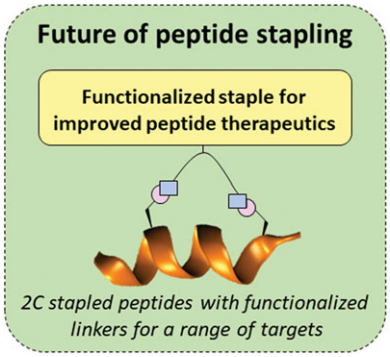
Peptide Stapling



An overview of the two-component peptide stapling chemistries that led to the discovery of biologically active macrocyclic peptides is provided. This progress report aims to highlight recent advances, challenges, therapeutic potential, limitations, and future opportunities for the field of peptide stapling.

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Two-Component Stapling of Biologically Active and Conformationally Constrained Peptides: Past, Present, and Future

*Jessica Iegre, Josephine S. Gaynord, Naomi S. Robertson, Hannah F. Sore, Marko Hyvönen, and David R. Spring**

Dedicated to Sir Jack E. Baldwin on the occasion of his 80th birthday

Peptides are an emerging class of therapeutics in the pharmaceutical world. Whilst small molecules have dominated the therapeutic landscape for decades, the design and application of peptide drugs is emerging among the pharmaceutical industries and academia. Although highly selective and efficacious, peptides are characterized by poor pharmacokinetic properties and amelioration of their bioavailability remains a major hurdle. Incorporation of conformational constraints within the peptide (such as peptide stapling) has been extensively used to improve the bioavailability of these molecules; consequently, it is not surprising that a plethora of stapling techniques has been developed and has had a significant impact on the development of peptide therapeutics. Among the numerous stapling techniques known, two-component methodologies allow facile and divergent functionalization of peptides. The authors have pioneered a stapling technique that makes use of the double Cu-catalyzed azide–alkyne cycloaddition between di-azido peptides and functionalized di-alkynyl staples. In recent years, the authors have created biologically active, conformationally constrained peptides functionalized with cell-penetrating peptides, fluorescent tags, and photo cross-linking moieties, demonstrating the wide applicability of this methodology. Herein, the impact, advantages, limitations, and future applications of this technology and other two-component peptide stapling techniques on the development of clinically relevant peptides are highlighted.

1. Introduction

Small molecules (MW <500 Da) are a well-established class of drugs commonly used to treat a variety of diseases by targeting deep and well-defined pockets of proteins, enzymes, receptors, ion-channels, etc. However, most of the signalling pathways that regulate important physiopathological mechanisms are characterized by molecular interactions at shallow and undefined pockets, such as those found at the interface of many protein–protein interactions (PPIs).[1] Indeed, more than 80% of proteins are considered undruggable with small molecules due to the nature of the binding pockets.[2,3] Despite several recent successes in targeting PPIs with small molecules,[4,5] the unsuccessful attempts are numerous. It is clear that molecules able to interact with these shallow pockets may unlock a number of diseases that can be investigated and treated. Peptides are an ideal alternative to small molecules, considering that they are able to mimic the endogenous portion of the interacting proteins. Moreover, being made of proteinogenic amino acids, peptides show low toxicity and can be easily synthesized. Several peptides have been successfully developed and used to target intracellular and extracellular PPIs over the past couple of decades, although only a few of them appear to have been stable in a living organism.[6] The development of peptide therapeutics that can reach the target intact is hampered by their poor physicochemical properties.[7] Specifically, peptides are characterized by poor plasma stability and rapid renal excretion resulting in a short half-life and poor membrane-permeability. These undesired properties have disadvantaged the growth of peptides as therapeutics in the pharmaceutical world. However, the high efficacy, selectivity, low toxicity, and low production costs compared to other biotherapeutics[8] have encouraged scientists to investigate new methods to overcome the intrinsic limitations of peptides. Moreover, the number of peptides that nature provides is enormous, validating their capabilities to interfere with extra and intracellular pathways. Indeed, new methodologies that improve the stability of peptides have emerged in the last couple of decades and made peptide therapeutics one of the fastest growing fields in the pharmaceutical industry.

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Josephine Gaynord was born in the United Kingdom and obtained her M.Sci. in chemistry from the University of Nottingham in 2016. During this time, she spent a year at Takeda Cambridge as a medicinal chemistry placement student and completed a collaborative GlaxoSmithKline medicinal chemistry masters project. In 2016, she started her Ph.D. under the supervision of Professor David Spring at the University of Cambridge, researching the use of stapled peptides to inhibit protein–protein interactions and novel bioconjugation methods.

David Spring is currently a professor at the University of Cambridge within the Chemistry Department and a fellow of Trinity College. He received his D.Phil. (1998) from Oxford University under Sir Jack Baldwin. He then worked as a Wellcome Trust Postdoctoral Fellow at Harvard University with Stuart Schreiber (1999–2001), after which he joined the faculty at the University of Cambridge. His research program is focused on synthetic chemistry and chemical biology.

Among the different strategies used to improve the pharmacokinetic properties of peptides, cyclization is probably the most widely used, and at least 125 cyclic peptides which are orally absorbed have been reported.[9] Unlike *N*-alkylation or the introduction of amide isosteres (peptidomimetics), cyclization does not necessarily affect the structure of the peptide and therefore its function is preserved. Cyclization of peptides can be achieved in different ways—for example, head-to-tail, side chain to side chain—and the reader is directed to several excellent reviews for a comprehensive description of these different approaches.[10–13] In this progress report, we will focus on cyclization obtained via stapling.

Peptide stapling is a cyclization technique that joins two amino acid side chains of the same peptide to constrain it conformationally with the aim to stabilize the bioactive form. Although the term stapling is generally applied to α-helical peptides, in this progress review, it will be used to describe secondary structures beyond α-helices as well (generally referred to as conformationally constrained peptides). Stapling methodologies can be divided in two main groups: one-component peptide stapling (1C-PS) and two-component (2C-PS). The former utilizes intramolecular reactions to staple together two amino acids of the linear sequence while the latter exploits intermolecular coupling involving a staple and two amino acid side chains.[14]

2C-PS techniques are the main topic of this progress report with emphasis on those that allow functionalization of the staple and, in particular, on the CuAAC stapling technique developed in our group. Unlike most of the 1C stapling techniques, the 2C-PS can involve the use of a multifunctional staple: it may constrain the peptide in the bioactive conformation, protects it from protease-mediated degradation, and, at the same time, can be used to introduce cell-permeable motifs, fluorescent tags, or functionalities useful for biological assays.

2C-PS methodologies have enabled the creation of a toolbox of functionalized stapled peptides targeting specific intracellular PPIs and is therefore of interest to the medicinal chemistry community. Herein, we aim at discussing recent advances in the field, specifically highlighting the impact of our methodology and other 2C-PS chemistry when applied to the development of new functionalized peptide therapeutics and medical tools, including their limitations and future direction.

2. Macrocyclic Peptide Therapeutics: What is Out There?

Even though the pharmaceutical industry has traditionally invested in small molecules, the number of drug discovery programs involving peptides and other new modalities—for example, oligonucleotides, molecular conjugates, hybrids—has increased considerably in the last few decades.[15] We can rationalize this trend, by considering that 80% of the biologically relevant targets can more easily be targeted using biologics rather than small molecules, due to the nature of the binding sites.[2,16] Peptides represent a valuable solution as they show selectivity and affinity comparable to other biologics (such as antibodies); however, they are more easily synthesized and avoid immunogenicity and tissue penetration issues.[3,6] Moreover, the cost of the production of peptides is comparable to small molecules and as such much cheaper than the production of antibodies, for example.

Today, there are more than 500 peptide therapeutics in preclinical studies and some 150 in clinical trials, highlighting the importance of this emerging field in the pharmaceutical landscape.[17,18]

2.1. Peptides in Advanced Phases of Drug Discovery Programs

There are a number of linear peptides and peptidomimetics on the market (such as Lupron Depot, Byetta, Lantus, Victoza among others) but optimization of their in vivo stability has not been trivial. Furthermore, despite carefully developed formulations, they cannot be administered orally due to their weak

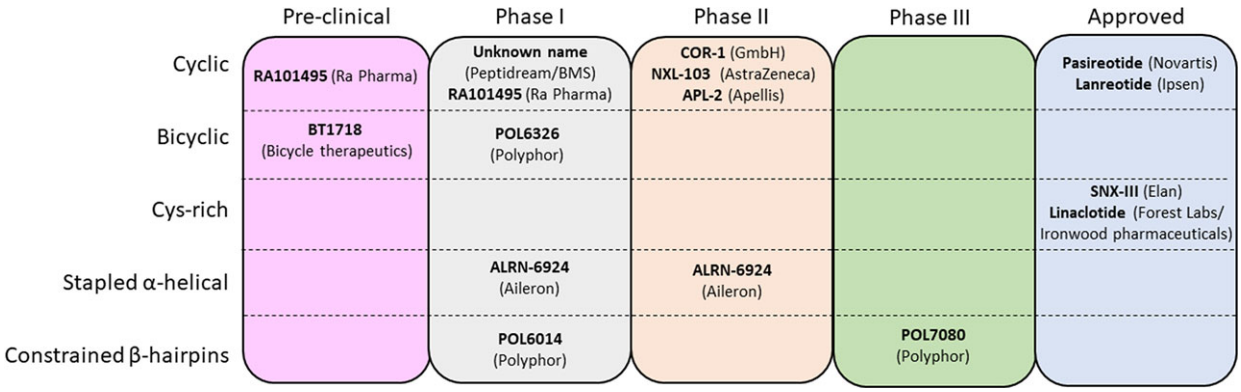


Figure 1. Overview of notable macrocyclic peptides from preclinical studies to the market, this is further segmented by the type of macrocycle. This information is correct up to April 2018 and is not a comprehensive list. Peptidomimetic drugs are not included in this figure.

or poor oral bioavailability. Therefore, many pharmaceutical companies and academic groups have focused their efforts on the development of peptides with increased in vivo stability and ideally oral bioavailability. This is the case for macrocyclic peptides, which have been reported to show increased plasma stability, cell-permeability, and the potential to be administered orally—encouragingly, a recent review reported the development of 125 orally bioavailable cyclic peptides.[9] With the term macrocyclic peptides, we refer to all the peptides that see at least two amino acid residues of the linear sequence linked together, with or without functionalities, to form a macrocycle. Cyclic, bicyclic, cysteine rich, stapled α-helical peptides, and constrained β-hairpins belong to this class[15] and an overview of some notable cyclic pharmaceutical peptides with public structures currently in preclinical, clinical studies, and approved drugs is shown in **Figure 1**.

To witness the success of cyclic peptides as therapeutics and medical tools, it should be noted that nowadays there are several companies specialized in the development of pharmaceutically relevant conformationally constrained peptides—Aileron Therapeutics, Pepscan, Peptidream, Bicycle Therapeutics, Ra Pharma, and Cyclogenix, among others. Moreover, even global pharmaceutical companies traditionally focused on small molecules have expanded their pipelines to accommodate cyclic peptides and "new modalities" in general.[15]

2.2. Strategies to Discover Biologically Active Cyclic Peptides

The strategies used to develop macrocyclic peptides into therapeutics can be broadly divided into three classes: natural products, screening of peptide libraries, and target-based rational design (**Figure 2**).

Natural product cyclic peptides and semisynthetic cyclic peptides were the first cyclic peptides to be used as drugs. It should be noted that nine cyclic peptides approved by the FDA and EMA between 2005 and 2015 were derived from natural products.[19] A few examples of this class of molecules are Caspofungin,[20,21] Cyclosporin A,[22] Daptomycin[23] and the more recent Anidulafungin,[24] Romidepsin,[25] Linaclotide, and Pasireotide[26] (some of which are shown in Figure 2). These are fermentation products of microorganisms and offer the advantage of being highly selective and stable. In addition, natural cyclic peptide hormones and hormone derivatives such as vasopressin, octreotide, and oxytocin should not be forgotten as they represent important drug molecules (Figure 2).[19]

Peptide libraries developed for screening can either be derived from biological or chemical sources. Cyclic peptide libraries from biological sources are synthesized with the aid of bacteriophages (phage-display) or using cell-free display technologies (mRNA display).

The cyclization of peptides from biologically derived libraries can be obtained in different ways (Figure 2):

(a) cysteine amino acids can be added randomly in the sequence—displayed on the surface of a phage[27]—to allow disulfide bridge formation.[28–31] An attractive variant of this methodology has been termed "CLIPS" cyclization technology and generates bicyclic peptides via conjugation between three cysteine residues and a tris-(bromomethyl) benzene (TBMB) core.[32] More recently, other trivalent thiol-reactive linkers have been developed for the same purpose allowing diverse conformations of the bicyclic peptides. This class embraces *N,N′,N″*-(benzene-1,3,5-triyl)tris(2-bromoacetamide) (TBAB), *N,N′,N″*-benzene-1,3,5-triyltrisprop-2-enamide (TAAB), and 1,3,5-triacryloyl-1,3,5-triazinane (TATA).[33]

(b) head to tail cyclization performed intracellularly exploiting the protein splicing capability of split inteins (SICLOPPS technology).[34,35] This technology implements a reverse two-hybrid system to allow functional and binding assays.

(c) mRNA encoded libraries of linear peptides are translated in vitro and then cyclized later by using reagents such as disuccinimidyl glutarate, which connects the N-terminus and a side chain of a Lys residue or forms disulfide bridges across Cys residues.[36,37]

Although phage display peptide libraries offer high library diversity, they may have low affinity for the target and there could be problems associated with the use of live cells and phages. mRNA encoded libraries may overcome these limitations and therefore represent a valid alternative.[38]

Incorporation of unnatural amino acids into biological libraries is challenging but new genetic technologies have made engineering of mRNAs and tRNAs easier.[39,40] In the recent

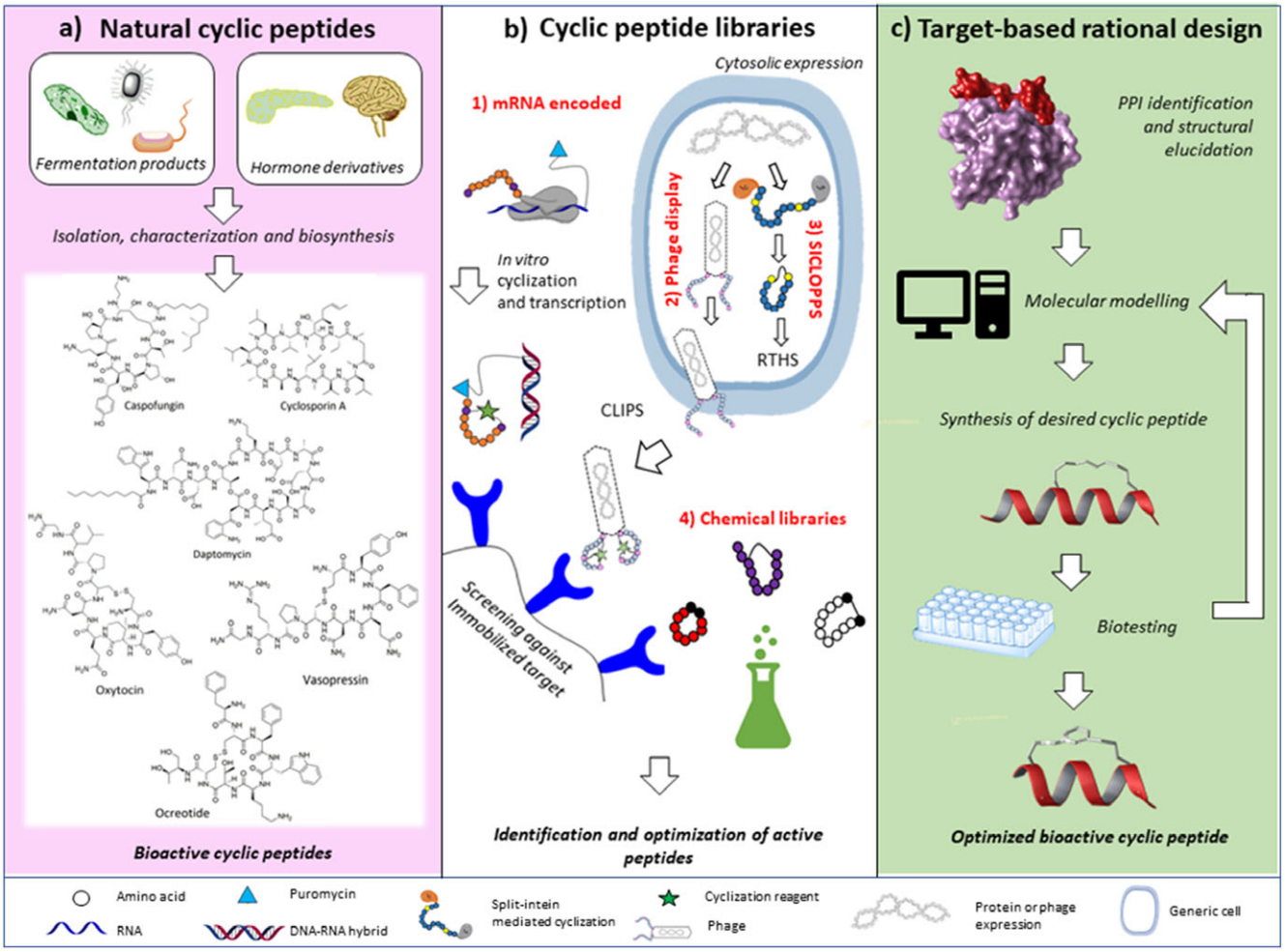


Figure 2. Schematic representation of the some of the technologies used to access biologically active cyclic peptides. a) Natural products obtained from microorganism or biosynthesis of hormone-like compounds. b) Cyclic peptide libraries: 1) mRNA encoded libraries expressed in vitro and cyclized using specific reagents; 2) phage-display libraries expressed by bacteriophages in host cells and cyclized after phage release either by disulfide bridge formation or by CLIPS technology; 3) genetically selected cyclic peptides expressed in bacteria, cyclized in cell using the SICLOPPS technology, and tested in functional assays in cells; 4) chemical libraries of cyclic peptides synthesized in parallel or using combinatorial chemistry. c) Target-based rational design of cyclic peptides: a generic PPI is shown as pink and red surface, designed peptides as red helices, and staple in grey sticks. RTHS, reversed bacteria two-hybrid system.

years, a number of biological cyclic peptide libraries including unnatural amino acids have been reported.[41] Noteworthy, are the mRNA-encoded peptide libraries developed in the Suga group: thioether macrocyclic peptides were obtained by incorporating unnatural *N*-(chloroacetyl)-D-Trp or *N*-(chloroacetyl)-Tyr into mRNA encoded libraries followed by spontaneous cyclization with Cys residues.[42,43] In addition, 5-hydroxytryptamine and benzylamine amino acid derivatives were assembled in vitro into linear peptidic sequences and cyclized using a photogenic oxidative coupling to obtain fluorescent cyclic peptides.[44]

Chemical libraries of cyclic peptides can be obtained by parallel or combinatorial synthesis. While parallel synthesis gives access to a limited number of macrocycles, combinatorial methods allow the generation of a greater number of compounds. Once assembled, the chemical peptide libraries can be screened against a selected target before or after cleavage from the resin support and the peptide sequence will then be identified after the screen.

The target-based rational design approach aims at developing cyclic peptides for a specific validated target. This approach is more suitable for research groups with a deep understanding of the structural features of the target protein and does not require access to a library of biomolecules. Whilst this approach gives access to a limited number of peptides, it allows more flexibility for peptide modification. Moreover, it should not be forgotten that a target-based rational design approach can be used to optimize peptide hits coming from library screenings. In a typical example, an X-ray crystal structure of the target protein or of the native PPI is desired (Figure 2). Once the part of the interaction that can be replaced by a constrained synthetic peptide has been identified, computational modelling can guide the design of the peptide.[17] Subsequently, the designed peptide is synthesized and assessed in vitro. Iterative cycles of enzymatic, cellular, structural assays and synthesis will allow understanding of the structure–activity relationship (SAR) and thus further redesigns until the optimal

peptide is made prior to in vivo testing. Being target-based, this approach offers the advantages of knowing the target in advance and hence can generate novel synthetic peptides with a high affinity for the desired target. Moreover, functionalization of the peptide to tune its pharmacokinetic properties is possible from the start and, if structural information is available, the functionality will be placed in such a way that it does not impact the binding affinity. Among the disadvantages, there is the necessity of structural information to guide the design. Molecular modelling on its own can be challenging and unreliable when dealing with flexible macrosystems and hence X-ray crystal or protein NMR structures are preferred, but can be difficult to obtain.

In summary, there are many different approaches that can lead to the discovery of bioactive cyclic peptides and the choice of the one to use depends on resources and needs. In the following sections, we will focus on the description of macrocyclic peptides obtained with 2C stapling methodologies.

3. Two-Component Peptide Stapling (2C-PS) Techniques at a Glance

Since the advent of peptide stapling as a tactic to overcome the limitations of linear peptides as therapeutics, there has been a plethora of different stapling methodologies reported. The very first methods involved the intramolecular linkage of endogenous amino acid side chains, which is known as one-component peptide stapling (1C-PS). The principle of 1C-PS involves the very straightforward intramolecular chemical linking of reactive amino acid side chains, for example, a lactamization reaction between lysine and either aspartic or glutamic acid. From the early days of peptide stapling, many 1C methods have been developed using both natural and unnatural amino acids. To this day, the "gold standard" technique of 1C-PS remains the all-hydrocarbon staple, formed by ring-closing metathesis (RCM) of alkene bearing amino acids. This approach was originally exemplified by Verdine[45] based upon prior work by Blackwell and Grubbs.[46] This method has been utilized in the development of many peptide therapeutics over the past 20 years including ALRN-5281, the first stapled peptide to enter clinical trials.[47] For a focused discussion on all-hydrocarbon stapled peptides and other macrocyclization strategies developed thereafter, the reader is redirected to a number of excellent reviews.[14,48–52]

2C-PS, the reaction of a bifunctional linker with two side chains on the linear peptide, was developed to overcome some of the intrinsic issues with 1C-PS. The next paragraphs will focus on some of the recent research into 2C-PS methods, which are of interest because of their novel application of synthetic chemistry or alternative approach to developing stapled peptides useful in medicinal chemistry.

3.1. Why Use 2C-PS?

In any medicinal chemistry campaign, a large number of analogues must be generated to determine the structure–activity relationships and optimize the physicochemical properties. This is also the case when developing stapled peptides, as there are many different variables to consider, including the primary amino acid sequence, position of the staple, length of the staple linkage, and the chemical composition of the staple. In addition to this, to aid biophysical assays and improve the stability of the biomolecules, the addition of functional groups such as fluorescent tags, affinity labels, and groups to improve solubility or permeability might be necessary. A limiting factor of 1C-PS is the amount of synthetic effort involved to modify or functionalize the stapled peptides. Any modifications to the staple or to the sequence would require resynthesis of the modified amino acid, incorporation of this new amino acid into the linear peptide sequence, and re-stapling. Significant optimization may be required for each of these steps, including the insertion of the new amino acid in solid-phase peptide synthesis (SPPS) procedures. Furthermore, any alteration of the peptide primary sequence may alter binding to the target.[53] It was to address these limitations that 2C-PS was developed, as modification of a 2C stapled peptide simply requires synthesis of the modified linker and re-stapling of the same linear peptide. This allows rapid generation of a diverse library of stapled peptides from one linear sequence in a combinatorial manner. Moreover, the creation of a toolbox of functionalized staples for 2C chemistries would facilitate the development of any stapled peptide, regardless of the target. An overview of the main 2C-PS techniques developed so far is given in **Figure 3**.

The majority of 2C-PS techniques were developed from their 1C counterparts, for example, the bis-lactamization performed by Phelan and coworkers[54] (refer to **Table 1** for a comparison of 1C and 2C-PS methods).

3.1.1. Natural Amino Acids

Natural amino acids are much easier to use than unnatural; they are more cheaply and widely available and hence simplify the synthesis of the linear peptide. Using natural amino acids also means that the alterations made to the wild-type (WT) peptide sequence can be kept to a minimum, rather than introducing structurally different unnatural amino acids which could have an adverse effect on binding. The use of natural amino acids, unlike unnatural amino acids, could potentially lead to problems with orthogonality and chemoselectivity. The most reactive amino acid side chains present themselves for use in stapling, and therefore many of the 2C-PS approaches utilizing natural amino acids are based around lysine and cysteine, and, to a limited extent, tryptophan.

3.1.1.1. Tryptophan. The use of tryptophan represents an under-explored area and it is worth mentioning as its use provides opportunities for staple functionalization. Johannes et al. have developed a method wherein two tryptophan residues at $i,i+4$ positions were reacted with a para-substituted benzaldehyde in a condensation reaction (Figure 3c). This resulted in a conformationally rigid non-helical model peptide which had vastly improved proteolytic stability toward proteases compared to the linear analogue (100% intact stapled peptide after 100 h incubation vs $t_{1/2}$ of 69 min for the linear analogue).[76] Tryptophan offers the advantage of being present in an extremely low abundance in natural proteins, therefore, reducing the potential

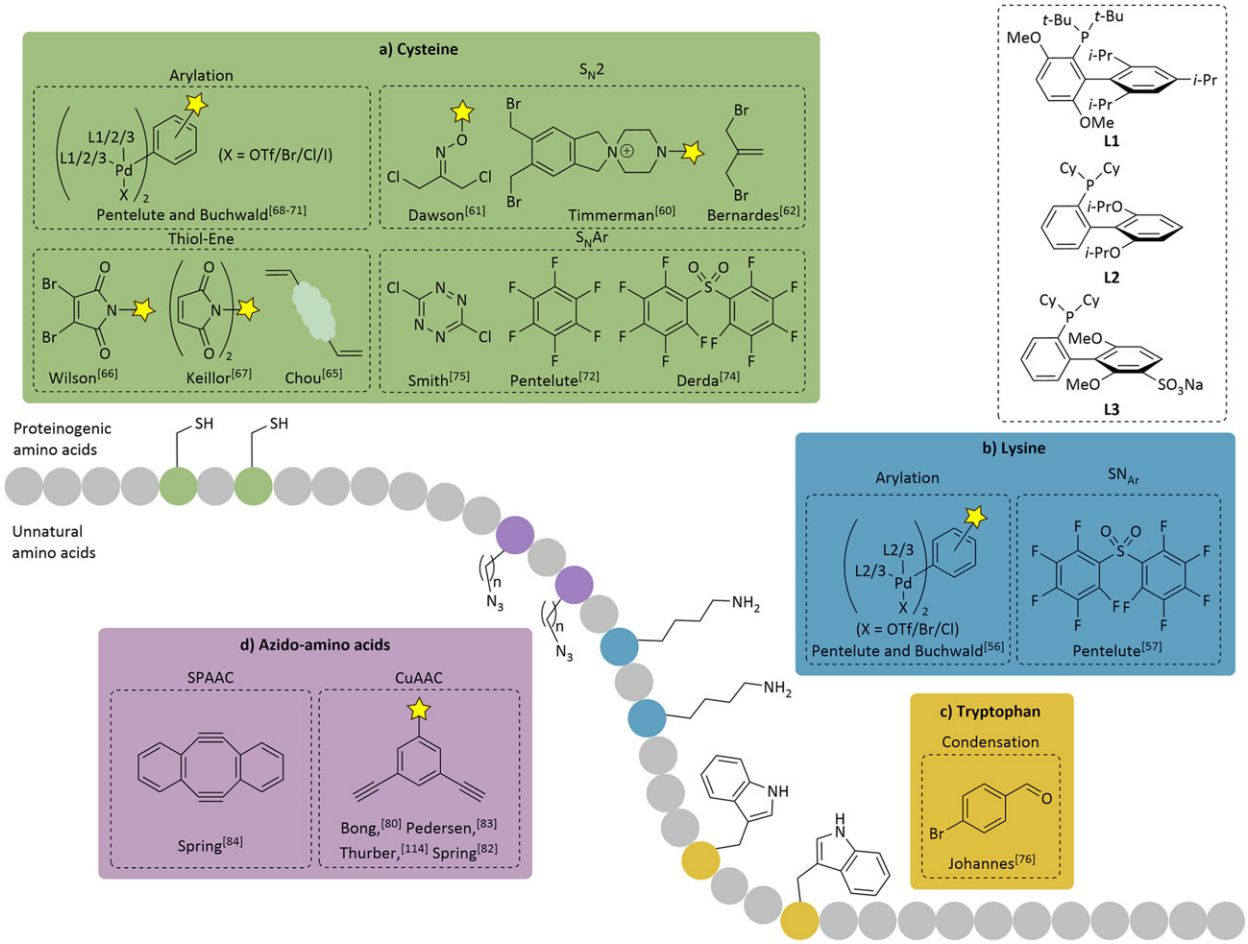


Figure 3. An overview of the 2C-PS methods described in this progress report utilizing: a) cysteine residues, b) lysine amino acids, c) tryptophan residues, d) azido amino acids. Methods involving proteinogenic amino acids are shown in the top half, and those utilizing unnatural amino acids are shown in the bottom half of the figure. The stars represent methods where the linkers have been functionalized, either with fluorescent tags, cell-permeable motifs, drugs, or affinity labels. Cy, cyclohexyl.

Table 1. A table outlining some 2C-PS methods discussed in this section.

Amino acid	Method	1C	Ref.	2C	Ref.	Functionalized 2C linker?
Lys	Lactamization	Yes	[55]	Yes	[54]	No
	Pd-arylation	No		Yes	[56]	Yes
	S_NAr	No		Yes	[57]	No
Cys	S_N2	No	[63]	Yes	[58–62]	Yes[60,61]
	Thiol-ene	Yes		Yes	[64–67]	Yes[64,66,67]
	Pd-arylation	No		Yes	[68–71]	Yes
	S_NAr	No		Yes	[72–75]	Yes[75]
Trp	Condensation	No		Yes	[76]	No
Azido	CuAAC	Yes	[77–79]	Yes	[80–83]	Yes
	SPAAC	No		Yes	[84]	No

for chemoselectivity issues. This is an excellent example of how relatively simple and robust chemistry can be effectively applied to peptide stapling.

3.1.1.2. Cysteine. Cysteine has been extensively exploited for protein and peptide modification[85,86] due to the high nucleophilicity of the sulfhydryl group[87] and its relatively low occurrence in living systems.[88] The transformations of conjugate addition ("thiol-ene") and S_N2 are the most common reactions for the application of cysteine to peptide stapling. The simplest 2C cysteine-based peptide stapling method is bis-alkylation using alkyl or benzyl halides. Separate work by Greenbaum[58] and Waters[59] investigated the optimal linkers for bis-alkylation of cysteine at $i,i+4$ and $i,i+3$ positions.

Over the past few years, many examples of cysteine stapling have been published which highlight beautifully the ease of linker modification with 2C-PS (Figure 3a). Regarding peptide stapling using a S_N2 reaction, Timmerman and coworkers have more recently developed novel functionalizable, water-soluble peptide staples derived from the aryl-halide linkers used in work by Greenbaum and Waters.[58–60] Additionally, in an elegant and simple method, Dawson et al. used 1,3-dichloroacetone (DCA) to staple between cysteine (or homo-cysteine) residues at $i,i+4$ positions via a S_N2 reaction. The use of cysteine or homo-cysteine on the same model sequence resulted in different

degrees of helicity as determined by circular dichroism (CD) with homo-cysteine being the most effective at enhancing helicity (53% vs 35%). They then used the ketone moiety to install functionality via oxime linkages.[61] Similarly, Bernardes et al. have recently reported a biocompatible and chemoselective methodology which exploits the S_N2 reaction between cysteine and isobutylene to give cell-permeable grafted peptides.[62] Moving on from S_N2, Jiang and coworkers published a thiol-ene approach using divinyl sulfonamides as linkers for 2C-PS with cysteine.[64] Finally, Chou and Wang published a light-mediated thiol-ene 2C stapling method, utilizing bis-alkene linkers of different lengths to successfully staple between the $i,i+4$ and $i,i+7$ positions.[65] In a more unusual application of cysteine, the Pentelute group have developed an S_NAr cysteine stapling technique based on their previous studies into the reactivity of sulfhydryl groups toward perfluoroaryl compounds.[72] A similar method was developed by Derda and coworkers using decafluorodiphenylsulfone.[74] Whilst the linkers were not functionalized, Pentelute and coworkers highlighted the unusually lipophilic nature of the perfluoroaryl linkers, and recently published on the subject of their potential application in the development of peptides able to penetrate the blood–brain barrier.[73] This work is especially interesting as it highlights the ability of unusual linker constructs to influence the overall properties of the peptide, and thus open up a new area of application for stapled peptides.

Transition-metal-catalyzed reactions usually require very specific reaction conditions, often not biocompatible, therefore eliminating a wide range of interesting synthetic transformations from being applied to complex biomolecules. The groups of Buchwald and Pentelute have jointly developed a biocompatible method for cysteine-arylation using palladium catalysis.[70,71] This methodology was applied to peptide stapling at both $i,i+4$ and $i,i+7$ positions and was shown to be rapid, facile, and compatible with many potentially interfering amino acids. As a 2C method, the modular construction of these stapled peptides allowed a systematic investigation into the effect of the linker on a range of physicochemical properties (such as lipophilicity and plasma-protein binding), which are rarely considered until a later stage in the drug discovery process.[69] As part of investigations into a protein cross-linking system, this work has recently been developed to a step-wise stapling strategy using the cysteine-arylation followed by urea formation with a lysine residue to form functionalized macrocycles.[68]

Finally, due to the nature of the reactivity of the sulfhydryl groups in cysteine, it has been applied in reversible stapling methods. Reversible stapling systems are those where the stapled peptide is able to either conformationally change, or the staple linkage is able to reopen to allow the peptide to gain flexibility. Reversible systems which efficiently pass between rigid and flexible states in a biorthogonal manner would in theory allow researchers to elucidate the relationship between target binding, biological activity, and secondary structure (or rigidity). Wilson and coworkers developed a reversible method using the reaction between dibromomaleimides and cysteine.[66] The constrained peptides showed increased helicity and improved proteolytic stability compared to the linear peptide, and the linkers could be functionalized. The authors then made use of the instability of thiol-maleimide linkages, thus the stapling was reversible when subjected to aqueous solutions of a variety of thiols, including glutathione (GSH). These conditions are comparable to those found in cancer cells, which could allow application of this reversible stapling method to targeted drug delivery. The authors comment on how the peptide may become unconstrained upon entry into the cell and would therefore be less likely to be transported back out of the cell. A similar method was developed by Keillor and coworkers using functionalized maleimides.[67]

Another cysteine-based reversible 2C-PS method was developed by Smith et al.[75] In an elegant reaction cycle, 3,6-dichloro-1,2,4,5-tetrazine was introduced via an S_NAr reaction at a variety of positions. The stapled peptide, when subjected to UV radiation, liberates molecular nitrogen to yield the uncyclized peptide bearing cyano groups on the cysteine residues. This modified linear analogue could be returned to the unmodified starting peptide via treatment with excess cysteine. The authors also demonstrated the successful functionalization of the linker via inverse-electron demand Diels–Alder reaction, and were able to attach a fluorescein dye. The conjugation was performed under biocompatible conditions on a protein. This work allows rapid transformations between cyclized and linear peptides, and if all the reaction conditions could be developed to be biocompatible, it could reveal the extent of the link between secondary structure and biological activity for individual targets.

3.1.1.3. Lysine. The 2C chemical linkage of lysine formed the basis of the first 2C-PS method, bis-lactamization. This was developed by Phelan and coworkers from the 1C method and involved an intramolecular amide bond formation. Despite its simplicity, a dual-protection strategy was required to ensure high purity and conversion to the desired product.[54]

Fast-forward 20 years, and the groups of Buchwald and Pentelute have applied palladium-mediated arylation of lysine, making use of modern synthetic organic chemistry for peptide stapling. This was to complement their reported cysteine-arylation methodology[56] to address some of the issues with using cysteine for stapling, namely the scarcity of cystine in native proteins and peptides (requiring alteration of the peptide primary sequence in the majority of cases) and the potential for instability with some thiol-linkages. They have developed a system using air-stable Pd(II)-aryl complexes under mild conditions, although the reaction suffered due to interference of some unprotected amino acid residues. The groups showed that a wide range of complex biomolecules and affinity tags could be attached to a lysine residue on a model p53 peptide followed by palladium-mediated macrocyclization.

Pentelute and coworkers were also involved in the development of an unusual stapling technique where perfluoroaryl sulfone linkers were applied to lysine stapling.[89] This methodology was developed to overcome the problems associated with cysteine oxidation and disulfide formation, which can hamper cysteine-arylation reactions. Both of these recent examples of 2C lysine stapling chemistry are impressive examples of a nontrivial organic transformation performed on a complex biomolecule.

3.1.2. Unnatural Amino Acids

The use of unnatural amino acids for peptide stapling requires the purchase or synthesis of non-proteinogenic amino acids, and their incorporation into the peptide synthesis procedure. This

can introduce a high monetary and time cost to this process. However, the use of unnatural amino acids can also provide excellent orthogonality and alternative staple compositions. The advantages of using unnatural amino acids can massively outweigh the disadvantages, and it is illuminating that the most commonly used peptide stapling method, ring-closing metathesis, uses unnatural amino acids yet still finds high levels of usage in industry and academia for the development of pharmaceutically relevant stapled peptides.[52]

3.1.2.1. Cycloadditions. A widely used reaction that allows 2C-PS using unnatural amino acids is the cycloaddition. Meldal and Sharpless widely hailed the Huisgen 1,3-dipolar cycloaddition of alkynes and azides as the premier example of "click chemistry," an umbrella term used to describe reactions of high yields, wide scope, mild conditions, and facile setup.[90,91] The CuAAC fits this bill completely, and thus was successfully applied to 1C stapling methods.[77,78,92] Bong and coworkers first developed the 2C CuAAC peptide stapling method.[80] They introduced azidoalanine residues in $i,i+4$ positions of a GCN4 leucine zipper peptide, and reacted with a bis-alkyne linker. Whilst this stapling method induced helicity in the peptides, the linkers were not functionalized.

We were inspired by the advantages of the 2C-PS approach to develop our own methodology, namely the incorporation of the CuAAC and strain-promoted azide–alkyne cycloaddition (SPAAC) as $i,i+7$ 2C stapling techniques, which will be discussed later. The application of our stapling methodology in the development of a diverse range of PPI inhibitors, involving helical, constrained peptides and a toolbox of functionalized staples will be discussed later.

3.2. Which Staple is the Right Staple?

There are very few comparative studies wherein one linear peptide is subjected to multiple different stapling techniques, with the biological and chemical properties assessed. This is presumably due to the amount of work required to optimize several different stapling systems. Numerous studies exist comparing different 1C methods;[93–95] however, only one, by Fairlie and coworkers, directly compares two cysteine-based 2C-PS methods on one model peptide.[96] Even then, this gives limited information to a researcher wishing to choose the best 2C-PS method.

How is the appropriate staple chosen? The synthetic feasibility of most amino acid stapling techniques, which utilize natural amino acids has allowed researchers to perform screens to investigate the effect of properties such as length and rigidity on the final stapled peptide. There are several factors involved with the development of a stapled peptide, including the choice of stapling method, the length of "staple" required and the position of the staple attachments. An interesting way of approaching this dilemma is to allow the linear peptide to kinetically select the best linker. If a peptide is naturally inclined to adopt a helical structure, then the staple which best fits the gap between the reactive amino acid side chains will have the fastest, most efficient reaction. Greenbaum et al. performed such a screen, looking at the reaction between a model peptide and a range of commercially available alkyl and benzyl halides[58] (a similar screen was performed by Lin et al.).[97] Other screens have been performed looking at different effects, namely those reported by Fujimoto and coworkers[98] and Woolley and coworkers.[99]

As discussed in this last section, there are a vast number of different 2C peptide stapling methods, each with disadvantages and advantages to suit any target peptide and biological application. The most significant examples are those that allow facile functionalization of the stapled peptide to allow researchers to assess the utility of their peptides in biophysical assays.

4. 2C CuAAc Stapling: Where do we Stand?

4.1. CuAAC 2C-PS: Advantages and Limitations

We became interested in the 2C CuAAC, inspired by the work by Bong and coworkers on the 2C $i,i+4$ peptide stapling method,[80] based in turn on the 1C version developed by D'Ursi[78] and Wang.[79] The main aim during the development of our novel methodology was to expand the existing method to include $i,i+7$ stapling and beyond, and to introduce functionalization on the linker. In recent years, we have developed a toolbox of bioactive helical and non-helical peptides and functionalized staples.

The 2C CuAAC stapling approach (commonly referred to as the "double click") involves an intermolecular coupling between two azido side chains of a linear peptide and a bis-alkynyl linker to give the cyclized peptide. However, competing alternative reaction pathways and relatively high peptide concentration could lead to undesired products (**Figure 4**). As a result, the optimized macrocyclization reaction is typically carried out in aqueous conditions, at relatively low concentrations of peptide and with close to one equivalent of the linker.

Despite the wide range of stapled, functionalized peptides that can be accessed with this methodology, there are some significant drawbacks to be overcome before it can be truly appreciated as the method of choice for peptide stapling. Non-proteinogenic amino acids containing azido side chains are required for the CuAAC approach and can be easily synthesized in a few steps from commercially available Fmoc-protected amino acids, with minimal purification required.[100] However, the handling of azido amino acids may be a limiting factor for their application. An inverse 2C-PS methodology using acetylene-alkyl amino acid and di-azido staples would be fully compatible with SPPS; however, it would require the rather challenging synthesis of Fmoc-protected alkyne-containing amino acids and hence would not provide advantages over the classic CuAAC methodology.[101]

In addition, the CuAAC methodology requires the use of a copper(II) catalyst. This has the associated limitations of air-sensitivity of the catalytic copper-complex, requiring all solvents to be degassed[81] and the risk of toxicity arising from any residual copper in the final stapled peptides. Both of these issues have been addressed with the development of the copper-free SPAAC (see Section 4.2) pioneered by Bertozzi and coworkers.[102]

Unlike some other stapling methods, the 2C CuAAC reaction takes place in solution as opposed to on-resin possibly due to on-resin site isolation issues.[14] As a result, a major drawback to this chemistry is the potential requirement for multiple purification steps; firstly of the linear peptide, and then the final stapled

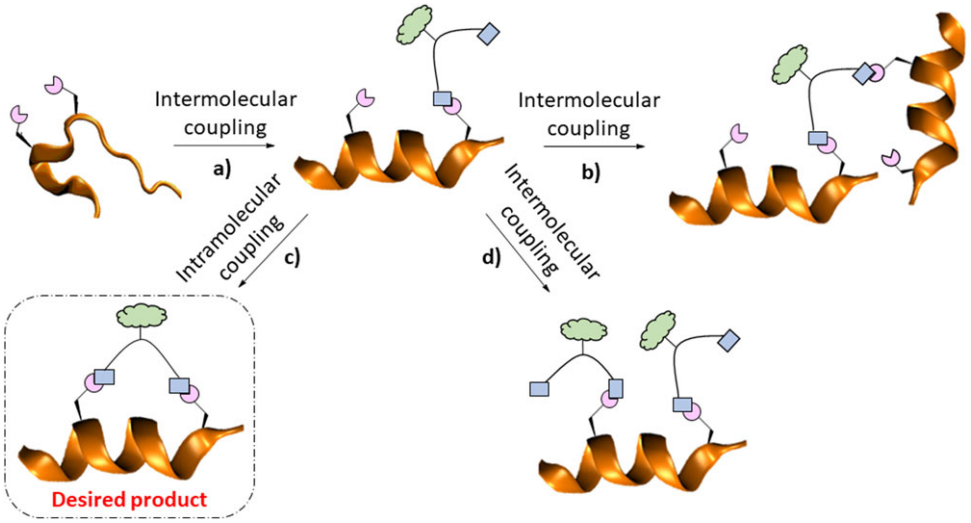


Figure 4. a) Potential reaction pathways that can occur during 2C-PS after the initial intermolecular coupling. c) Intramolecular coupling to give the desired product macrocycle or b,d) a number of different undesired products due to multiple intermolecular coupling reactions.

peptide resulting in loss of material. The possibility of carrying out the 2C CuAAC stapling reaction on resin may overcome these issues. An on-resin variation of this stapling has been reported since the original Spring Group work was published,[83] and if conditions could be developed to give consistent success for the CuAAC on-resin reactions, this would vastly increase the impact of this work.

The main use of the 2C CuAAC PS approach focuses on $i,i+7$ stapling of helical peptides (that is stapling across two turns of the helix). However, an $i,i+4$ and two $i,i+6$ peptides have also been reported.[104–106] It is clear that linkers suitable for stapling at other positions, aside from the widely exploited $i,i+7$, need to be validated.

Nevertheless, there are a number of advantages that outweigh the drawbacks of this methodology. In particular, azido amino acids are compatible with SPPS and the use of this type of unnatural amino acids avoids chemoselectivity issues often associated with proteinogenic amino acids.

In addition, the conditions used in the stapling are mild and functionalization of the linkers is relatively easy allowing for the fast generation of diverse stapled functionalized peptide libraries. Considering the nature of the staple, using the 2C CuAAC results in stapled peptides that contain a bis-triazole motif. As with any stapling technique, these linkers give the final compound distinct properties and could potentially interact positively at the PPI surface, thus improving binding.[110] Overall the advantages of the double CuAAC 2C-PS are highlighted by the number of PPIs successfully disrupted in cells (see Chapter 5).

4.2. An Improved Methodology: Strain-Promoted Macrocyclization of Peptides

We sought to address some of the issues with the $i,i+7$ 2C CuAAC stapling method discussed previously, namely the biocompatibility due to the toxicity and air sensitivity of the copper catalyst complex, and the lack of on-resin stapling success. This creates a major bottleneck in screening for stapled peptide inhibitors, as when many peptide variants are required, each linear peptide must be purified, stapled, and repurified. We wanted to develop a stapling technique that was so simple and biocompatible that it could be performed in assay media on a large peptide library, to quickly identify the strongest binding peptides out of an array of variants. For this we turned to the strain-promoted azide–alkyne cycloaddition (SPAAC), which is a highly versatile reaction and is ideal for these demands as it is catalyst-free, does not require an inert atmosphere, and is fully orthogonal to the reactivity of other amino acid side chains. The identification of this reaction and its application to bioconjugation was pioneered by Bertozzi and coworkers.[103,107,108] The 2C $i,i+7$ CuAAC methodology was easily modified to accommodate this new reaction. Azide-containing amino acids were introduced at $i,i+7$ positions in a p53-derived peptide and stapled cleanly and efficiently with a strained diyne in the presence of p53 reporter cell assay medium. All of the stapled peptides were formed in good yields, and the order of activity was confirmed by ex situ resynthesis and testing of the same stapled peptides.[84] Currently the reported diyne linkers used in the SPAAC stapling have not been functionalized; however, this is an active area of research in our group. Due to this limitation, improving factors such as solubility and permeability must be performed by alterations to the peptide sequence or by the addition of groups to the termini of the peptide. Consequently, the SPAAC stapling methodology is best used as a high-throughput technique for the identification of the best linear peptide variant; however, in order to impart the desired properties on the stapled peptide, the CuAAC stapling may be required. Additionally, in situ stapling often gives lower cell-based activities than pure, pre-stapled peptides, likely due to the formation of side-products (such as isomers). Consequently, whilst the in situ SPAAC stapling method gave very quick results, in order to validate these results, the stapled peptides need to be resynthesized. A comparison of the SPAAC with the 1C and 2C CuAAC peptide stapling is shown in **Figure 5**.

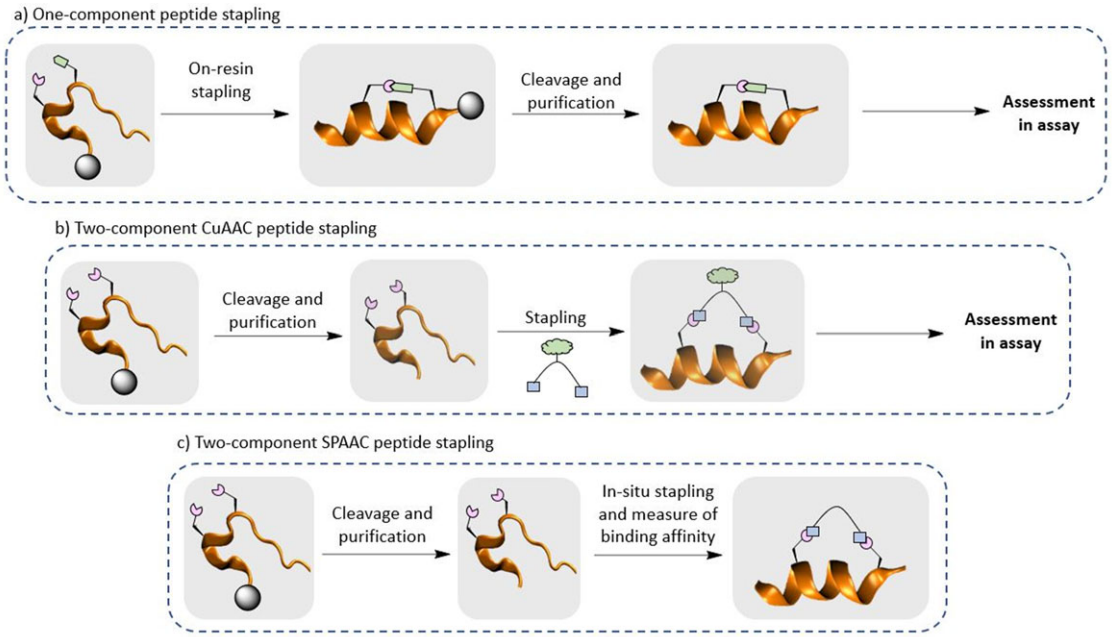


Figure 5. A diagram comparing the length of the processes involved to synthesize, staple, and functionalize peptides for analysis using biophysical assays via a) one-component peptide stapling, b) 2C CuAAC peptide stapling, and c) the 2C in situ SPAAC stapling.

It is worth noting that other 2C-PS systems have the potential to be fully biocompatible, namely the reversible oxime staples developed by Haney, Horne, and coworkers[109] and photoswitchable linkers.[110] The advancement of other stapling techniques to be fully biocompatible is necessary to improve the efficacy and efficiency of high-throughput inhibitor identification.

5. Rational Design of Conformationally Constrained Peptides in 2C-PS

The design of conformationally constrained peptides obtained via 2C-PS relies heavily on the availability of high-resolution X-ray crystal structures of the PPI of interest. In fact, molecular modelling of the desired PPI is usually the starting point for rational design. Computational alanine scanning (CAS) allows the determination of hot spots and simultaneously gives an indication of the residues that can be varied.[111] Moreover, the X-ray crystal structure of the native PPI shows the complex in its binding conformation and hence it is of crucial importance to determine the stapling position and staple type to use to lock such a conformation. If the part of the protein that is to be mimicked is an α-helix, the interest goes to the amino acids on the same face of the helix. Amino acid side chains spaced by 4, 7, and 11 residues are known to point toward the same face of an α-helix. Once the binding conformation and stapling positions have been identified, molecular modelling will guide the design of a suitable distance-matching staple and amino acid side chain length ("staple design set" from now onward) to use to avoid or minimize disruption of the binding conformation and molecular dynamics will assess their suitability. Ultimately, a series of constrained peptides are made in order to understand whether the staple introduced is the right one to stabilize the conformation and potentially increase the binding affinity for the targeted protein. Increase in binding affinity could be obtained by a reduction in entropic loss upon binding following conformational constraint explicit by the staple and/or by favorable interactions between the staple and the protein. x-ray crystallography or NMR studies could then help further design and understand binding modes.

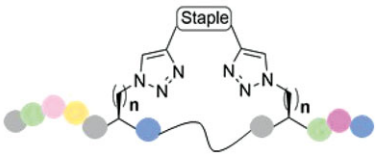
Unlike 1C-PS, in the 2C-PS field, extensive comparative studies in which the same staple design set has been applied to different peptide sequences are still missing. Various combinations of the stapling design used on different sequences and that resulted in stabilization of the secondary structure are shown in **Table 2A** (for 2C CuAAC and SPAAC PS) and **Table 2B** (for 2C stapling methodologies using Cys and homo-Cys as amino acids) with the aim of facilitating the design of new 2C conformationally constrained peptides. It should be noted that only the combinations resulting in the greatest stabilization of the secondary structures are reported. The reader is redirected to the literature cited in the tables for more combinations.

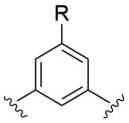
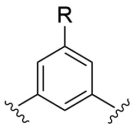
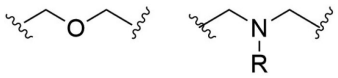
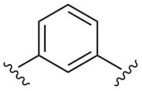
From the data reported, it appears that the only combination that has been applied to different sequences embraces azido ornithine or homo alanine azido amino acids to be used in $i,i+7$ stapling in the presence of 1,3-dialkylbenzene. Such combinations resulted in enhanced helicity of all the stapled peptides considered (p53, G$s\alpha$, GLP-1-derived peptides) compared to the linear sequence as determined by CD (Table 2A).

The amount of data reported in literature to date makes a similar comparison impossible when it comes to 2C-PS methodologies that use Cys (or hCys) considering the vast number of different reactions applicable.

It is therefore clear that more comparative studies are needed to set out design guidelines that could help scientists in the field to develop stapled peptides without having to optimize each single case.

Table 2A. A summary of the reported 2C CuAAC and SPAAC stapled peptides with structural information.



Position	*n*	Staple	Structure	Sequence	Structure stabilization	Ref.
i,*i*+4	2		Extended	TNKS (Ac-REXGDGXE-NH_2)	Enhanced	[103]
	3		Helix	Bim-BH (Ac-IWIAQELDRXGDXFNAYYARR-NH_2)	Equal	[106]
i,*i*+6	3		Helix	Cft4 (Ac-MDIXIDDILXELDKETTAV-NH_2)	Equal	[105]
i,*i*+7	2, 3		Helix	Model peptide (Ac-AAEAWAXAEAAEAXEA-NH_2)	Enhanced	[112]
				Gsα (Ac-RDIXQRN$_L$HLRXYELL-OH)	Enhanced	[83]
	3		Helix	p53 (Ac-LTFXHYWAQLSX-NH_2)	Enhanced	[113]
	2		Helix	GLP-1 (Ac-HAEGTFTSDVSSYXEGQAAKXFIAWLVKGR-NH_2)	Enhanced	[114]
				Exedin (Ac-HGEGTFTSDLSKQXEEEAVRXFIEWLKNGGPSSGAPPPS-NH_2)	Equal	

R, functional group.

6. Combinatorial Synthesis of Macrocyclic Peptides: A Toolbox of Functionalized Stapled Peptides

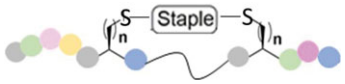
In this chapter, we describe the variety of functionalized stapled peptides developed with different 2C-PS techniques as well as a few successful examples of target-based development of 2C-CuAAC stapled peptides.

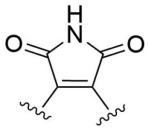
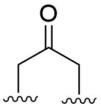
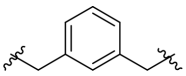
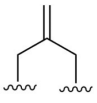
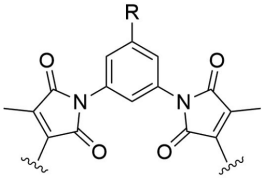
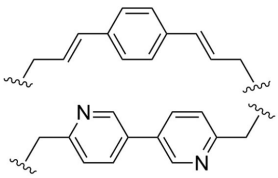
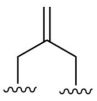
6.1. Functionalized Staples

Peptide stapling techniques have been mainly used to constrain a linear sequence into its bioactive conformation. The macrocyclization may result in an improvement in cell membrane–permeability and increased stability to proteases as well documented by many stapled peptides obtained via 1C-PS techniques.[52,115,116] Although it is widely accepted that stapling increases the proteolytic stability of the macrocyclized peptide compared to the linear analogue, data for stapled peptides obtained via 2C-PS is very limited and there is a lack of consistency in the methodologies used to analyze such properties. Therefore, more consistent analysis that relies on the use of realistic conditions—that is, plasma or serum—rather than single proteolytic enzymes are needed to draw definitive conclusions.

When developing a stapled peptide for biological uses, the introduction of several functionalities may be required. However, most of the stapling methodologies reported do not allow easy functionalization of the macrocyclic peptide. Indeed, the extra functionalities such as fluorescent tags, cell-permeable motifs, affinity tags, etc. are added onto the peptide sequence, either at the *N*- or *C*-terminus. This could reduce the already poor yield of SPPS drastically, considering that the coupling efficiency diminishes with the increasing number of amide couplings. Moreover, there is always the risk of affecting the conformation of the biologically active peptide if the spacer placed between the functionality and the peptide itself is not long enough, resulting in reduced binding affinity for the desired protein. Therefore, stapling methodologies that introduce extra functionalities in positions other than the peptidic sequence offer

Table 2B. A summary of the reported stapled peptides obtained with 2C PS methodology using Cys or homo-Cys.



Position	*n*	Staple	Structure	Sequence	Structure stabilization	Ref.
	1		Helix	BID-BH3 (Ac-EDIIRNIARHLAXVGDXNLDRSIW-NH_2)		[66]
	2			RNAase (Ac-KETAAXKFEXQHMDS-NH_2)		
i,*i*+4	2			Model (Ac-YGGEAAREAXAREXAARE-NH_2)	Enhanced	[61]
	1			Axin (Ac-ENPEXILDXHVQRVM-NH_2)		[65]
	1			Model (Ac-YGGEAAREAXAREXAARE-NH_2) Calpain (Ac-IPXKYRXLLA-NH_2)		[58]
			Extended	Model (Ac-XAAAX-NH_2)	Equal	[62]
				p53 (Ac-QSQQTFXNLWRLLXQN-NH_2)		[65]
i,*i*+7	1		Helix	Model (Ac-LSAAEXAAREAAXREAAARAGGK-NH_2)	Enhanced	[67]
				NOXA (Ac-AAXLRRIGDXVNLRQKLNN-NH_2)		[97]
i,*i*+11	1		Extended	Somatostatin (Ac-AGXKNFFWKTFTSX-NH_2)	Equal	[62]

R, functional group.

an advantageous alternative. Stapling techniques that provide this alternative include the previously described 2C CuAAC,[82,84] cysteine-functionalized maleimide,[66,67] cysteine-functionalized dibromobenzene,[117] "CLIPS,"[60] cysteine-dichloro acetone (DCA),[61] and cysteine-divinylsulfonamides.[64] In all of these peptide stapling chemistries, the staple becomes multifunctional: it constrains the peptide in the bioactive conformation, improves its stability in serum, and contains a chemical handle, which is used to insert additional functionality. The linear peptide sequence and the staple are elaborated separately, and then joined together to afford the functionalized stapled peptide in a combinatorial manner. In this chapter, we aim at providing

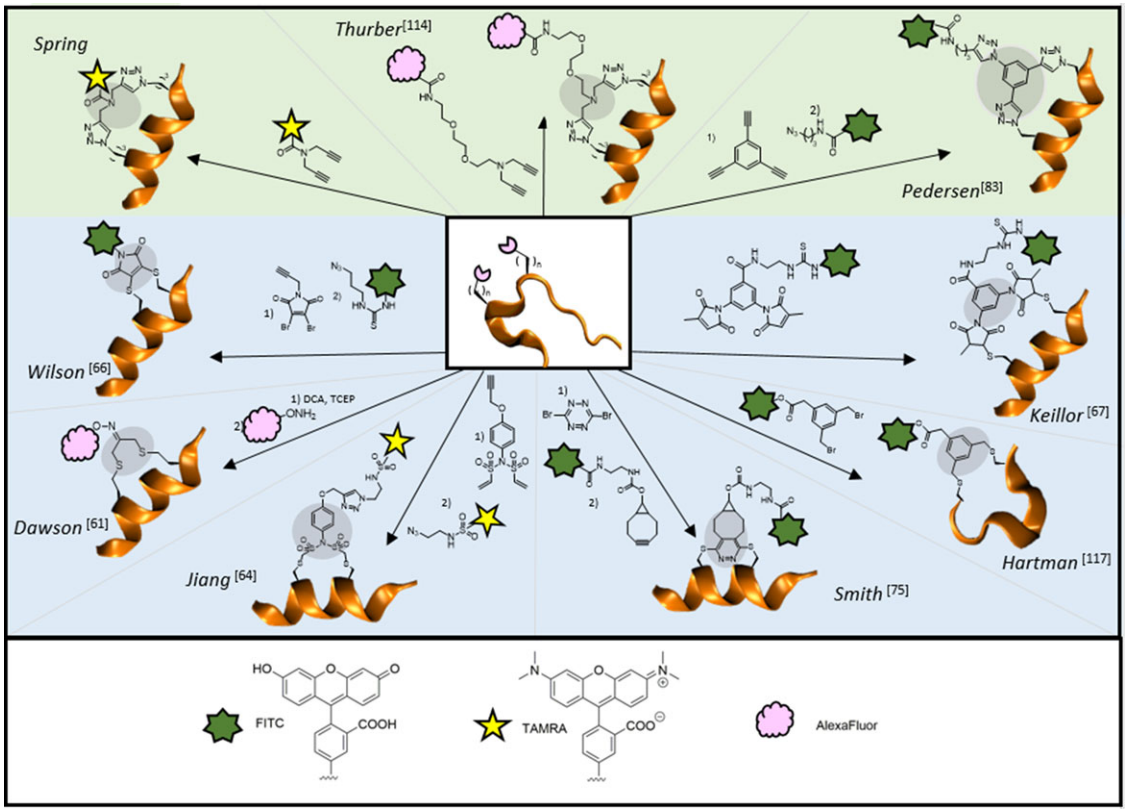


Figure 6. Schematic of the different methodologies developed by various groups to access functionalized stapled peptides bearing fluorescent tags on the staples. Top (green): methodologies that use azido amino acids. Bottom (blue): chemistries that use Cys residues. Staples are highlighted in grey. DCA, 1,3-dichloroacetone, dichloro acetone; TCEP, tris(2-carboxyethyl)phosphine.

an overview of the functionalities that have already been used and a few examples of motifs that could be introduced in the future to broaden the scope of the 2C-PS.

6.1.1. Tags Useful for Biological Assays

The appendices that can be added to peptides could not only improve the pharmacokinetic properties of the biomolecules but also serve as functionalities useful for biological assays. For instance, fluorescent tags can be added to monitor cellular uptake and subcellular localization, biotin may be useful in pull-down assays, and cross-linking motifs might be incorporated to assess target selectivity and potential off-target effects of a given peptide.

Incorporation of such motifs is traditionally done at the *N*-terminus of the peptides or by means of an unnatural amino acid. As mentioned previously, this could disrupt the interaction of the peptide with the target protein and therefore adversely affect the binding affinity. 2C-PS techniques that allow the functionalities to be placed on the staple may overcome this limitation and therefore offer an advantageous alternative.

Staples that carry fluorescent tags are among the most exploited functionalities in the peptide field. This is not surprising if we think about the poor membrane-permeability observed with most of peptides. The fluorescent tag allows visualization of the labelled peptide either on the cell membrane or in intracellular locations. Indeed, several groups have demonstrated the validity of this approach by using both unnatural and proteinogenic amino acids, as mentioned herein.

In our group, we have shown that the CuAAC staples bearing an amine are a suitable handle for the attachment of fluorophores, such as TAMRA, in one step via simple amide coupling (unpublished results, **Figure 6**). Similarly, Thurber et al. attached an AlexaFluor fluorescent tag to the staple of a GLP-1 stapled peptide.[114] In addition, Pedersen et al. made use of CuAAC chemistry to access a variety of fluorescently labelled stapled peptides—incorporating TAMRA and FITC—via a tri-alkyne staple which allows peptide stapling first and labelling in an additional step (Figure 6).[83]

Among the stapling techniques that allow stapling and functionalization using natural amino acids, the use of cysteine residues is very popular. Dawson et al.,[61] Keillor et al.,[67] Wilson et al.,[66] Hartman et al.[117] have incorporated several fluorescent tags—that is, TAMRA, FITC, AlexaFluor—onto the staple using 2C-PS chemistry described above (Chapter 3.1). An overview of some of the fluorescently labelled stapled peptides is shown in Figure 6.

In addition, Smith et al. have also introduced fluorescent tags into the staple by stapling two cysteine residues on the linear peptide with a 3,6-dichloro-1,2,4,5-tetrazine (Figure 6). The staple contains a handle for an inverse electron demand Diels-Alder reaction with the appropriately modified fluorescent tags.[75] Another example of peptide stapling using functionalized linkers

was reported by Jiang et al.: the group conjugated proteinogenic cysteine side chains with divinylsulfonamides bearing an alkyne handle. Using click chemistry, the handle was then attached to fluorescent dyes (Figure 6).[64]

As peptides are particularly useful for targeting PPIs, the elucidation of intracellular pathways in the presence or absence of the peptide is of interest in this field. The addition of tags to stapled peptides that would aid in the determination of a certain PPI is therefore particularly useful: this is the case for biotin (or vitamin H). Biotinylation is the covalent modification of a peptide (or proteins/nucleic acids) with biotin to exploit the high affinity that the latter has for avidin. The strong biotin–avidin interaction has many research applications including the enzyme-linked immunosorbent assay (ELISA), Western blot analysis, immunohistochemistry (IHC), immunoprecipitation (IP), cell surface labelling, and flow cytometry/fluorescence-activated cell sorting (FACS).

The incorporation of biotin into the peptide requires a spacer between the two parts to avoid the peptide interacting with avidin and the biotin tag interacting with the targeted protein. Once again, incorporation of the biotin tag onto the staple may be a suitable solution. Pedersen et al.,[83] Wilson et al.,[66] Hartman et al.,[117] Timmerman et al.,[60] Jiang et al.,[64] and Dawson et al.[61] have all showed that incorporation of the biotin tag into the staple can be achieved via the stapling techniques described above on an analytical scale. Similarly, incorporation of a FLAG epitope tag (octapeptide with sequence DYKDDDDK often used for protein purification or protein localization in living cells) was also achieved with the DCA (dichloro acetone) ligation stapling.[61] However, since no cellular data is reported on the peptides developed, the validation of this concept remains to be seen.

Photoaffinity labelling is another powerful technique that can be used to investigate protein–ligand interactions and a linker that functions as both a peptide staple and a cross-linking reagent could be particularly useful. Although cross-linking motifs have been incorporated into peptide sequences as amino acids,[118] their synthesis can be complicated. On the other hand, the synthesis of photo cross-linking staples such as a benzophenone photo cross-linking staple represents an easier alternative. We recently developed a benzophenone staple with multifunctionality: the 3,5-dialkyne benzene motif constrains a p53 peptide in the helical, bioactive conformation, the benzophenone group allows cross-linking with the desired mdm2 protein and the terminal alkyne on the benzophenone motif permits further functionalization (**Figure 7**).[104]

6.1.2. Cell-Penetrating Peptides

Cell-penetrating peptides are one of the most useful functionalities that can be appended onto peptides. Peptides are often characterized by poor membrane-permeability and therefore can require functionalization to reach the target intact and validate their cellular activity. It is not fully understood whether peptide stapling on its own is enough to provide cell-permeability and indeed, the staple type, stapling position, and peptide sequence seem to affect the ability of the peptide to enter into cells. Several examples of all-hydrocarbon stapled peptides have demonstrated that stapled peptides are able to translocate into the cytosol;

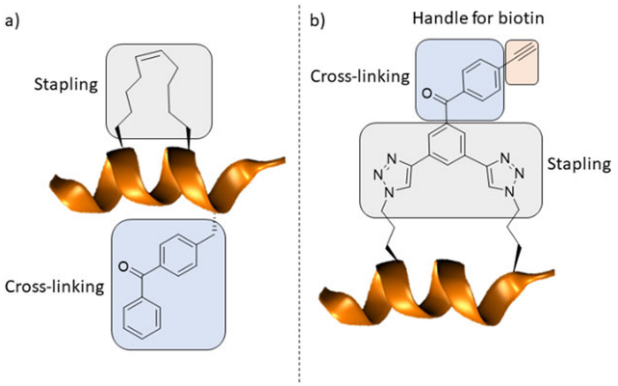


Figure 7. Differences between photo cross-linking functionalities: a) added as amino acid[118] and b) on the multifunctional staple.[104]

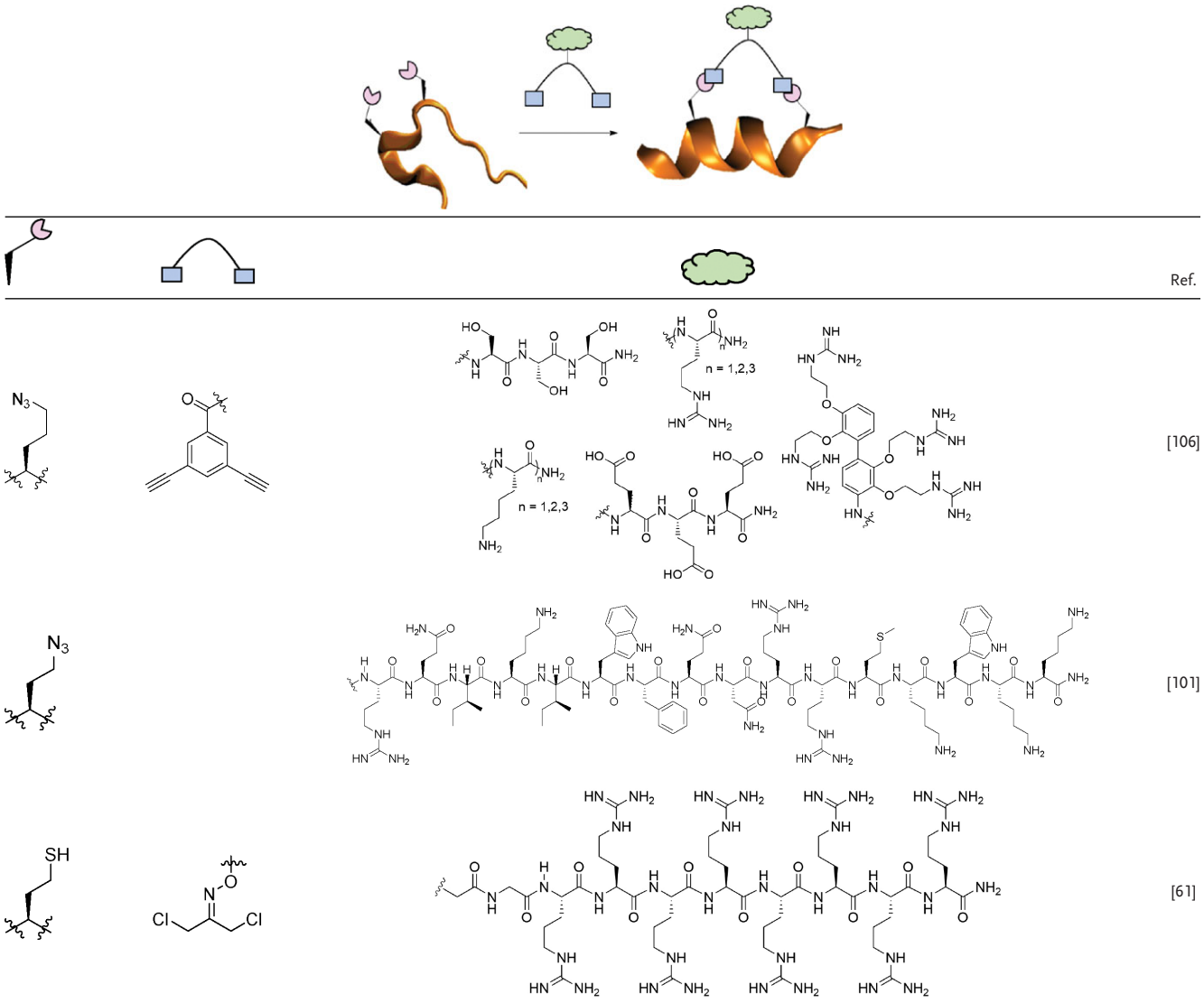
however, there is an enormous number of stapled and cyclic peptides that require a cell-permeable motif to reach their intracellular target.

The use of motifs composed solely of arginine residues has been shown to increase the cellular uptake of the peptides in several occasions. Poly(Arg) cell-penetrating peptide (CPP) motifs are traditionally composed of 6–9 arginine residues generally incorporated into the linear sequence.[119] The number of Arg residues necessary to translocate the peptide into the cytosol depends on different factors including the peptide sequence, cellular uptake mechanism, and cell type. Work by many research groups on p53-based peptides has shown that an increase in net positive charge of the peptide is necessary for cell penetration. We therefore included a poly(R_3) tag onto the staple and coupled it with a sequence based on the $p53_{17\text{-}29}$ peptide and successfully obtained cell activity. Interestingly, when the sequence was changed to the phage-display-derived sequence named PDI sequence (LTFEHYWAQLTS), cell penetration was achieved using an aliphatic linker for the double CuAAC stapling and therefore an increase in net positive charge was not required.[82]

The use of the 2C CuAAC stapling chemistry came particularly to hand when investigating peptide uptake into platelets.[106] A wide range of peptidic chains were incorporated onto the staple of p53-based fluorescently labelled peptides and cellular uptake monitored by flow cytometry and confocal microscopy. Among the chains screened, some of which are shown in **Table 3**, the poly(R_3) staple appears to be the most promising in penetrating into the cytosol without causing toxicity in platelets and it was chosen thereafter to translocate Bim-BH3-derived peptides (AcIWIAQELDOrn(N_3)IGDOrn(N_3)FNAYYARR-NH_2) intracellularly and hence exert bioactivity.[106]

Another example of functionalization of the staple with CPPs is provided by the work done by Itzhaki et al. in which a macrocyclized extended peptide targeting tankyrase showed cell-permeability when the Penetratin motif (RQIKIWFQNRRMKWKK) was incorporated into the aromatic staple (Table 3).[103] Once more, the peptide sequence was optimized independently from the staple, allowing the two optimized entities to be combined to obtain a potent, stable, and cell-permeable peptide. Similarly, in the Dawson lab, a polyarginine tag (R_8) was attached onto the ketone handle of a macrocyclized peptide via a chemoselective ligation methodology (Table 3).[61]

Table 3. A list of CPPs incorporated into the staples.



The staple sets shown were chosen to stabilize the binding conformation (see Chapter 5).

Most of the CPPs reported in the literature so far are linear peptidic sequences composed of natural amino acids. It is surprising that despite the numerous efforts toward the development of biologically relevant peptides with stability in vivo, very few attempts of stabilizing the CPPs are reported in the literature to date. Considering the many peptides that necessitate a cell-permeable motif to get into cells, more research aiming at improving the stability of these peptides in vivo is of crucial importance.

This can be achieved by replacing the natural L- amino acids with the D- stereoisomers, or via stapling. Therefore, an understanding of how stapling affects the cell-permeating ability of different CPPs is fundamental to this goal; only two examples of all-hydrocarbon and lactam-stapled CPPs have been published thus far.[120,121] The development of a diverse range of CPPs stapled with different techniques, the measurements of their pharmacokinetic properties and correlations with their membrane-permeability capabilities will pave the way to the development of stable CPPs, which could deliver plasma stable cargo peptides otherwise uncapable of reaching the cytosol of the targeted cells.

6.1.3. Staple to Improve Pharmacokinetic Properties of the Peptides

Stapling and macrocyclization of peptides may increase the stability of this group of biomolecules in plasma and hence extend their half-life by reducing the enzymatic degradation. However, the pharmacokinetic properties of stapled peptides can be improved further through increased binding to the plasma proteins and by slowing the globular filtration. This can be achieved in several different ways including fusion to serum proteins with extended half-lives such as albumin or the Fc fragment of an IgG, or by the introduction of polyethylene glycol (PEG) moieties or glycosylation sites. Many small molecules and proteins have been

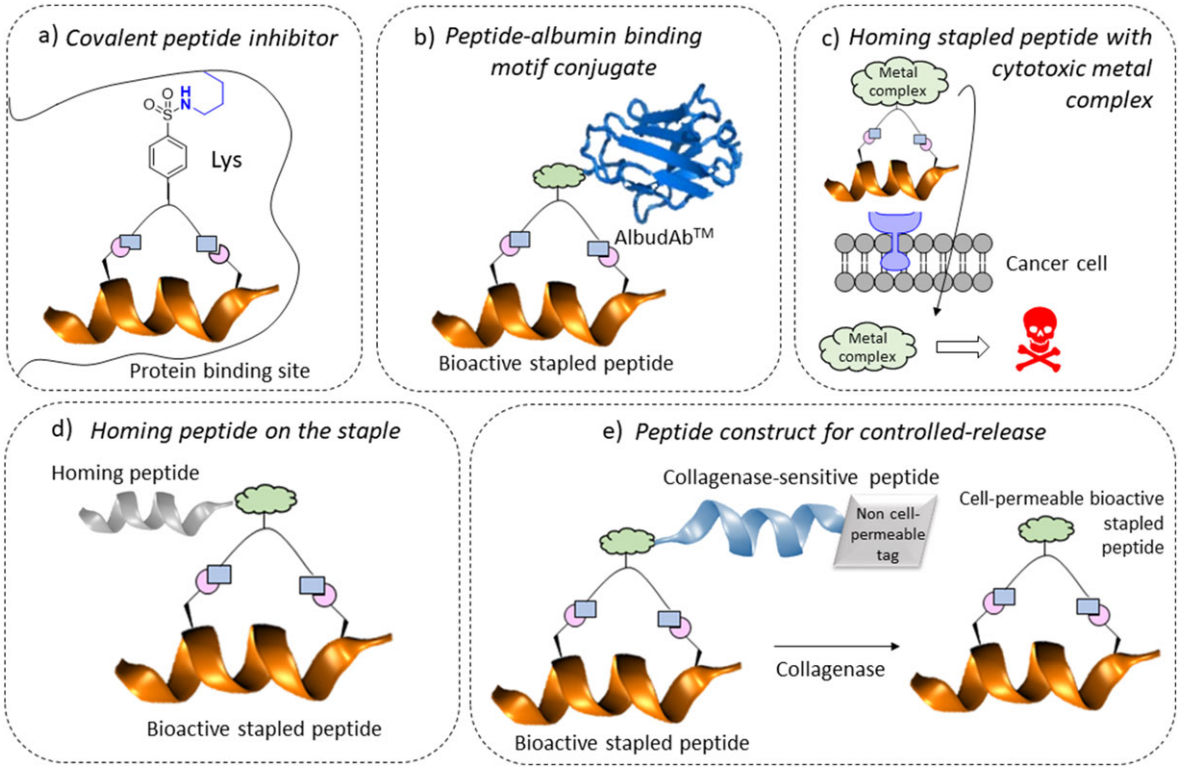


Figure 8. Overview of possible functionalized staples unexplored so far. a) Staple with electrophile incorporated for covalent Lys binding. b) Peptide-albumin binding motif attached onto the staple. c) Homing stapled peptide with cytotoxic metal complex incorporated in the staple. d) Bioactive peptide stapled with a homing peptide. e) Construct for controlled-release of peptides. Amino acid side chains used for stapling are shown as pink circles, staple as two blue squares connected by a black curve, and the functional handle on the staple as a green cloud with functionalities attached or incorporated.

successfully fused or conjugated to these half-life-extending motifs and showed an improved pharmacokinetic profile in vivo.

Combining the ability of the staple to increase the enzymatic stability of the peptide with functionalities which could maintain the peptide in circulation for longer or reduce the renal excretion represents an attractive strategy to prolong the peptide half-life. The 2C-PS techniques provide a promising alternative to this aim considering the variety of functionalities that can be incorporated onto the staple. The introduction of PEG chains onto the staple could easily be achieved with the functional handle being a carboxylic acid, a ketone, an amine, etc. In addition to prolonging the peptide half-life, PEGylation has been shown to improve solubility, reduce immunogenicity and antigenicity and represents a useful appendix to improve the properties of the peptides in general. There exist several examples of PEG–peptide conjugates in which the PEG chains are attached at the *N*- or *C*-terminus or to an amino acid side chain, and a very limited number of PEG-functionalized staples.[66,83,117] Moreover, PEGylation of the staple was performed to validate the stapling methodologies, and was therefore applied to model peptides rather than bioactive ones with the result that no in vivo testing has been reported to date.

Similarly, albumin-binding motifs—for example, AlbudAB[122] and albumin binding peptides such as DICLPRWGCLW—have been attached to peptidic chains to prolong the half-life of therapeutic peptides but conjugation onto the staple of a stapled peptide is still missing. Considering that PEG chains are flexible and albumin-binding molecules are relatively big, attachment onto the staple might avoid negative interactions with the targeted protein and represent an attractive and unexplored strategy (**Figure 8**b).

6.1.4. What Else can be Done?

The successful applications of functionalized stapled peptides in cells lay the foundations for the development of novel types of motifs that can be used to expand the scope of these molecules. Although tags that improve the properties of the peptide have been widely used, many others are still unexplored. For instance, the covalent targeting of residues located in proximity of the peptide binding sites is an attractive strategy to improve the overall binding affinity of the peptides. As peptides are typically highly selective and specific, when attached to a covalent binding motif they can reduce the binding of the latter to off-target proteins, an event which is known to cause toxicity.[123] Among the residues that could be targeted, non-solvent exposed lysine is one of the most commonly targeted residues and there are several examples of small molecules acting as lysine-targeting covalent inhibitors.[124,125] Recently, a stapled peptide was also equipped with an aryl sulfonyl fluoride amino acid within the linear peptide sequence to target a lysine residue located in the p53-binding site of mdm4.[128] However, when an unnatural amino acid is placed within the linear sequence, there is a risk that enzymatic degradation could lead to detachment of the electrophilic amino acid prior to reaching the desired target, and

hence the covalent inhibition would not be obtained. In addition, the synthesis of electrophilic, unnatural amino acids can be time-consuming and challenging. Therefore, we envision that a better alternative will be to equip the staple with a functional electrophilic group suitable for covalent targeting of lysine residues. Examples of electrophiles that could be used are aryl sulfonyl fluorides or aryl sulfonate activated esters which have been used to assess lysine reactivity in the human proteome.[129] Such functionalities have been shown to exert good reactivity toward lysine residues and an aryl group represents a suitable staple for $i,i+7$ stapling, for instance, in the double CuAAC stapling (Figure 8a). For this purpose, a protein with a low turnover which contains basic lysine residues near the binding site must be identified to allow the covalent complex to be formed and remain in circulation for a significant amount of time.

Additionally, a functional handle on the staple could be used to obtain a controlled-release device. Attachment of cell-impermeable groups to motifs that are substrates for specific enzymes could result in selective cleavage and hence cell-permeability when the local concentration of enzyme increases resulting in permeability changes. Collagenase sensitive linkers[128–130] are an example of a sequence that is recognized by the homonymous enzymes that are highly expressed in specific conditions such as cancer (Figure 8e).

Another application of functionalized stapled peptides that may be useful for selective cell-targeting is the conjugation with peptide sequences recognized by receptors expressed on the surface of specific cell types such as cancer cells (also referred to as "homing peptides"). The functional handle of a staple would act as the homing peptide and lead to the incorporation of the biologically active stapled peptide into the targeted cell types (Figure 8d). Tumor "homing peptides" have been extensively studied and include RGD, NGR, LSD, RSR, and KRK peptides among others.[131,132]

Alternatively, the stapled peptide could serve as the plasma stable cell-recognizing homing peptide and the staple could be functionalized with cytotoxic metal complexes such as copper, platinum, zinc, and gold complexes that have been shown to cause cancer cell toxicity (Figure 8c).[133,134] Although homing peptides have been conjugated with cytotoxic metal complexes in the past,[135–137] they have shown low to moderate cell activity due to endosomal trapping, degradation, and low uptake.[138] Therefore, a stable stapled peptide able to recognize specific receptors or enzymes would allow the cytotoxic motif to be internalized by specific cell types, only affecting the mortality of these cells. If a multifunctional staple was used, the physicochemical properties of the peptides could be modulated further to optimize uptake and hence provide selective cytotoxicity.

Ultimately, the end goal of peptide stapling is the development of a pluri-functional staple. The design of novel staples that could incorporate more than one of the functionalities described above, in addition to stabilizing the active peptide conformation and increasing stability toward proteases is what researchers in the field should ideally focus on in order to expand the scope of 2C-PS even further. The functionalities could be added in a sequential manner to the only functional handle present on the staple or alternatively, a staple bearing more than one functional group could allow the simultaneous introduction of several property-modulating functionalities, one on each functional handle.

6.2. Bioactive Stapled Helical Peptides and Beyond

Our 2C CuAAC stapling technique has been applied to the development of stapled peptides, either to promote α-helix formation or to merely constrain the peptides, as inhibitors of a broad range of PPIs. With our technique, we have produced successful peptide inhibitors for some well-studied PPIs and some less-explored targets. Herein, the main examples are described, giving overviews of the targets and features of the stapled peptides, as well as highlighting the significance of the work in each case.

6.2.1. Functionalized Stapled Peptides Targeting the p53/mdm2 PPI

It was during the development of 2C stapled peptides to target the p53/mdm2 interaction that our 2C $i,i+7$ CuAAC methodology was first developed in our laboratory. The roles of the p53/mdm2 interaction are fully known and understood in healthy and cancerous cells, making it an attractive PPI to target. When we became interested in the p53/mdm2 interaction as a potentially druggable target, some small molecular inhibitors were known, the most notable example being Nutlin-3[139] and a hydrocarbon-stapled peptide (ATSP-7041),[140] both targeting the *N*-terminal region on mdm2 to which an α-helix of p53 binds.

The p53/mdm2 project was used as a proof-of-concept for the development of the different 2C CuAAC methodologies. Previous work had shown that p53/mdm2 stapled peptides suffer from poor cell-permeability, requiring changes to the amino acid sequence to introduce cationic species for improvement.[141] When first developing the 2C CuAAC $i,i+7$ stapling, the initial aim was to improve the cell-permeability of the wild-type sequence by functionalizing the staple, rather than the linear peptide sequence. This was easily achieved thanks to the functional handle on the staple, and a stapled peptide was developed which compared favorably to the all-hydrocarbon stapled peptide in a competitive fluorescence polarization assay and showed cellular uptake by confocal microscopy.

Later on, we applied our CuAAC methodology to develop linkers and p53 peptides that could be used in photoaffinity labelling.

The application of the SPAAC as a 2C-PS technique for the development of p53/mdm2 stapled peptides led to the development of a potent mdm2 binder (**Figure 9**a). Crystallographic data of the most potent stapled peptide showed that it was bound to the protein in a helical conformation and identified a beneficial interaction between the linker and the mdm2 protein.[84] This was highly unusual, and was an effect that had only been observed previously with all-hydrocarbon stapled peptides.[142]

Through the development of the CuAAC and SPAAC methodologies, we have synthesized many potent stapled peptides to drug the p53/mdm2 PPI, as well as designed a wide range of possible linkers.[82,106,107]

6.2.2. Functionalized Stapled Peptides Targeting the cft4 Hub

Another stapled peptide developed using our CuAAC approach was synthesized to target the interactions between a genome stability hub, ctf4, and its client proteins.[105] It was believed that disrupting this PPI would affect the genomic stability, thus

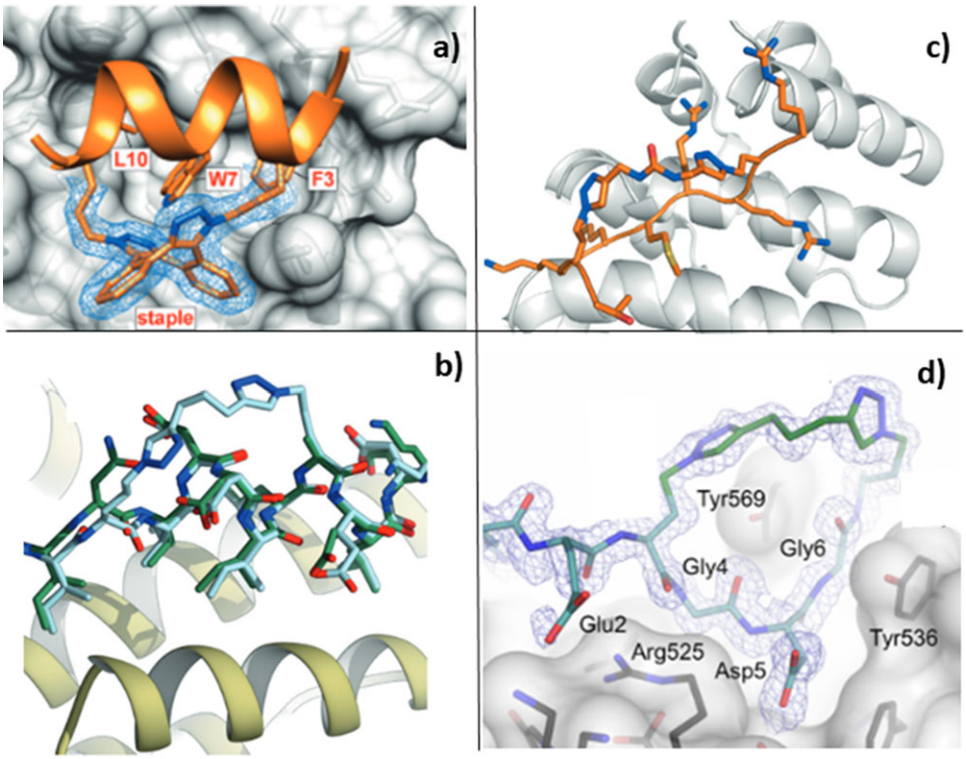


Figure 9. Structures of stapled and conformationally constrained peptides bound to their protein. a) X-ray structures of p53 stapled peptide (orange) bound to mdm2 (PDB:5AFG). Reproduced with permission,[84] Copyright 2015, Wiley-VCH. b) Two stapled peptides (cyan and green) bound to ctf4 protein (yellow) (PDB: 4C95). Reproduced from Y. Wu et al.,[105] Copyright 2017, Wiley-VCH. c) Modelled structures of a conformationally constrained peptide (orange) bound to mImportin α1ΔIBB (grey). Reproduced under the terms of CC-BY 4.0,[143] Copyright 2017, The authors. d) Crystallographic structure of a macrocyclized extended peptide (green) bound to TNKS2 ARC4 (grey) (PDB: 5BXO). Reproduced with permission.[103] Copyright 2017, American Chemical Society.

providing a novel target for cancer therapy. Stapled peptides were developed using the 2C CuAAC methodology employing an unorthodox $i,i+6$ staple positioning and an un-functionalized aliphatic linker. Canonical stapling positions are $i,i+4$ (1α-helix turn), $i,i+7$ (2α-helix turns), $i,i+11$ (3α-helix turns). With the term unorthodox we refer to stapling positions different from the canonical ones and hence having amino acid side chains for stapling not pointing towards the same face of the helix. The best stapled peptide gave a tenfold increase in binding affinity compared to the wild type and was confirmed to bind in a similar manner by X-ray crystallography (Figure 9b). In addition to this peptide, others were identified which gave a 2.7-fold increase in binding over the wild-type peptide and included functionalizable linkers. This work highlighted the druggability of the ctf4 protein hub with stapled peptides and brought to the fore this chemotherapeutic approach (targeting of a genome stability hub), which may be applicable to other PPIs in the human replisome as a valid anticancer tactic (Figure 9b).

6.2.3. Functionalized Stapled Peptides as Imaging Agents for the GLP1 Receptor

The ability to functionalize the linker without disrupting the amino acid sequence is of vital importance when peptides are employed as imaging agents.[114] Thurber et al. utilized the 2C double CuAAC stapling technique for the construction of $i,i+7$ stapled peptides to be used as imaging agents for GLP1 receptors. They were able to use bis-alkynyl linkers modified with imaging agents and no observed loss in binding. Thurber and coworkers also investigated the bioavailability of sub-cutaneously administered fluorescently labelled 2C CuAAC stapled peptides. They found that their stapled peptide exhibited higher bioavailability than their linear analogues, due to the increased proteolytic stability of the macrocyclized peptides.[144]

6.2.4. Beyond Helical Peptides

The following examples are not about peptide stapling to increase the α-helicity of a peptide, but constraining an unstructured peptide in the binding conformation.

6.2.4.1. Targeting the PPI Between HNF1β and Transportin-α. Peptides constrained using the CuAAC strategy were developed for the inhibition of a key PPI which could potentially lead to the treatment of ovarian clear cell carcinoma (OCCC).[143] OCCC is a subtype of ovarian cancer that has an extremely poor prognosis for patients with advanced forms of the disease. This is due in part to an intrinsic resistance to platinum-based therapeutics, as well as a lack of targeted therapies specifically for the treatment of OCCC. We developed a first-in-class peptide-based inhibitor,

which targeted the PPI between HNF1β transcription factor (TF) and nuclear transport protein transportin-α. HNF1β contains a nuclear localization sequence (NLS), a short peptide sequence that binds to transporter proteins and aids the transport of the TF to the nucleus, where it exerts its biological effect. By designing a constrained peptide to mimic the NLS of HNF1β, the peptide could potentially out-compete HNF1β for binding to importin, and consequently the peptide would be preferentially imported into the nucleus. Around 49% of transcription factors contain intrinsically disordered domains,[145] hence the focus of stapling was to constrain the peptide in its binding conformation, rather than to increase α-helicity. The best constrained peptide gave a 2.5-fold increase in binding compared to the wild-type linear peptide (as measured by a direct fluorescence polarization assay), and all the constrained peptides displayed reasonable cell-permeability (Figure 9d). This work represents the first example of the downregulation of a transcription factor by targeting a nuclear import pathway using a constrained NLS peptide.

6.2.4.2. Targeting TNKS. On a similar line to the previous example, conformationally constrained peptides were developed as inhibitors for tankyrase (TNKS), a protein involved in the Wnt signalling pathway which is found mutated in several diseases, including many types of cancer. Small molecular inhibitors have been developed to target the poly(ADP ribose) polymerases (PARP) domain of TNKS; however, they suffer from poor selectivity and the potential for resistance mechanisms to emerge.[103] To circumvent these issues, we applied the 2C CuAAC peptide stapling to the development of a constrained peptide inhibitor for a different domain of TNKS. The ankyrin-repeat unit recognizes and binds to Axin, which in turn promotes the PARPylation and degradation of the TNKS. It was the PPI between the ankyrin-repeat unit on TNKS and Axin that we sought to inhibit with stapled peptides. Despite the unique substrate-recognition domain of TNKS compared to the other PARP family members, it remains to be seen whether this approach will prevent development of resistance. A combinatorial approach was used to screen the best length of azido-containing unnatural amino acids and bis-alkynyl linkers against the binding affinity, made synthetically facile by the modular nature of our 2C CuAAC stapling approach. To improve cell-permeability, cell-penetrating peptides were easily incorporated via functionalization of the linker. This design approach highlighted peptides with sub-micromolar TNKS binding affinities that could disrupt the TNKS–Axin interaction in a dose-dependent manner (Figure 9c). The macrocyclized peptides also showed increased proteolytic stability compared to linear analogues (100% intact peptide observed after 4.5 h incubation with AspN protease while linear peptide was completely degraded after 1 h). This is the first reported example of stapled peptides that target the TNKS–Axin binding PPI, and the success of these inhibitors is an excellent proof of concept for this pathway, paving the way for the development of more TNKS inhibitors.[103]

This last chapter has shown how successful the applications of 2C stapling methodologies have been to the development of PPI inhibitors in a variety of biological settings. Specific benefits of the 2C methodologies, which enabled the research discussed above, were the synthetic tractability of the stapled peptides (allowing fast synthesis of analogues) and the facile introduction of functionalized groups (including motifs to promote cell-permeability and tags to aid biochemical and biophysical assays).

7. Conclusions and Outlooks

Peptide therapeutics represent an emerging area in the pharmaceutical field. Considering their selectivity, efficacy, low toxicity, and cost of goods, peptides are the ideal molecules to target shallow areas of proteins, such as the interface of PPIs, thus unlocking a large number of extra and intracellular pathways that can be disrupted. The main drawbacks of this class of molecules are their inherent instability in vivo and their weak bioavailability. Significant progress has been made in increasing the resistance of the peptide to proteases and improving their cell-permeability and hence, tissue penetration. Macrocyclization of linear peptides is certainly one of the most successful methodologies to overcome many of the limitations of peptides, and a plethora of different techniques have been developed to this end. In this progress report, we have highlighted the recent advances made in peptide stapling, the advantage of the 2C-PS techniques over 1C and, in particular, the achievements obtained with the rational design of 2C CuAAC stapled peptides. The 2C-PS methodologies involve an intramolecular reaction between the two amino acids on the linear peptide and a staple or linker that can bear a functional handle for further functionalization. After merging the two components together, the peptide may be constrained in the bioactive conformation minimizing the entropic penalty upon binding of the peptide to the targeted protein and increasing its stability to enzymatic degradation. This methodology has been successfully applied not only to constrain α-helical peptides, but also to random coils, therefore expanding its scope. Using the CuAAC 2C-PS technique developed in our group, we have created a toolbox of staples not only suitable for constraining different secondary structures of selected sequences, but also stapling at different positions and conditions while also bearing different functionalities. In addition, our 2C-PS methodologies have been proven to be compatible with all the biogenic amino acids that may be present in the peptidic sequence. The toolbox includes staples carrying fluorescent tags, cell-permeable motifs, biotin tags, nuclear localization sequences, and photoaffinity labelling moieties, showcasing the wide applicability of this system. Moreover, stapled peptides obtained were often found to be more active against the set target than the unstapled variants and wild-type peptides. Most importantly, they showed improved cell-permeability and in vitro stability. Encouraged by the positive results obtained so far, we envisioned that the scope of the 2C-PS can be expanded further by adding more functionalized stapled peptides to the toolbox.

To date, 2C-PS has mainly been used for targeting PPIs. Much of the focus of PPI inhibition at the moment is related to oncology research; however, there are still a vast number of other PPIs that could be targeted.[146] The main effort of PPI inhibition has been on the use of stapled peptides to mimic α-helical "hot-spot" binding regions in PPIs between globular proteins. Another class of PPIs involves the interaction between short peptides and proteins. One example of this is the interaction between peptide hormones and their receptors. ALRN-5281, the first stapled peptide

to enter clinical trials, is a stapled hormone peptide.[47] This illustrates how any signalling event involving a peptide or protein hot-spot can be manipulated using stapled peptides to generate more stable, permeable therapeutic derivatives.

For the progression of stapled peptides to ultimately be drugs, it is imperative that the scope of the linker composition is expanded as much as possible, as the linker constructs can greatly alter the properties of a stapled peptide. For instance, the staple could be functionalized with a group with the potential for covalent binding to reactive residues in the binding site such as lysine, cysteine, and methionine or alternatively, tags that could result in controlled-release of the biologically active peptide. In addition, extra functionalities may be introduced via the linker, which could result in prolonged peptide half-life in vivo by binding to plasma proteins such as albumin or reduced renal excretion. Moreover, 2C-PS techniques have not been applied to cell-penetrating peptides and could therefore provide a way to improve the stability and half-life of these cell-permeable tags in the blood stream. Furthermore, there are opportunities to develop suitable staples which could stabilize β-turns and similar secondary structures, as well as stabilizing α-helices at stapling positions beyond the $i,i+4$, $i,i+6$, and $i,i+7$.

One of the main limitations of the 2C PS is the fact that the cyclization is done in solution and therefore, an extra purification step is necessary prior to macrocyclization. Despite a limited number of examples of on-resin 2C CuAAC, a robust method to perform this chemistry on-resin is still missing. It is therefore clear that optimization of the macrocyclization conditions for on-resin stapling is necessary to increase the impact of the methodology. Moreover, even though the azido amino acids used in the CuAAC stapling are relatively cheap and easy to synthesize, their usage could represent a limitation to the use of this technology. A wide range of functionalized 2C stapled peptides have been synthesized using cysteine, offering a proteinogenic alternative to azido amino acids. Further reports regarding the stability of this class of stapled peptides in cells and in vivo are needed to validate their potential as therapeutic agents. It is clear that on-resin peptide macrocyclization techniques utilizing biogenic amino acids need to be developed in order to make peptide stapling feasible to a wider range of research groups. Moreover, thorough in vivo assessment of functionalized stapled peptides developed using 2C-PS methodologies is still missing and it is indeed necessary to validate the peptides made with these methodologies. In addition, comparative studies utilizing the same set of stapling conditions (amino acid side chain length, staple, stapling position) on different sequences are missing. Such studies are needed in the field as they could result in guidelines for the structural design of stapled peptides.

Finally, we should note that the chemical linkage of amino acid side chains in peptides was originally developed to improve their potential as drugs. It is feasible that appropriately stapled peptides can succeed in any situation in which a native peptide or protein is involved in a biological system.

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Conflict of Interest

The authors declare no conflict of interest.

Keywords

new modalities, peptide therapeutics, protein–protein interactions, stapled peptides, two-component stapling

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