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New insights into the kinetic target-guided synthesis of protein ligands

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The kinetic target-guided synthesis (KTGS) strategy is an unconventional discovery approach that takes advantage of the presence of the biological target itself in order to irreversibly assemble the best inhibitors from an array of building blocks. This strategy has grown over the last two decades notably after the introduction of the *in situ* click chemistry concept by Sharpless and colleagues in the early 2000s based on the use of the Huisgen cycloaddition between terminal alkynes and azides. KTGS is a captivating area of research offering an unprecedented and powerful strategy to probe the macromolecular complexity and dynamics of biological targets. After a brief introduction listing all chemical ligation reactions reported to date in KTGS, this review focuses on the last five years' progress to expand the repertoire of the click or "click-like" tool box targeting proteins, as well as to overcome limitations arising in particular from false negatives, *i.e.* potent ligands that are not formed, or formed in undetectable trace amounts. Furthermore, we wish to analyze the new twists and novelties described in some of these applications in order to better understand the conditions that govern this strategy and the extent to which it can be developed and generalized for a more efficient process.

1. Introduction

The discovery of bioactive compounds is a long, expensive, and complex process. During the last two decades, major improvements have been made aiming at replacing or improving the long-lasting strategy for developing one target molecule at a time. The pharmaceutical industry has developed new strategies that speed up and limit the cost of hit identification while trying to produce better clinically relevant candidate molecules.[1] These strategies include parallel and combinatorial synthesis, automated systems and high-throughput screening. Nonetheless, the major drawbacks of such strategies remain the necessity to synthesize the extended libraries and the limited success rate of bioactive hits.

In order to improve the drug discovery process, new methodologies have recently been developed addressing its time, cost and success limitations, such as *in vivo* screening, structure- or fragment-based drug design.[2,3] Furthermore, these methodologies complement and extend each other and are most likely combined to provide better potential drugs.[4] They are generally based on the knowledge of the targeted biomolecule owing to the large availability of biochemical data.[5]

Specifically, the target-guided synthesis (TGS) strategy has gained popularity in recent years due to its original concept: using the target biomolecule itself to assemble its best mutli-site ligands directly from a pool of fragments bearing complementary reactive functional groups. The process is divided into two major concepts: the thermodynamically controlled reactions involving reversible reactions also known as dynamic combinatorial chemistry (Fig. 1A)[6,7] and the kinetically controlled reactions involving irreversible bond formation.[8,9] Both have proved their efficiency in drug discovery. Particularly, in the kinetic TGS (KTGS) approach, best fragments from each binding site of the biomolecule would preferentially react together due to their spatial proximity, and lead to the corresponding dimeric ligand displaying synergistic bioactivity (Fig. 1B). Irreversible reactions to connect these fragments as well as reactants and products should be inert towards biomolecules and compatible with physiological conditions. Moreover, the rate difference between the reactions conducted with or without the biomolecule should be significant enough to unambiguously conclude that it is a biologically accelerated process. Besides, the isolation of products is virtually impossible as they are formed in trace amounts mainly due to target poisoning. Consequently, expected templated products should also be readily accessible through standard catalyzed synthetic chemistry for comparison and analysis purposes.

Due to these stringent criteria, only a few reactions have been reported since the serendipitous discovery of the first example in 1969 (Fig. 2f). All reactions reported to date are listed in Fig. 2.[10–36]

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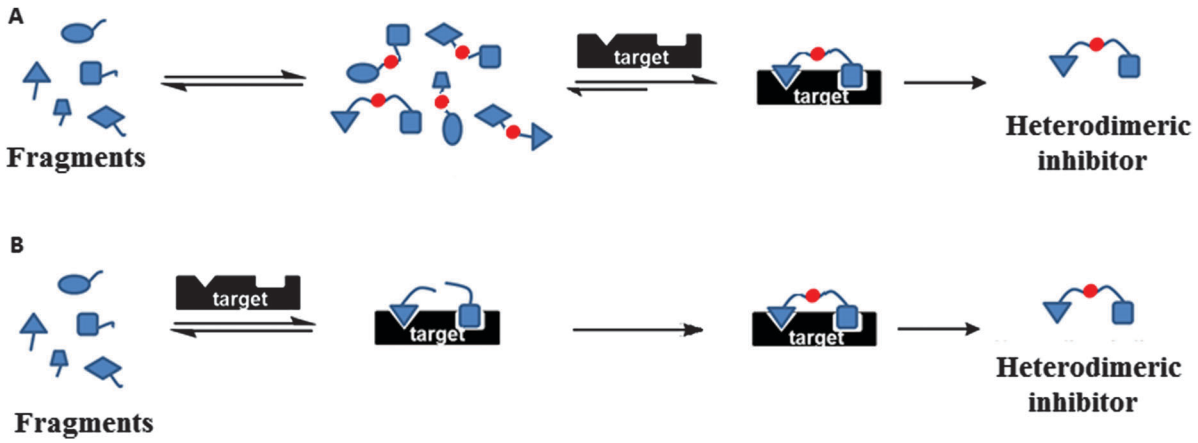

Fig. 1 (A) Thermodynamically controlled TGS or DCC principle (B) KTGS principle.

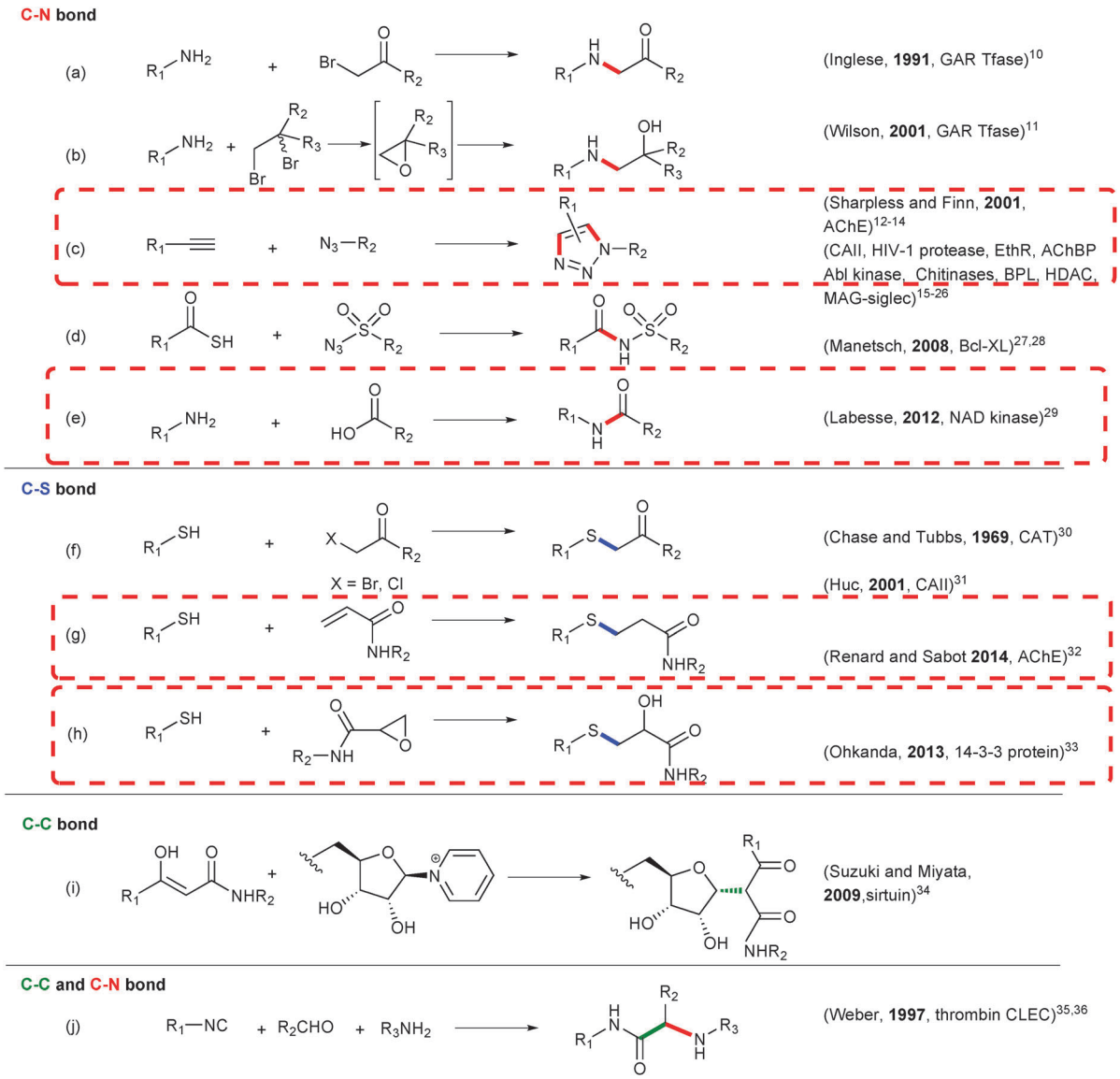

Fig. 2 KTGS reactions reported to date.

Notably, one variant of the KTGS developed by Sharpless *et al.* in 2002[12] relies on the use of click reactions, and particularly the 1,3-dipolar cycloaddition of organic azides and alkynes (Fig. 2c). This reaction proved to be remarkably well-suited for the discovery of ligands of various biomolecules such as enzymes,[6] RNA and DNA,[37–40] protein–protein interactions,[41] antibody-like protein-capture agents,[42,43] and the protein-targeted drug delivery mechanism.[44,45]

KTGS is a captivating area of research capable of probing the macromolecular complexity and dynamics of biological targets. It is a fundamentally important research field that undoubtedly has great potential for future drug discovery providing further developments. This review summarizes advances from the past five years aiming at expanding the repertoire of available reactions (Fig. 2e, g and h), to address limitations (*i.e.* false negatives), and to further develop the scope of this strategy through innovative applications involving proteins as biological targets. This review should call the reader's attention to the fact that KTGS is a multidisciplinary field of research that benefits from recent achievements in bioorganic chemistry, analytical sciences, and genetics.

2. The Huisgen cycloaddition

By far, the mostly used reaction for KTGS is the Huisgen reaction, *i.e.* the 1,3-dipolar cycloaddition of azide and alkyne moieties to afford the corresponding 1,2,3-triazole scaffolds. This reaction reported by Michael more than 100 years ago[46] requires harsh thermal conditions in its uncatalysed version to yield the two possible regioisomers of 1,2,3-triazoles (Fig. 3). Later on, Huisgen elucidated its concerted pericyclic mechanism in 1963.[47] In 2002 Meldal's and Sharpless' teams independently reported the regioselective synthesis of *anti*-triazole using copper(I) catalysis.[48,49] A few years later, Fokin *et al.* reported the regioselective reaction leading to the *syn*-triazole in the presence of ruthenium-based catalysts.[50,51] Among its long list of applications,[52] the azide–alkyne cycloaddition was found to be ideal for the KTGS approach that also came to be known as *in situ* click chemistry.

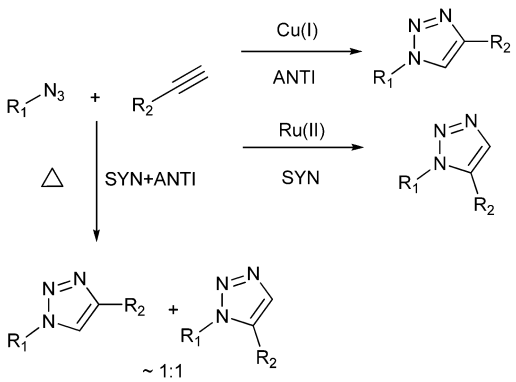

Fig. 3 Regioselectivity of the Huisgen reaction.

2.1. AChE: a new reaction site

Sharpless *et al.* validated the use of the azide–alkyne cycloaddition strategy in KTGS using acetylcholinesterase (AChE) as the target, a protein playing a key role in nerve impulse propagation, and one of the few validated drug targets for the treatment of Alzheimer's disease (AD).[12–14,53] It has a deep catalytic active site and a large peripheral site at its surface, both connected by a narrow gorge. Many potent dimeric inhibitors of mouse and electrical eel AChE were discovered *via* KTGS and were found to have synergistic activities. These experiments highlighted the importance of the following factors: (1) the overall spacing between the two ligands; (2) the triazole ring location within the enzyme; and (3) the affinity gain of the assembled bivalent inhibitor.

In this context, we questioned whether more than one reaction site could be exploited within a specific enzyme in order to extend the scope of the KTGS screening to various families of fragments.[26] Following the previous reports using tacrine derivatives as anchors for the *in situ* reactions, it was clear that the use of bulkier active site inhibitors, such as huprine, was unlikely to click within the constricted lower gorge site of AChE due to the presence of the bulky carbobicyclic system. Huprine derivatives, initially developed by Camps *et al.*[54,55] and later by our group,[56–59] are the best monomeric AChE active-site ligands reported to date, especially huprines *X* and *Y* that exhibit a 270-fold affinity gain towards AChE compared to tacrine. These inhibitors can be readily functionalized at position 12[56] and more interestingly at position 9 (Fig. 4).[57–59]

Docking studies showed that a four-carbon atom chain on position 9 sticks out into the middle of the gorge, making it ideal for a triazole ring to be positioned in that space. However, the concern is that the higher degree of enzyme flexibility at the mid-gorge may hamper the occurrence of the *in situ* click reaction. Thus, two huprine derivatives bearing a two or four-carbon alkyl azide chain (**(±)-9-HUPZm**) were incubated in the presence of alkyne-containing phenyltetrahydroisoquinoline (**PIQ-An**) derivatives of varying chain lengths.[20] From the ten possible regioisomeric heterodimers, only the *anti*-**9-HUPZ4PIQ-A2** and the regioisomeric mixture of *syn*- and *anti*-**(±)-9-HUPZ4PIQ-A3** (ratio ≈ 1 : 2) were observed after two and seven days of incubation with *m*-AChE and nine and thirteen days with rh-AChE, respectively (Fig. 5). Interestingly, **PIQ-A2** (with two carbon atom chain) is ideally located and only the optimal regioisomer was formed. Conversely, the addition of an extra carbon allows a more flexible folding of the

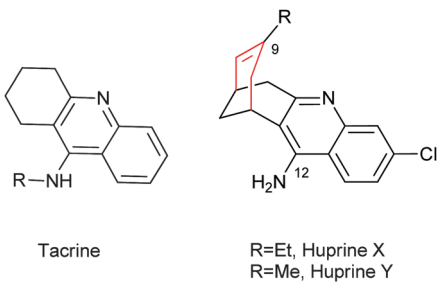

Fig. 4 Structure of tacrine and huprine.

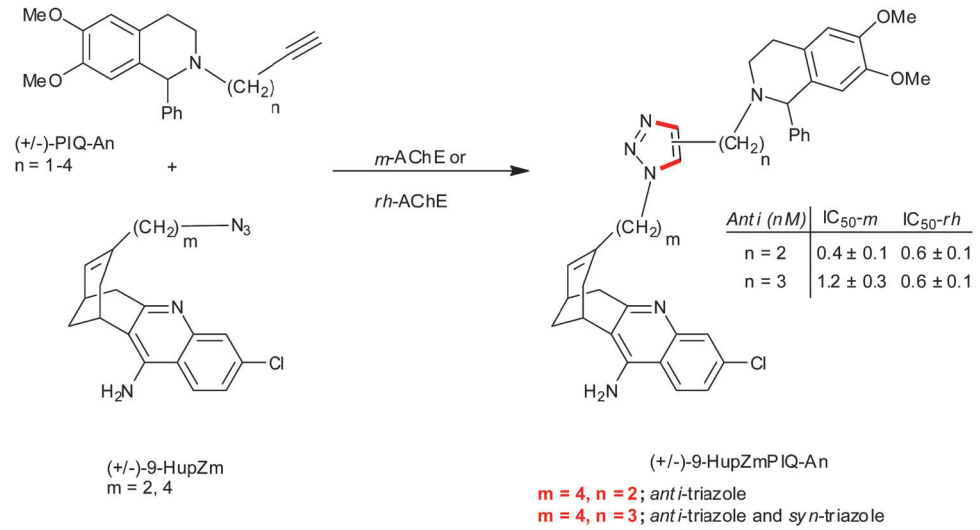

Fig. 5 *In situ* reaction of huprine and PIQ derivatives in the middle gorge region of AChE.

chain that leads to a mixture of isomers. As expected, no trace of **(±)-9-HUPZ2PIQ-A4** was detected. Despite having the same spacer length as **(±)-9-HUPZ4PIQ-A2**, the triazole unit would be unfavourably located at the gorge bottleneck where the space is limited by two tyrosine derivatives. These experiments were successfully conducted with both *m*-AChE and, for the first time, with the more flexible, less stable recombinant human rh-AChE. A novel click reaction site within the gorge of AChE was identified: it enables the use of bulky huprine inhibitors of the acylation site.

Furthermore, computational studies confirmed that the specific selectivity observed of **(±)-9-HUPZ4PIQ-A2** is thermodynamically driven and is primarily related to the protein internal energy difference between both conformations. Indeed, depending on the reaction site within the enzyme and the overall spacing between the two ligands dictating the reactive group orientation, a single enzyme could govern the absolute regioselectivity, either *syn* or *anti* isomer, of the Huisgen reaction.

2.2. AChBPs: click reaction at the flexible subunit interfaces

The acetylcholine binding proteins (AChBPs) are considered as extracellular domain structural surrogates of nicotinic acetylcholine receptors (nAChRs), important targets for the treatment of AD. To develop new ligands of AChBPs through KTGS, the authors hypothesized that substitution of a 1,2,3-triazole unit with the ester moiety of ACh would mimic its H bond acceptor character, while leaving the trimethylammonium moiety so as to keep the cation–π interaction with aromatic amino acid side chains of the target protein.[22,23] After an activity screen of different triazole-linked quaternary ammonium compounds against AChBPs from *Lymnaea stagnalis* (Ls), *Aplysia californica* (Ac), and the Y55W *Aplysia* mutant (AcY55W), the best hit **1** was identified (Fig. 6).

In order to prove the viability of the methodology, the corresponding alkyne and azide constituents of **1** were incubated in the presence of Ls, Ac and AcY55W AChBP for 3 days. Analysis showed that Ls selectively accelerated the formation of **1** (Fig. 6). No ligand was formed in the control experiment run in the presence of a nicotinic antagonist methyllycaconitine thus demonstrating that the flexible subunit interface of AChBP can serve as a template. Unfortunately, the regioselectivity of the bioprocess has not been determined, the *anti*- and *syn*-triazole being inseparable by LC/MS analysis.

Following this proof of concept, two multicomponent *in situ* assays were conducted using two libraries of azide building blocks A1 and A2 containing a diversity of scaffolds bearing a quaternary nitrogen atom, and alkyne **4** (Fig. 7, screenings 1 and 2). The *in situ* synthesis of **1** was not as effective as in the binary mixture. Instead, new ligands with better affinities were formed in significantly higher quantities, the best of which is triazole **2**. The optimization of the alkyne scaffold was consequently carried

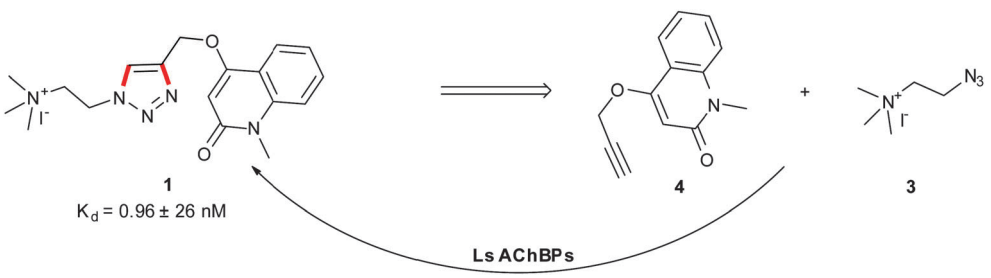

Fig. 6 The best hit **1** identified by an activity screening was efficiently assembled by AChBPs.

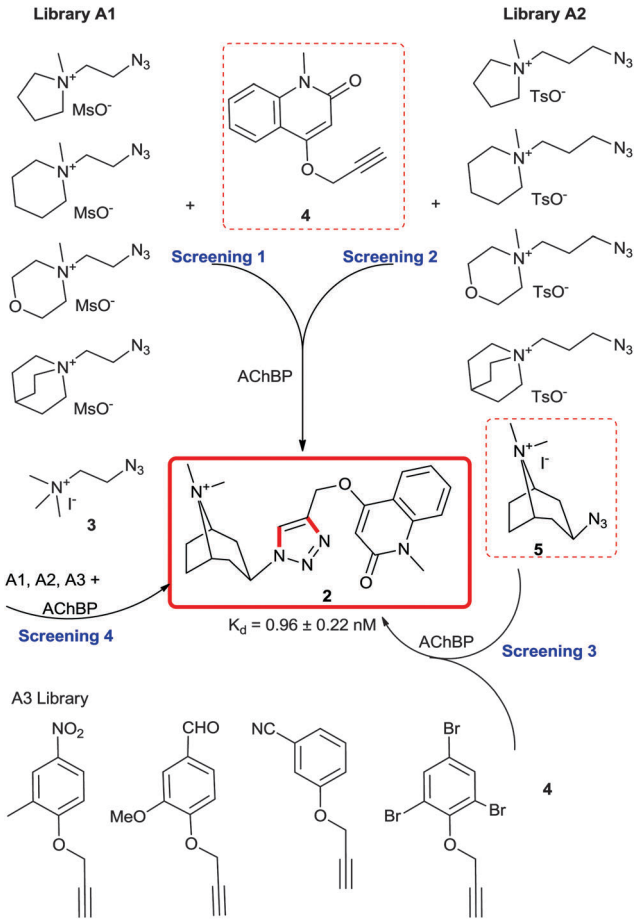

Fig. 7 Screening strategy for the discovery of AChBP ligands. Screening **1** and **2**: two libraries A1 and A2 of quaternary ammonium azide compounds were screened with alkyne **4**. Screening **3**: screening of the best azide hit **5** with alkyne library A3 including **4**. Screening **4**: screening of all azide and alkyne compounds.

out by screening a new library A3 of five structurally related alkynes along with the best azide partner **5** (screening 3). All of the five possible combinations were detected with Ls AChBP, none of which had a better affinity than triazole **2**.

Furthermore, a final multicomponent experiment was conducted, including all 15 individual compounds. Despite new triazole compounds being identified, triazole **2** with the highest affinity was again formed in greater amounts. Importantly, this example illustrates that *in situ* click chemistry could provide not only qualitative but quantitative information.

2.3. Abl: a pool test

Passarella *et al.* recently confirmed the capacity of Abl (tyrosine kinase), an important target for the development of specific antileukemic cancer therapies, to selectively favour the formation of the best inhibitor from a pool of complementary derivatives.[24] The two fragments of a previously reported triazole inhibitor[60] **FA030** were incubated in a mixture with an additional azide and four other alkynes. As expected, only the peak of **FA030** was detected after analysis (Fig. 8). The other possible compounds were known to be ineffective inhibitors of the Abl activity. The *in situ* and control experiments were tested for the presence of residual copper, which was found at 0.04% (usually at 1–5 mol% of Cu(I) for the CuAAC reaction in the presence of sodium ascorbate). Besides, the authors presumed that, under the reaction conditions, copper would be in its Cu(II) oxidized form and thus would not be able to act as a catalyst. Hence, the possibility of a residual copper catalysis was eliminated.

2.4. CAII: an enzyme-tethered reaction

Tethering is an approach that uses reversible disulfide bonds between a cysteine and a thiol-containing fragment to identify weak binding fragments.[61] Among its many applications,[62,63] tethering was coupled to KTGS.[21]

A CAII mutant enzyme H64C, where histidine 64 was replaced with a cysteine, was used to perform a tethered *in situ* click reaction. The tethering method would enable the use of weak inhibitors by covalently attaching them to the protein. Additionally, it would facilitate the monitoring of the cycloaddition process by X-ray crystallography. For that purpose, the thiol-containing azido components with different chain lengths were tethered to the enzyme surface *via* a disulphide bridge with the mutant cysteine.

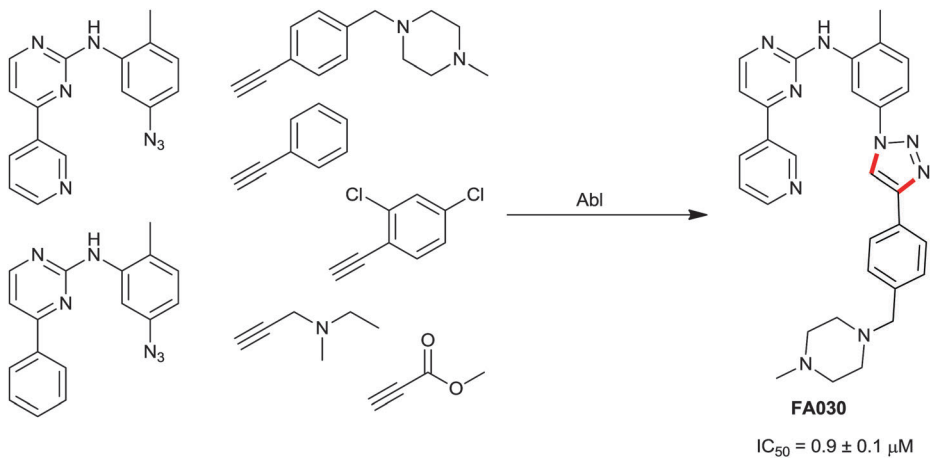

Fig. 8 *In situ* generation of the best affinity ligand using Abl as the target.

The alkyne-containing sulfonamide anchors were reversibly bound to the zinc ion in the active site of CAII. The click reaction was monitored by both LCMS and X-ray crystallography. Using LCMS, the regioselective formation of the *syn*-triazole heterodimer in the binding pocket was detected after two hours of incubation at 37 °C. Using X-ray crystallography, the pre- and post-click conformations of the ligand–enzyme complexes were analysed. The crystals were obtained after a 2-month soaking period at 18 °C. Interestingly, in the crystallographic state, the protein induced a stereo specificity where only the slightly more potent *R*-alkyne component reacted to give the 1,5-*S*-product (Fig. 9). The authors attributed this to the slow exchange of the *S*-alkyne to the *R*-alkyne that has a better spatial orientation for the cyclisation reaction.

2.5. EthR: a fragment-based approach

Transcriptional repressors are proteins that bind to specific sites on DNA and prevent transcription of nearby genes. In the particular case of EthR, it controls the expression of EthA, a mycobacterial monooxygenase that plays an important role in the treatment of multidrug-resistant tuberculosis by activating the antibiotic Ethionamide. Deprez *et al.* hypothesized that inhibition of EthR would increase the transcription of EthA and therefore improve the efficacy of Ethionamide.[20]

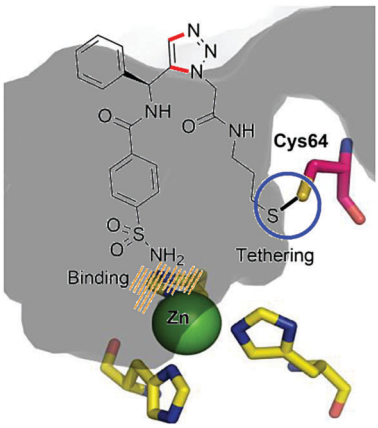

Fig. 9 Tethered *in situ* generated compound in the active site of CAII.

Following a screening of a large library of EthR inhibitors and subsequent hit-to-lead studies monitored by X-ray crystallography, the authors found that **BDM31381** adopted a different orientation than the initial hit. This observation led to an *in situ* study in order to probe the binding capabilities of the protein especially since a new binding pocket was revealed. Hence, the azide analogue **BDM14801** was synthesized and incubated with 6 libraries of 10 alkynes, each chosen for their potential ability to interact with the hydrophobic portion of the binding pocket (Fig. 10). The analysis of the reaction mixtures confirmed the formation of a predominant product in the presence of EthR, *anti*-4-iodobenzenesulfonamide triazole derivative **BDM14950**. The X-ray structure of **BDM14950** showed that the azide containing part of the molecule occupied the same space in the binding site as the azide fragment of **BDM14801**, whereas around the alkyne-part of the molecule, structural modifications of the deeper part of the pocket could be observed, probably to accommodate the bulky derivative. Importantly, the triazole derivative displayed a 10-fold higher inhibitory activity than that of the parent azide **BDM14801**.

2.6. MAG, Siglec-4: unknown binding site

A fragment-based approach was combined with *in situ* click chemistry in order to develop high-affinity ligands of unknown binding sites of the myelin-associated glycoprotein (MAG, Siglec-4), a sialic acid binding immunoglobulin-like lectin (Siglec).[18] A three-step strategy was designed to achieve this goal: (1) random screening of libraries and/or physiological ligands would determine a first binding site that would serve as a starting point; (2) the search for second binding site ligands involves an NMR identification based on the change of their transverse magnetization decay upon binding; (3) from the identified hits, the ones adjacent to the first site are identified by their enhanced paramagnetic relaxation caused by the spin-labelled first-site ligand. Once the closest sites are identified, the target protein is incubated with libraries of complementary ligands for both sites.

This strategy was successfully applied to MAG, Siglec-4 using a library of four alkynes and three azides. Theoretically, 24 different triazoles (*syn*/*anti*) could be obtained. Using LC-MS and MS-MS fragmentation data, only one triazole-compound was identified in

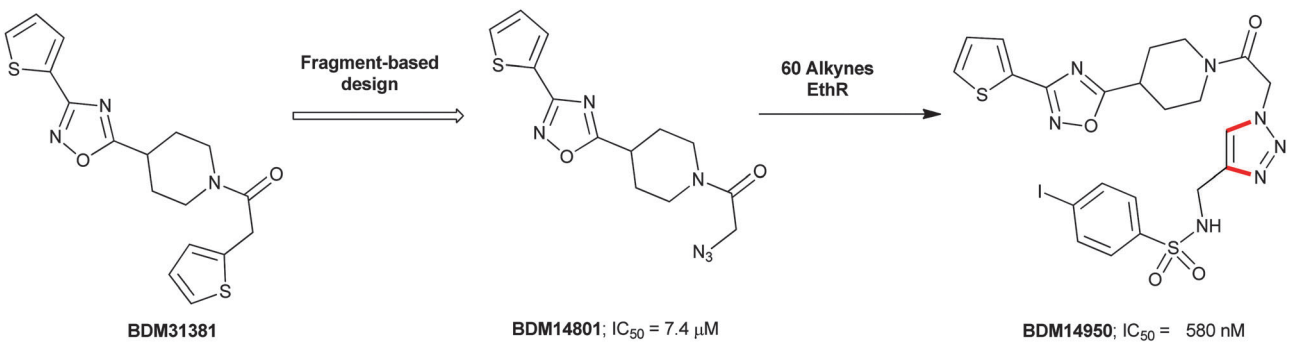

Fig. 10 **BDM31381** identified in the hit-to-lead study as adopting a different binding orientation in X-ray crystallography; **BDM14801**, an azide-containing analogue of **BDM31381**; **BDM14950**, the EthR templated compound.

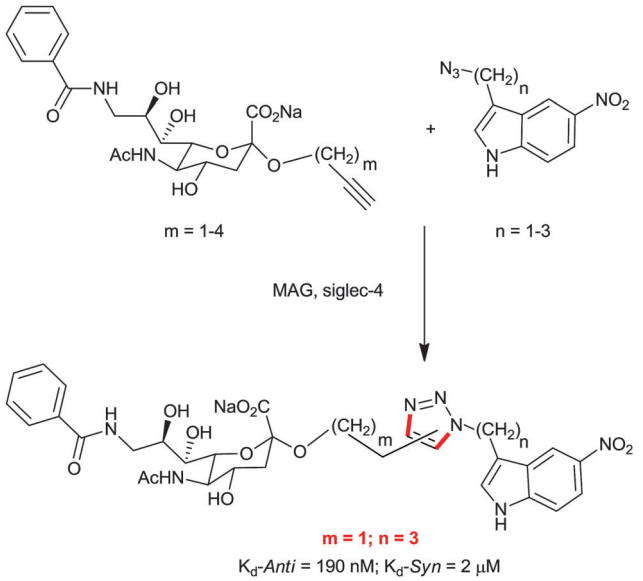

Fig. 11 The library of compounds identified by screening and the corresponding hit.

the *in situ* reaction mixture. Unfortunately, the regiospecificity of the reaction could not be proved. Importantly, a 1000 fold synergistic effect was observed for the triazole product relative to the parent azide and the alkyne derivative (Fig. 11).

2.7. HDAC: an active Cu–enzyme complex

In situ click chemistry uses solely the target macromolecule to template the formation of the best affinity ligand, without any external activation. However, in the example reported by Miyata *et al.*,[19] a Cu–histone deacetylase (HDAC) complex proved to catalyse the reaction. Most inhibitors of this zinc metalloenzyme consist of a Zn-binding group located in the active site, a capping region that interacts with amino acids at its rim, and a linker that connects the two parts.

Based on the clinically used HDAC inhibitor Vorinostat, the authors synthesized a library of two alkynes with anchoring hydroxamic acid, a zinc binding moiety, and 15 alkyl azides as matching building blocks (Fig. 12). Each binary mixture was prepared with the alkyne at a concentration approximately equivalent to its IC_{50} with a large excess of the azide and HDAC. Then subsequent fluorometric assays for HDAC activity were directly carried out on the reaction mixture. Only one resulted in a significant alteration in the fluorescence signal, hence indicating the formation of a triazole-linked inhibitor. The *in situ* and regiospecific formation of the *anti*-triazole was determined, whereas no product was formed in the control experiments. However, some aspects of this successful *in situ* click chemistry reaction were unusual: (1) the triazole formation in their study was as much as 50%, while ordinary *in situ* click chemistry enables the formation of the bivalent ligand in only a small percentage; (2) only *anti*-triazole (IC_{50} = 4 μM) was formed *in situ*, even though the *syn*-isomer (IC_{50} = 0.51 μM) proved to be more active. These observations were consistent with the hypothesis of the presence of a small amount of enzyme containing Cu(I). Indeed, ICPMS revealed the presence of both 0.95 μM of Zn and 0.1 μM of Cu. Various control experiments determined the necessity of having the assistance of Cu(I) to enable the *in situ* click chemistry to occur specifically within the enzyme's active site.

2.8. *Sa*BPL-R122G mutant: turnover efficacy

The slow turnover rates of the enzymes involved in *in situ* reactions are due to the higher affinity of the assembled inhibitors, leading to a much slower dissociation from the binding sites. The saturation of active sites with the new inhibitor is generally the limiting step of the reaction. In order to circumvent this problem, Abell *et al.* have mutated the amino-acid of the enzyme that helps retaining it in its "closed" conformation.[25] Arginine R122 of the biotin protein ligase (BPL) from *Staphylococcus aureus* (Sa) was replaced with a Glycine G. The R122G mutated enzyme still had the same

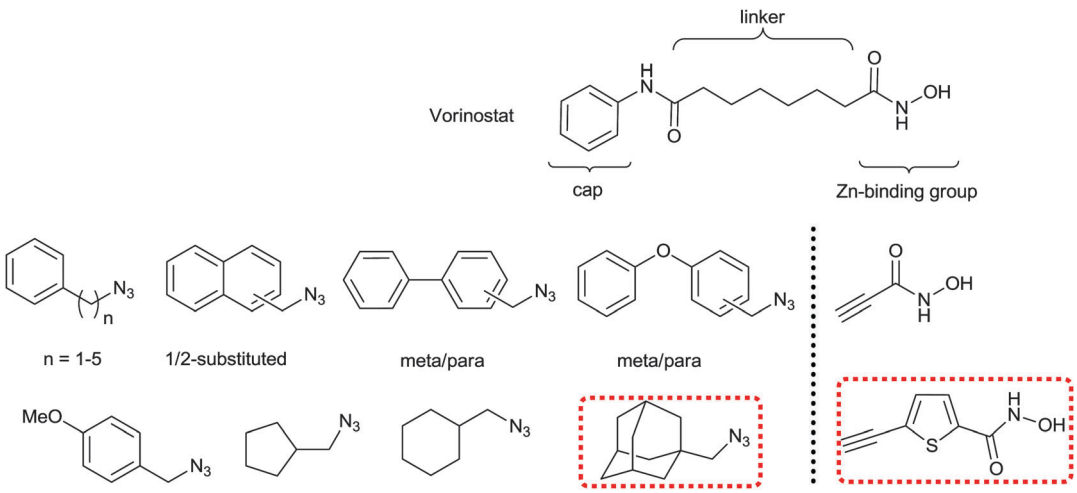

Fig. 12 Vorinostat, HDAC inhibitors. To the right, alkyne moieties as zinc-binding groups, to the left, azide compounds as the cap. The triazole combination of the two molecules in the red boxes was detected *in situ*.

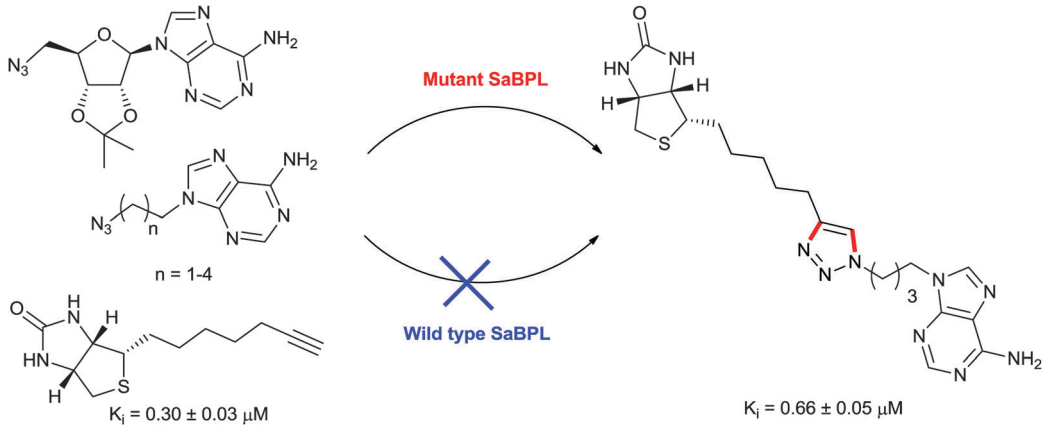

Fig. 13 *In situ Sa*BPL-R122G assembly of a biotin-based inhibitor from an array of azide.

secondary structure and activity as the wild-type enzyme, making it a good model for the discovery of inhibitors. When subjected to multicomponent *in situ* experiments including a library of five azides and a biotin-linked alkyne, the mutated enzyme showed greatly improved efficacy and sensitivity compared to the wild-type enzyme to form the triazole derivative (Fig. 13). The turnover rate of the reaction with the mutated enzyme was considerably higher probably due to the 40-fold increase in the dissociation constants of the triazole compounds. Moreover, the potency profile of the triazoles against the wild type enzyme matched the results from the *in situ* experiments in the mutated enzyme thus confirming the success of this approach.

3. NAD Kinase: direct amidation reaction of a carboxylic acid with an amine

The NAD Kinase from *Listeria monocytogenes* (LmNADK1) has been unexpectedly found to promote amide bond formation between 5′-amino-5′-deoxyadenosine and carboxylic acid groups, without prior activation of the carboxylic acid moiety.[29]

This allowed the assembly of two adenosine derivatives that bind to the NAD binding sites of two human pathogens in the micromolar range. The active site of this enzyme is mainly composed of two subpockets occupied by the adenosine moiety (A subsite) and the nicotinamide riboside moiety (N subsite) of its natural substrate and product, NAD and NADP, respectively. Knowing the structure of the enzyme, a stepwise approach was used by Labesse *et al.* in order to understand the orientation of a series of adenosine derivatives soaked in the active site. In almost all of the X-ray structures obtained, two molecules of the adenosine derivatives were found to bind simultaneously to the two subpockets of the active site. Furthermore, these structures highlighted the short distance (≈5 Å) between the 5′ adenosine substituent of the N subsite and the C8 adenosine derivative occupying the A subsite as shown with ligand **6** (Fig. 14a).

As a result of these observations and with the aim of connecting the C8 adenosine substituent in subsite A to the 5′ adenosine substituent in subsite N, the amino derivative **7** was soaked in the presence of the thioglycolated adenosine derivative **8** and the enzyme. The X-ray structure showed the *in situ* formation of the amide bond between the two adenosine derivatives (Fig. 14b) to give diadenosine **9**. Interestingly, His223 of the N site has been found responsible for the amidation reaction by stabilizing the tetrahedral intermediate.

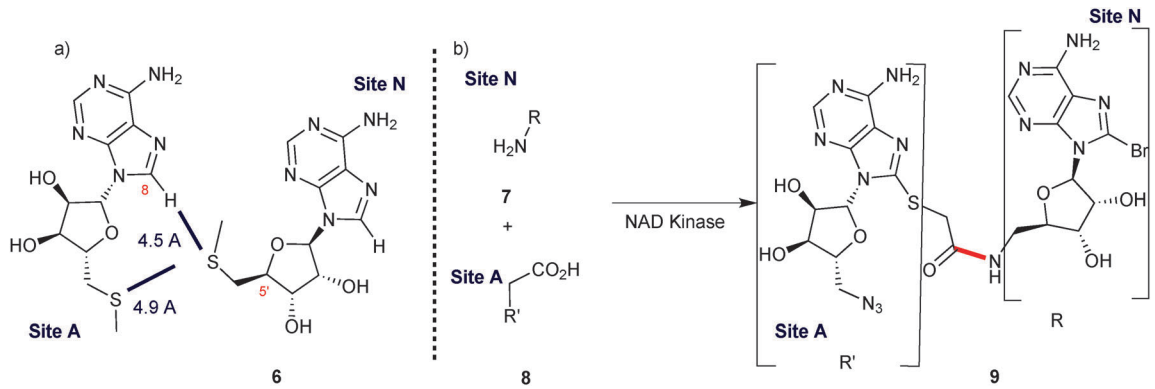

Fig. 14 (a) Representative spatial orientation of the two bound ligands 6 with the corresponding distance between the substituents of the 5′A–5′N position (4.9 Å) and the 8′A–5′N position (4.5 Å); (b) the dimeric 8′A–5′N adenosine **9** formed in the active site of LmADK1.

4. 14-3-3 protein: PPI inhibitor through epoxide ring opening

Protein–protein interactions (PPIs) are very important targets for therapeutic purposes as they are at the centre of most biological processes. Despite the challenges of developing small molecules as PPI inhibitors, recent progresses and advances has made it possible for some to reach clinical trials.[64,65] Manetsch *et al.* have validated the efficiency of the KTGS approach for the assembly of PPI inhibitors of Bcl-XL using the sulfo-click reaction (Fig. 2d)[27,28] More recently, Ohkanda *et al.* have reported the template effect of the recombinant human (rh) 14-3-3 protein, implicated in cancer and neurological diseases, for the *in situ* generation of PPI inhibitors.[33] The fungal phytotoxin fusicoccin **10** (Fig. 15) binds near the phosphopeptide-binding pocket of plant 14-3-3 proteins in a hydrophobic cavity. Along with the phosphopeptide QSYpTV (H-Gln-Ser-Tyr-phosphoThr-Val-OH) and **10**, the enzyme forms a stable ternary complex. The authors thus hypothesized that analogues of **10** and the phosphopeptide could probably react *in situ* if they have complementary reactive functions. Hence, four analogues of **10** (**11–14**; 300 μM) with different spacer arms containing an epoxide (Fig. 15) were incubated in the presence of the rh-14-3-3 protein (300 μM) and QSYDC (H-Gln-Ser-Tyr-Asp-Cys; 300 μM) a cysteine-containing analogue of the phosphopeptide (Fig. 16). Even though the background reaction in the absence of the enzyme was always present, the authors noticed that when the spacer was too short (**11**), the conjugate production was inhibited to 37%; which means that both ligands were bound to the enzyme but were unable to reach the functional groups. Whereas with compound **12**, the conjugate formation was enhanced by almost 200% compared to the control. Conjugate formation with **13** and **14** were 169 and 129%, respectively (Fig. 16). This example of KTGS highlights

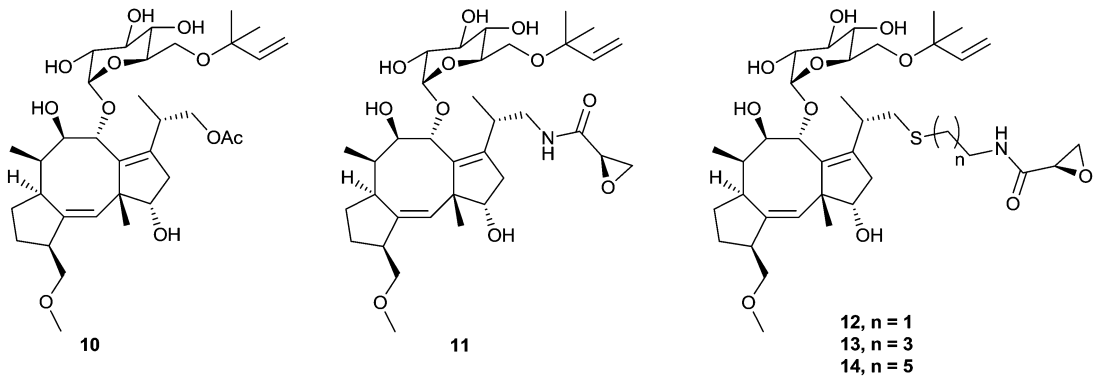

Fig. 15 Structure of inhibitor **10** and the epoxide analogues **11–14**.

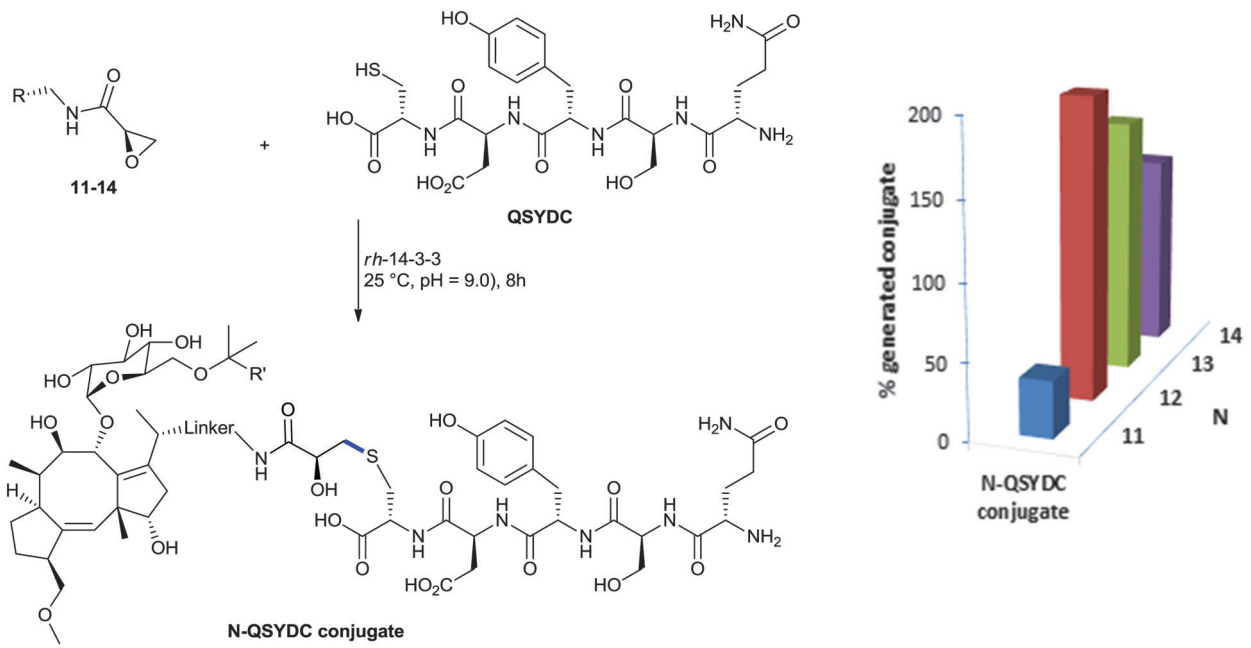

Fig. 16 Epoxide ring-opening templated reaction by the 14-3-3 protein. The chart on the right depicts the percentage of the templated conjugate of the different compounds compared to the blank reaction in the absence of the enzyme.

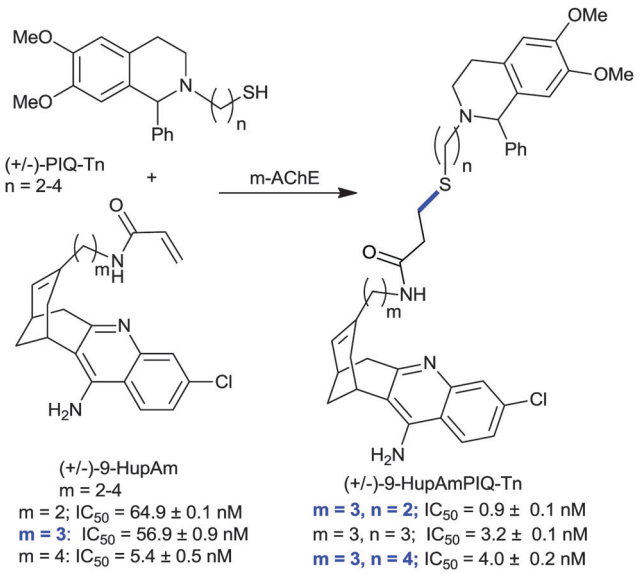

Fig. 17 Thio-Michael adducts templated by AChE.

again the importance of the spacer length for *in situ* click chemistry and reinforces its application for the discovery of PPI inhibitors.

5. AChE-accelerated thio-Michael addition

The Michael addition of thiol to maleimide derivatives is a common reaction in bioconjugation. In fact, maleimides undergo fast, chemoselective biothiol addition. This inspired us to use thiol derivatives with less reactive acrylamide partners to avoid or at least limit the formation of non-biocatalyzed adducts in KTGS experiments, while remaining irreversible. Assisted by molecular docking studies, a library of three acrylamide-containing huprine HUPAm derivatives and three thiol-containing PIQ-Tn derivatives was incubated in binary mixtures in the presence of *m*-AChE.[32] Two compounds, (±)-9-HupA3PIQ-T2 and (±)-9-HupA3PIQ-T4, were assembled exclusively within the binding site of AChE after 6 hours, out of nine possible heterodimeric inhibitors (Fig. 17). Interestingly, the templated heterodimers were 14- to 57-fold more active towards *m*-AChE with IC_{50} values in the low nanomolar range compared to the starting huprine. Strangely, the non-assembled (±)-9-HupA3PIQ-T3 has a 17 fold in activity. The other six compounds of the library either have lower affinities or have a very low synergistic effect. The irreversible Michael addition of thiols to acrylamides thus proved to be efficient for the synthesis of templated inhibitors and satisfyingly, about 8 times faster than the Huisgen reaction. This is probably due to a lower activation energy of the thio-Michael addition and a better use of space which would certainly be beneficial for unstable or more flexible enzymes. Moreover, this reaction could also be performed in tighter binding pockets, since the reaction is linear, and does not require accommodation of the relatively bulky triazole moiety. Furthermore, to limit handling of reactive thiol species subject to rapid disulfide bond formation, a cascade double catalysis assay was engineered starting from thioacetate PIQ derivatives to generate the thiol functions *in situ* before their templated assembly (Fig. 18). Indeed, AChE is a very efficient serine esterase that hydrolyses esters/thioesters into the corresponding alcohols/thiols, respectively. Starting from the thioester derivatives, AChE was capable of producing the final heterodimers through a cascade esterasic and templated catalyses. A set of control experiments confirmed that both the hydrolysis and the thio-Michael addition step were catalysed by *m*-AChE.

6. Conclusions

This Feature Article summarizes most of the recent advances regarding the use of the KTGS strategy for drug design. The KTGS strategy in general and the *in situ* click chemistry in particular have proved to be effective and versatile tools for the design of ligands of a variety of very different proteins, given that all the necessary precautions and control experiments are taken into account. Importantly, the KTGS could indifferently proceed either in favourable constricted active sites or in more flexible environments. Furthermore, more reaction sites than one are possible within a single protein, thus opening advantageously the screening to high molecular diversity.

The false negatives are probably the major weaknesses for the KTGS strategy. They could be attributed to multiple and sometimes interdependent reasons: (a) poisoning of the biocatalyst by the templated products themselves; (b) the low binding affinity of fragments despite their synergistic effect; (c) the requirement of long incubation times (from hours to days or weeks) at 37 °C to detect templated products that may dramatically alter the tertiary structure proteins and their catalytic activity; (d) the detection limits of analytical techniques.

These issues were tackled through multidisciplinary approaches. Mutant proteins were specifically designed either to increase the dissociation constant of the protein–ligand complex thus promoting multiple rounds of biocatalysis, (a) or to covalently tether one of the two reaction components so as to create artificially an anchor molecule promoting the reaction (b). Furthermore, the

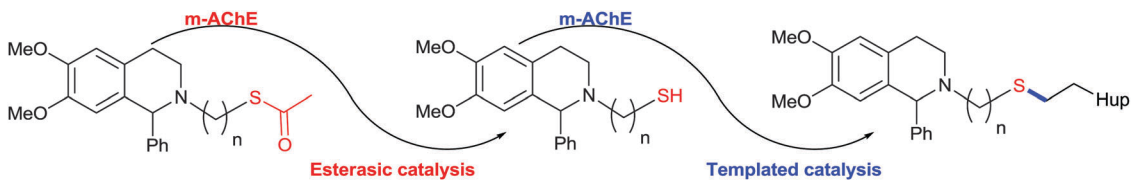

Fig. 18 Double catalysis process within AChE; 1-catalytic hydrolysis of the thioester; 2-templated synthesis of the dimeric inhibitor.

Cu(I)-doped protein approach has enabled the selective assembly of a triazole compound in detectable amounts (c). Besides, a handful of different analytical methods were employed to detect any traces of a templated product. These range from the usual HPLC analysis to MS coupling using the single ion monitoring mode or even the MS-MS fragmentation pattern when more compounds than one are expected to have the same molecular weight. Another solution also consists in indirect detection where fluorometric activity assays were conducted *in situ* using the enzymatic mixture. Moreover, X-ray crystallography has also provided unprecedented information regarding the regiochemistry and stereodiscrimination of the process (d).

Last but not least, the choice of the reaction is an important part of this strategy. In this context, the Huisgen reaction that presents important features (bioorthogonality, driving force, reagents/product stability) has proved to be highly effective. However, the emergence of novel click or ''click-like'' reactions can only be beneficial to the overall process of drug discovery. Indeed, due to the important structural diversity in the active site of proteins, and their relative stability over time, the discovery of complementary reactions is highly encouraged to offer new opportunities and improve the accessibility of this strategy to a wider range of functional groups and biological targets.

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