azido precursors of A*f*BPs of plasmepsins in malarial parasites with a library of aromatic azides which led to the discovery of a compound **G16** (**51**, Fig. 17), that showed good inhibition against all four PMs and parasite growth in infected RBCs with good membrane permeability and minimum cytotoxicity; a discovery that could provide an impetus in malarial drug developments.

Wong, Sharpless, Fokin and co-workers employed CC for the first time to generate inhibitors of HIV-1 protease.[90] The

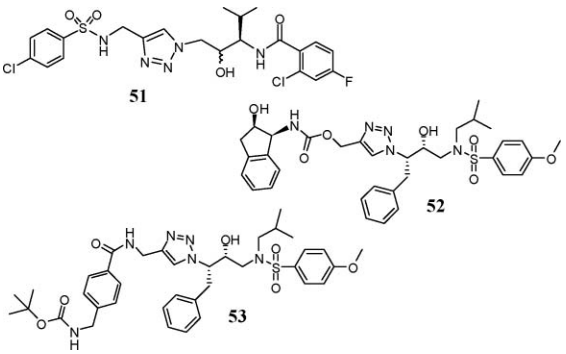

Fig. 17 Chemical structures of aspartic protease inhibitors. **51**: Compound **G16**, a potent inhibitor of Plasmepsins in the malaria parasite *Plasmodium falciparum with* EC_{50} of 1.04 μM. **52** and **53**: HIV-1 protease inhibitors with K_i values of 1.7 and 4.5 nM, respectively.

researchers first synthesized two azides equipped with hydroxyethyl transition state analogues of aspartic proteases and subsequently CC with a library of alkynes. Subsequent *in situ* screening in a microplate format revealed two potent inhibitors, **52** and **53**, of HIV-1 protease (Fig. 17).

In situ click chemistry

Ingenious methods such as molecular imprinting techniques[91] and dynamic combinatorial chemistry (DCC),[92] both based on proteins acting as a template for the assembly of molecules, have been evolved to identify tight binding partners of proteins. In a similar approach, based on Mock's finding that the very high kinetic barrier in the 1,3-dipolar cycloaddition reaction between azides and alkynes could be tremendously reduced when the two components are brought together into close proximity in a Cucurbituril (a nonadecacyclic cage compound) host structure,[93] Sharpless and co-workers designed *in situ* click chemistry (Fig. 18) as a powerful strategy towards kinetically controlled (unlike the thermodynamic control in conventional DCC), target-guided synthesis of enzyme inhibitors. In this approach, the non-protein host in Mock's original designs were replaced with the active site of an enzyme where the otherwise slow cycloaddition reaction gets heavily accelerated.

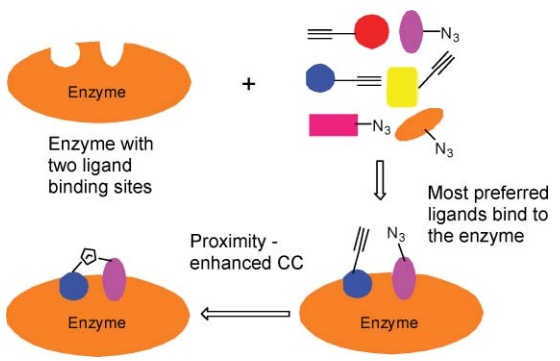

Fig. 18 Schematic of *in situ* CC. In the *in situ* CC, the enzyme is incubated with a mixture of alkyne- and azide-equipped ligand scaffolds from which the enzyme chooses the most preferred binders under the kinetic control. In the enzyme active site, the two components react to form the triazole linkage and hence a stable bidentate inhibitor due to proximity-enhanced CC.

Acetylcholine esterase (AChE)

AChE catalyzes the hydrolysis of neurotransmitter acetylcholine at the synaptic cleft, facilitating nerve impulse transmission across the synaptic gap. Sharpless and co-workers applied *in situ* CC, for the first time, *via* mixing 49 building block combinations of tacrine molecules equipped with alkyl azides and phenanthridinium molecules equipped with alkyl acetylenes (both were known inhibitor scaffolds of acetylcholine esterase) in the presence of the enzyme.[94] The researchers found that only one product, *syn*-TZ2PA6, which is a femtomolar inhibitor, was chosen by the enzyme. Kolb and co-workers further optimized this approach with the help of liquid chromatography-mass spectrometry in selected ion mode (LC-MS-SIM).[95] The same group, in a subsequent report,[96] employed a tacrine azide as an anchor molecule at concentrations sufficient to saturate the active site and this enzyme-inhibitor complex was found to recruit the most preferred peripheral binding group from a mixture of alkynes, leading to the identification of two compounds, TZ2PIQ-A5 (**54**) and TZ2PIQ-A6 (**55**) (Fig. 19), which are the most potent non-covalent inhibitors of AChE known to date.

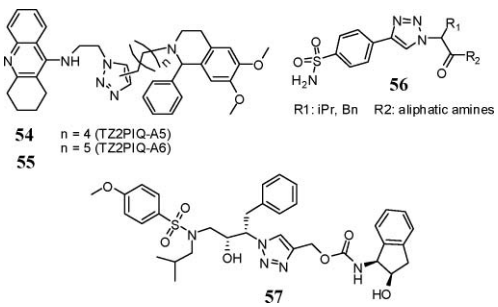

Fig. 19 Chemical structures of *in situ* click inhibitors against different enzymes: **54** and **55**: *In situ* click-hits of AChE formed from tacrine azide (TZ2) and alkyne incorporated phenyltetrahydroisoquinolines. **56**: The most potent *in situ* hits identified against CA II. **57**: Chemical structure of *in situ* hit identified against HIV-1 protease.

Carbonic anhydrase II

Carbonic anhydrases (CA) are metalloenzymes that catalyze the interconversion of CO_2 and HCO_3^-. They play critical roles in respiration, transport of CO_2 and protons, pH balancing of blood and other tissues, and many more.[97] Most CAs are zinc-dependant enzymes and the majority of their inhibitors are based on zinc-coordinating aromatic or heteroaromatic sulfonamides scaffolds. With saturating concentrations of acetylenic benzenesulfonamides as the core binding scaffolds and a mixture of 24 different azides, Kolb and co-workers performed *in situ* click chemistry on Bovine CA II (bCA II).[98] Analysis of the crude reaction mixture with LC-MS-SIM revealed that 12 out of the 24 reagent combinations led to triazole formation (**56**, Fig. 19) and all the observed triazoles were of an *anti*-regiochemistry, which is a consequence of the inhibitor selection by the enzyme active site.

HIV-1 protease

HIV-1 protease is an aspartic protease that plays a crucial role in the viral replication in AIDS, and hence has been identified

as one of the drug targets of this disease.[99] Although several potent inhibitors of HIV-1 protease have been developed (some of them are FDA-approved drugs), rapid viral replication and high error rates of HIV reverse transcriptase causes mutations that confer drug resistance at an alarming rate. In line with the *in situ* click inhibitor developments of AChE and CA II, Whiting *et al.* employed *in situ* click chemistry to generate potent HIV-1 protease inhibitors.[100] An azide with a moderate inhibition potency (IC_{50} ~ 4 μM) and five different alkynes with very low affinities (IC_{50} > 100 μM) were incubated with the protease and the crude reaction mixture was analyzed by LC-MS-SIM. The researchers identified the formation of only one triazole product, compound **57**, with *anti*-regioselectivity, in the presence of the enzyme (Fig. 19). Subsequent inhibition assays revealed that the compound possess good inhibition potency (IC_{50} = 6 nM) against the HIV-1 protease.

Conclusions

Several chemical and technological advancements in the very recent past have radically improved today's research fields in chemical biology and molecular biology. From the chemistry side, it is a set of bioorthogonal reactions that these developments mostly owe to. Of the different bioorthogonal reactions, the "click" reaction between azides and terminal alkynes, due to its exquisite chemoselectivity, sensitivity and bioorthogonality, has paved the way for great developments in catalomics in recent years. ABPP is one area that has heavily benefited from click chemistry. On one side, due to the modular and efficient nature of click reaction, the synthesis of probe libraries becomes greatly simplified and on the other side, it has led to the development of an entirely new generation of ABPs called clickable "label-free" ABPs. The incorporation of clickable tags into biologically relevant natural products/natural product-based drugs and subsequent *in vivo* labeling is emerging as a very useful approach towards the identification and validation of the potential targets of such important compounds.[101] In spite of all these advances, the real-time dynamic imaging of activity of a specific protein of interest in a live organisms remains highly challenging. This requires a highly delicate integration of different methods such as the development of highly specific and sensitive probes, non-toxic reaction conditions and detection methods with superior tissue penetrating capacities. This remains a great avenue for future research. The strain-promoted metal catalyst-free version of click reaction, due to its non-toxic nature, is making possible sensitive metabolic labeling experiments in living animals. It already shows the first glimpse of "dynamic imaging" of certain PTMs; an achievement surpassing all the other bioorthogonal reaction-based detections. The development of enzyme inhibitors is another important area where CC plays an active role. It has been identified as a convenient strategy towards fragment-based inhibitor assembly, where large libraries of potential bidentate inhibitors are generated with minimum synthetic efforts. Ingenious strategies such as *in situ* CC has so far shed some light into new ways of generating extremely potent inhibitors against certain enzymes. However, at present this technique is in its infancy that requires very large quantities of proteins and relatively poor amplification is observed except in some highly optimized cases. One of the novelties of CC that makes it highly popular not only in catalomics, but also in the fields of chemistry and biology in general, is that it can be readily integrated to both conventional biological techniques such as gel-based labeling, pull-down assay, etc, and many of the upcoming high-throughput bioassays and characterization techniques such as microarray, LC-MS/MS *etc.* Considering the important contributions this novel chemistry has made to the chemical and biological fields in such a short period from its birth in 2002, we could anticipate that it will continue to contribute significantly towards a better understanding of our life processes.

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