Reviews • KEYNOTE REVIEW

Computational techniques have an increasingly important role in the design of stapled peptides to target protein–protein interactions.

Stapled peptide design: principles and roles of computation

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Stapling is a key technique for stabilising peptides in an α-helical structure. The resultant stapled peptides are then able to compete efficiently for binding to protein targets involved in protein–protein interactions that are mediated by α-helices. Certain general design principles to optimise their binding and biological activity have emerged in recent years. This is accompanied by an increasing use of computational methods in stapled peptide design. In this article, we detail these design principles and review the contributions that computation has made to the field. We also highlight several pressing questions regarding the mechanism of action of stapled peptides, which could potentially be resolved by computational means.

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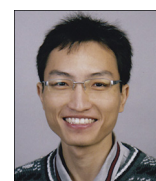
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Introduction

Protein–protein interactions (PPIs) mediate many important cellular functions and regulatory pathways. They have traditionally been considered an 'undruggable' class of molecular targets for drug discovery because of their relatively large and shallow interfaces, which are not optimal for the binding of small molecules [1]. High-throughput approaches for lead discovery have met with limited success when applied to PPIs [2]. This is not surprising, given that the chemical make-up of the small molecules that comprise high-throughput screening libraries is biased towards organic molecules that bind to the deeper pockets found in traditional protein targets, such as enzymes and G-protein-coupled receptors [3]. Nevertheless, there have been several recent successes in targeting PPIs with small molecules [4]. Some of these small-molecule inhibitors are now in clinical trials [5–7].

Peptides are an alternative class of drug molecules that have shown promise as therapeutic agents for the modulation of PPIs [8,9]. Being larger in size, they are able to span a large contact surface area, making them suitable for the inhibition of these attractive, yet challenging, drug targets. They are also able to bind with high specificity and potency to their targets, resulting in reduced off-target effects and improved safety [9]. Despite these advantages, peptides are limited

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in their pharmacological applications because of their low oral bioavailability, poor metabolic stability, poor membrane permeability, and rapid clearance [10]. Moreover, the unbound peptide is usually highly unstructured, with low intrinsic secondary structure in solution. It has to incur a potentially huge entropic penalty for binding because of the need for reorganisation into a conformation that mimics that of the target protein's binding partner, resulting in poor target affinity.

The α-helix is the most common motif found at protein–protein interfaces [11,12]. Peptides that are constrained into a helical structure and, hence, preorganised into their bound conformations, can bind to protein targets involved in α-helix-mediated PPIs with reduced entropic penalty. A variety of strategies to stabilise the α-helical structure have been developed in recent decades [13]. These include the introduction of hydrogen bonding or electrostatic interactions between side chains, incorporation of α,α-disubstituted residues [14] such as aminoisobutyric acid [15] into the α-helix, use of hydrogen-bond surrogates [16,17], and formation of side-chain-to-side-chain covalent bridges called 'staples' [18].

The stapling technique is rapidly emerging as one of the most widely adopted strategies for α-helix stabilisation. Stapled peptides are highly promising therapeutic agents for the inhibition of PPIs, because they generally exhibit enhanced helicity, protease resistance, and biological potency [19–22]. The development of stapled peptides promises to enable access to α-helix-mediated PPI drug targets [23,24]. Challenging targets, such as transcription factors, which were thought to be undruggable, have been successfully inhibited by stapled peptides [25,26]. It has also been shown that stapled peptides are able to disrupt enzyme [27], multidrug resistance efflux pump [28] and membrane receptor [29] dimers, thus opening up new possibilities for targeting traditional drug targets, which is becoming especially important because of the development of resistance against current drugs.

Given that there are 3.6 residues per α-helical turn, residues are found on the same side of the helix at the i, $i + 4$, $i + 7$, and $i + 11$ positions (where the residue C terminal to residue i is designated $i + 1$, and so on). Lys, Asp, Cys, and suitable unnatural residues may be incorporated into the peptide at any two of these positions for covalent crosslinking. There is an array of macrocyclisation chemistries for forming side-chain crosslinks, including the hydrocarbon [30], triazole [31], lactam [32], thiol-based [33,34], and azobenzene [35] staples. Comprehensive reviews of the different stapling strategies are available in the literature, and are not further discussed here [18,36]. Among the various staple types, the hydrocarbon and triazole staples are among the most stable chemically and the least susceptible to biological degradation. There is ample evidence to suggest that the hydrocarbon and triazole staples enable cell penetration [22,37], which is an important property that allows access to intracellular drug targets. The design principles presented in this review are derived mainly from case studies of these two highly promising classes of stapled peptides, but they can also be applied to the other types of staples.

Hydrocarbon staples

The hydrocarbon stapling technique combines two α-helix stabilisation strategies, namely α,α-disubstitution and macrocyclic bridge formation [30]. Two α,α-disubstituted residues bearing

olefin side chains of varying lengths are introduced into the peptide α-helix, followed by a ruthenium-catalysed ring-closing metathesis reaction [38] to form the staple across one or two α-helical turns (Fig. 1a). An i, $i + 3$ or i, $i + 4$ staple spans one α-helical turn, while an i, $i + 7$ staple spans two α-helical turns. Various studies have reported the therapeutic potential of hydrocarbon-stapled peptides in the treatment of cancer, specifically by inhibiting the NOTCH transcription factor complex [25], reactivating the p53 tumour suppressor pathway [39–42], and promoting B cell lymphoma 2 (Bcl-2)-mediated apoptosis [43]. They have also shown potential as therapeutic agents for various other diseases, such as HIV [44,45], diabetes [46], cardiovascular disease [47], and respiratory infection [48]. Two hydrocarbon-stapled peptides developed by Aileron Therapeutics are currently undergoing clinical trials for the treatment of orphan endocrine disorders and malignant tumours [49].

Triazole staples

Triazole staples are formed by the Cu(I)-catalysed cycloaddition 'click' reaction between azido and alkynyl functionalities on the side chains of residues in the i, $i + 4$ positions (Fig. 1b). They have been used to stabilise the helical structure of peptides derived from parathyroid hormone-related protein and Bcl9 [31,50]. The latter stapled peptide was shown to be more helical, protease resistant,
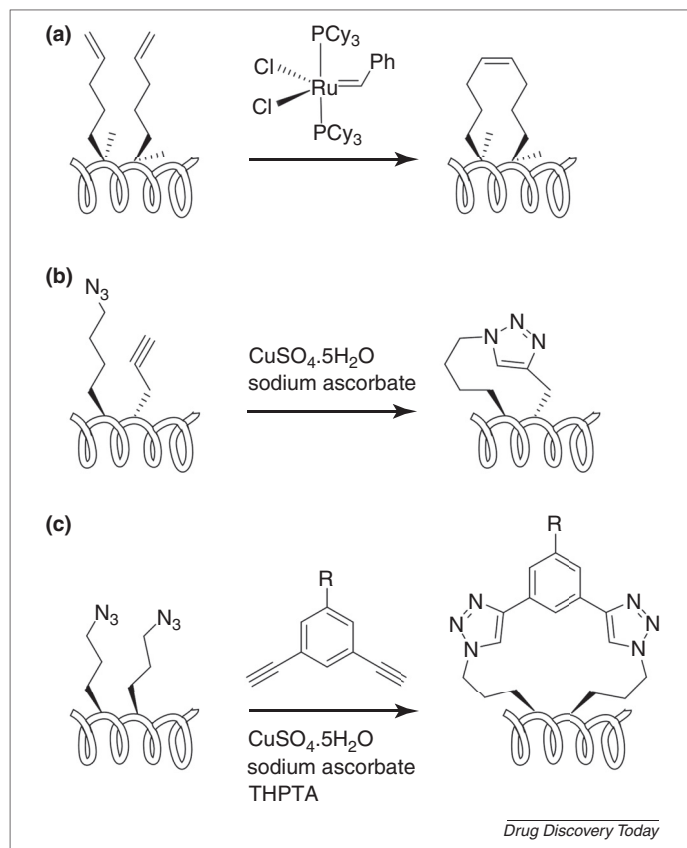

FIGURE 1

Macrocyclisation chemistries for forming side-chain crosslinks. **(a)** Ruthenium-catalysed ring-closing metathesis. **(b)** Cu(I)-catalysed azide-alkyne cycloaddition. **(c)** Double-click Cu(I)-catalysed azide-alkyne cycloaddition. Abbreviation: THPTA, *tris*(3-hydroxypropyltriazolylmethyl)amine.

and tighter binding than the corresponding linear peptide. Double-click reactions involving two side chains with azido functional groups at the i, $i + 4$ or $i + 7$ positions and a dialkynyl linker (Fig. 1c) have also been used to enhance helicity in peptides derived from the GCN4 leucine zipper and p53 transactivation domain [51,52]. These reactions allow for wider exploration of chemical space than hydrocarbon staples as different dialkynyl linkers can be installed. However, the double-triazole stapled p53 peptide did not exhibit any activity in cellular assays until its overall charge was altered by the use of positively charged dialkynyl linkers, while the cellular activity of the stapled GCN4 peptide was not evaluated. A follow-up study that carried out double-click triazole stapling on phage-derived sequences with fewer negatively charged residues than the p53 wild-type sequence also yielded cell-active peptides [22]. The effect of net charge on the cell permeability of stapled peptides is addressed later in this review.

The need for rational design

Hydrocarbon stapled peptides are at a relatively advanced stage of development, whereas triazole stapled peptides are emerging as promising alternatives with the possibility of customisable linkers that confer certain desirable properties. A wealth of data is beginning to provide important information regarding the effects of staple position, staple structure, and peptide sequence on the activity of stapled peptides. At the same time, computational tools are becoming an essential part of the design process because they help to rationalise empirical observations, provide insights into molecular mechanisms of binding, and identify promising candidate peptides through molecular models, thus reducing the need for screening extensive peptide libraries, which is both tedious and expensive. In this review, we summarise the general design principles that have emerged so far and provide insight into the important roles of computational techniques in stapled peptide design.

General design principles

Staple position

Residues to be stapled have to be placed on the same side of the helix for the macrocyclisation reaction to proceed efficiently. In theory, staples can be placed anywhere along the length of the peptide helix. In practice, however, not all staple positions are equal. The most important rule-of-thumb in the placement of staples is not to replace critical interacting residues, so that the entropic penalty for binding is decreased without compromising the enthalpy of binding. This core principle has guided the design of the majority of stapled peptides reported in the literature, such as those targeting BCL-2 [43], HIV integrase [27], and Rep Protein A [53]. In the last case, comprehensive alanine scanning [54] was carried out to identify important binding residues. Single-point alanine mutations were systematically introduced to the peptides and their binding affinities evaluated. This experimental technique is especially useful for targets with no available structural information of the protein–peptide complex.

Several studies have shown that staples that replace important binding residues invariably attenuate the binding affinity of the stapled peptides [55,56]. Staple scanning, which involves the sampling of all possible staple positions along the peptide helix, was carried out on the BIM BH3 sequence [55]. One of the stapled

peptides generated had two interface residues replaced by a staple and displayed a drastic reduction in cellular activity. Another study observed no improvement in eIF4E binding affinity when two interacting phenylalanine residues in the eIF4EG peptide were replaced by an i, $i + 4$ hydrocarbon staple [56].

Just as there are certain positions to avoid, there are also positions on the peptide helix that are desirable for staple placement. Given that staples should not be placed on the binding surface of the peptide, they can either point into the solvent on the opposite side of the helix (fully solvent exposed) or flank the binding interface (partially solvent exposed). There is a growing realisation that flanking staples are able to enhance binding affinity by both entropic and enthalpic means. Due to their proximity to the protein surface, additional favourable van der Waals contacts may be formed between the partially solvent-exposed staple and protein, provided that the fit between the staple and protein surface is complimentary and no steric clash occurs. This also reduces the entropic penalty for solvating the hydrocarbon staple [57].

The first evidence of such an interaction was provided by a crystal structure of a hydrocarbon stapled MCL-1 BH3 peptide bound to MCL-1 (Fig. 2a) [58]. A methyl group attached to the Cα atom of a stapled residue binds in a shallow hydrophobic groove formed by Gly262, Phe318, and Phe319. Weak hydrophobic contacts also exist for the hydrocarbon linker. These additional hydrophobic interactions conferred up to a fourfold improvement in MCL-1 binding affinity compared with peptides stapled at other positions. A computational study subsequently found that a similar situation exists in a set of hydrocarbon stapled peptides that were designed to inhibit the MDM2–p53 interaction [57]. Molecular dynamics (MD) simulations of the stapled peptides complexed to MDM2 demonstrated the positive contribution to the binding free energy by staples that lie close to and interact directly with the protein surface. The computational models were subsequently confirmed by X-ray crystallography, which revealed the intimate contacts between the hydrocarbon staple of the most potent stapled peptide and the MDM2 surface (Fig. 2b) [59]. Another crystal structure revealed that a stapled peptide developed by Aileron Therapeutics binds in a similar fashion to the structurally homologous MDMX, with the hydrocarbon staple engaging a flat binding platform beside the p53 binding pocket (Fig. 2c) [41]. This type of interaction is not unique to the hydrocarbon staple, as a recent crystal structure shows a triazole staple formed by double-click cycloaddition interacting with MDM2 in a similar manner (Fig. 2d) [22].

Oestrogen receptors (ERs) are another class of protein targets that has been shown to interact directly with hydrocarbon staples. In the case of ERα, aliphatic side chains that are part of the peptide's recognition motif and partially solvent exposed when bound to the protein were replaced by a hydrocarbon staple, leading to a sevenfold improvement in the binding affinity (Fig. 3a) of the stapled peptide (SP2) over that of the unstapled peptide [60]. Solvent-exposed positions were also selected for stapling to generate an alternative stapled peptide (SP1). The structure of its complex with ERβ shows that rotation of the peptide helix has occurred relative to the unstapled peptide, allowing the staple to collapse onto the protein surface to form extensive hydrophobic contacts (Fig. 3b). This study illustrates the
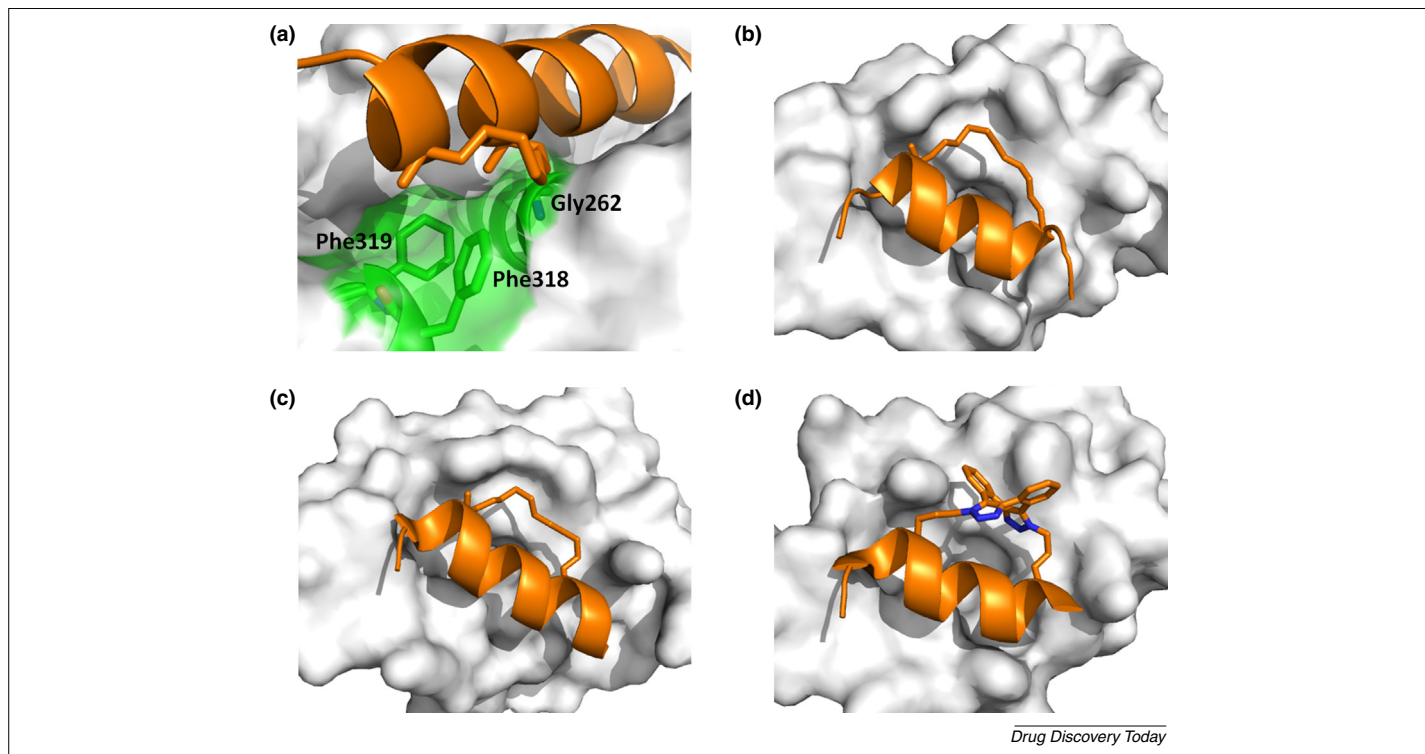
FIGURE 2

Staple binding sites on target proteins (white). **(a)** MCL-1 complexed with MCL-1 SAHB$_D$ (PDB 3MK8 [58]). **(b)** MDM2 complexed with SAH-p53-8 (PDB 3V3B [59]). **(c)** MDMX complexed with ATSP-7041 (PDB 4N5T [41]). **(d)** MDM2 complexed with triazole stapled peptide E1 (PDB 5AFG [22]).
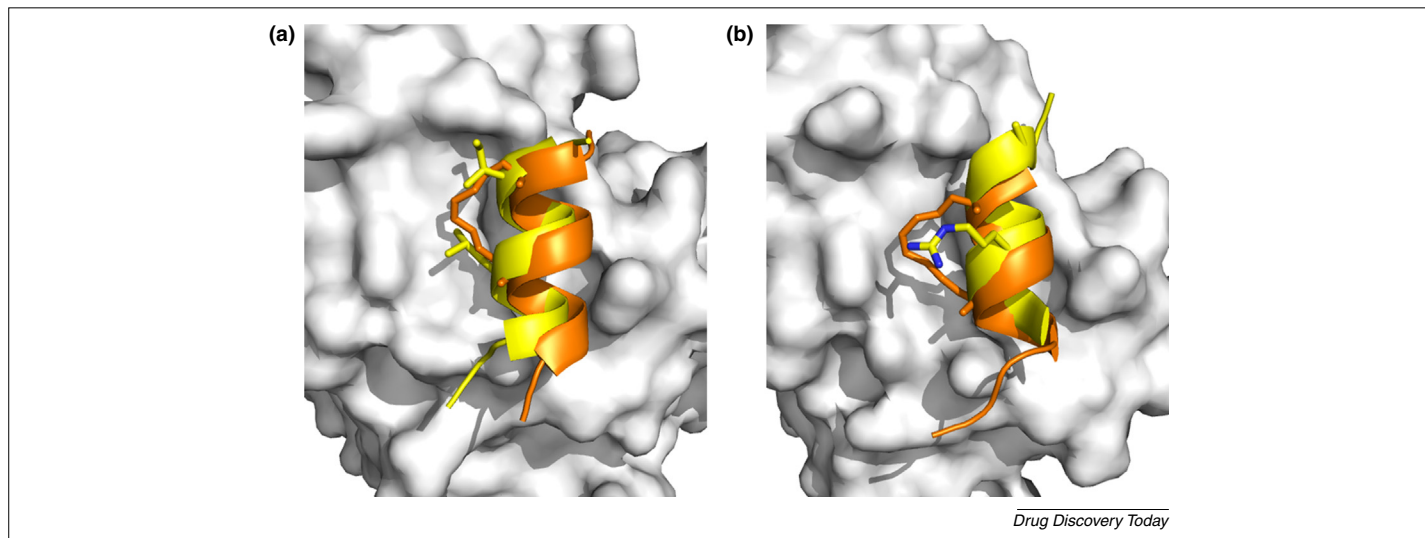
FIGURE 3

Structures of estrogen receptors (ER; white) bound to stapled coactivator peptides (orange), with the corresponding linear peptides (yellow) superimposed. The staples and residues that they replace are shown in sticks. **(a)** Structure of ERα bound to SP2 (PDB 2YJA [60]). The staple replaces residues that interact with the protein surface. **(b)** Structure of ERβ bound to SP1 (PDB 2YJD [60]). The staple induces rotation of the peptide helix, such that it is able to interact with the protein surface.

importance of direct interactions of the hydrocarbon staple with the protein and their potential to dictate the binding conformation and affinity of the stapled peptide.

Staple stereochemistry

Once the optimal staple positions have been determined, whether they are at the $[i, i + 3]$, $[i, i + 4]$, or $[i, i + 7]$ positions, the appropriate staples can then be installed. Each staple architecture can vary in its stereochemistry and linker length. These two variables are tightly coupled to each other and, more often than not, there is only one optimal combination of stereochemistry and linker length for a particular staple architecture (Fig. 4). The two unnatural residues forming a hydrocarbon staple can adopt either the S or R absolute configuration at the Cα carbon, and are referred
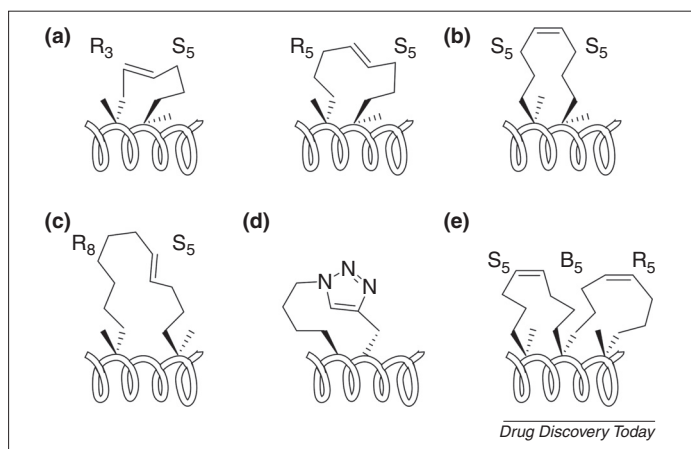
FIGURE 4

Stapled peptide architectures. Amino acids required for the formation of the hydrocarbon staples are indicated as either R_n or S_n, representing their absolute configuration as well as the number of carbon atoms (n) in their alkenyl side chain. **(a)** i, i + 3 hydrocarbon staples with six (left) and eight (right) carbon atoms. **(b)** i, i + 4 hydrocarbon staple. **(c)** i, i + 7 hydrocarbon staple. **(d)** i, i + 4 triazole staple. **(e)** i, i + 4 + 4 stitch.

to as either S_n or R_n, representing their absolute configuration as well as the number of carbon atoms (n) in their alkenyl side chain.

There are four possible stereochemical combinations for a staple: (R,R), (S,S), (R,S), and (S,R). For the i, i + 3 hydrocarbon staple, the most favourable stereochemistry for metathesis reaction and α-helix stabilisation is (R,S) [61]. However, it is not as effective at α-helix stabilisation as the most widely used staple architecture, the i, i + 4 hydrocarbon staple, whose optimal stereochemical combination is (S,S). The stereochemically opposite (R,R) version of the i, i + 4 hydrocarbon staple is formed by metathesis just as efficiently, but it is significantly less effective at α-helicity stabilisation and promoting cellular uptake and, hence, seldom used [62]. The next most popular staple architecture is the i, i + 7 hydrocarbon staple and, similar to its i, i + 3 counterpart, favours the (R,S) stereochemical configuration. Conversely, there have been no reports of the use of i, i + 11 hydrocarbon staples. Such long staples are likely to be too flexible to impart any noticeable entropic benefit, thus precluding their development and utilisation.

In the case of triazole staples, only i, i + 4 and i, i + 7 architectures have been described. Limited investigations into the effect of stereochemistry indicate that the (S,R) configuration might be more favourable than (S,S) for the i, i + 4 triazole staple [63], although a more exhaustive study is required to examine and characterise all possible stereochemical combinations. Only the (S,S) configuration has been adopted for the i, i + 7 triazole staple in double-click stapling, and the effect of stereochemistry has not been examined [52]. So far, the unnatural residues used to form the triazole staples are monosubstituted, and do not have an α-methyl group. Monosubstituted α-alkenyl amino acids were also used to staple BID BH3 peptides, and they were found to have comparable helicity, protease resistance, and binding potency to those stapled by the α,α-disubstituted analogues [64], which calls into question the necessity for α,α-disubstitution in peptide stapling.

Staple length

Linker lengths for hydrocarbon staples have to be optimised for maximal crosslinking efficiency and helicity. If the linker length is too short, metathesis cannot proceed efficiently, whereas if the linker length is too long, it might not be rigid enough to provide any helix stabilisation effects. There are two possible linker lengths for the i, i + 3 hydrocarbon staple, featuring either six or eight carbon atoms. The former is used if less hydrophobicity is desired, whereas the latter is more helix stabilising [65]. The six-carbon linker is formed by the amino acids R_3 and S_5, whereas the eight-carbon linker is formed by R_5 and S_5 (Fig. 4a). The six-carbon variant of the i, i + 3 staple has been successfully used to increase the helical and proteolytic stability of peptides derived from the HIV-1 envelope glycoprotein gp41 [66]. Curiously, the $R_5 + S_3$ combination, which differs from R_3 and S_5 only in the position of the carbon double bond, resulted in a lower yield of the cross-linked product and reduced helix stabilisation and, therefore, is not recommended for i, i + 3 stapling. By contrast, there is only one optimal linker length each for the i, i + 4 and i, i + 7 hydrocarbon staples, involving the use of an eight-carbon linker ($S_5 + S_5$) and 11-carbon linker ($R_8 + S_5$ or $R_5 + S_8$), respectively (Fig. 4b,c). No study has yet compared the two variants of the 11-carbon linker in i, i + 7 staples.

There have also been attempts to optimise the linker length for triazole staples, as well as the position of the triazole moiety within the linker. D'Ursi and coworkers determined that five or six methylene units in the triazole staple are optimal for helix stabilisation [31]. Wang and coworkers went a step further by screening different linker lengths and triazole positions. They concluded that the most suitable triazole staple for helix stabilisation comprises a triazole moiety flanked by four methylene groups at its N-terminal side and one methylene group at its C-terminal side (Fig. 4d) [50]. This particular linker has been used to generate triazole stapled peptides that inhibit the formation of the β-catenin–BCL9 and shelterin complexes [63].

Multiple staples

In cases where the helical portion of the peptide is very long, a single staple may not be adequate to protect it from proteolysis. For example, both single hydrocarbon and lactam staples were shown to be ineffective in enhancing the protease resistance of the 30-residue vasoactive intestinal peptide [67]. Multiple staples may then be required to protect the entire length of the peptide from proteolytic degradation.

Double i, i + 4 hydrocarbon staples were used to stabilise a 37-residue peptide from the gp41 envelope glycoprotein, improving its resistance to proteolytic degradation by at least threefold compared with the singly stapled peptides [68]. In a later study, [i, i + 4], [i, i + 3] double staples were used to stabilise a peptide derived from another region of gp41 [66]. These doubly stapled peptides demonstrated comparable binding affinity and enhanced proteolytic stability relative to the equivalent singly stapled peptide. A fusion peptide derived from the respiratory syncytial virus and stabilised by two i, i + 7 staples was also found to be exceptionally protease resistant and highly effective in suppressing viral infection in mouse models [48].

Stitches are a unique form of multiple stapling, in which a hydrocarbon staple is immediately followed by another

hydrocarbon staple. This technique requires the use of the amino acid *bis*-pentenylglycine (B$_5$), which acts as a junction point that allows two staples to emerge from a common residue in the peptide. There are many possible combinations of stereochemistry and linker length in such a system. A rigorous study of the various stitch permutations was carried out, and two systems, *i*, *i* + 4 + 4 (S$_5$ + B$_5$ + R$_5$) and *i*, *i* + 4 + 7 (S$_5$ + B$_5$ + S$_8$), emerged as the most effective for helix stabilisation (Fig. 4e) [69]. A peptide with the latter stitch architecture was found to have superior helicity and cell penetration compared with an *i*, *i* + 7 stapled analogue. Stitched peptides appear to be promising alternatives to the singly stapled versions, and further investigation is required to assess the effect of stitching on binding affinity and cell activity.

Sequence modifications
One of the advantages of installing a hydrocarbon staple to a peptide is its ability to enhance cell permeability. However, the introduction of a hydrocarbon staple alone is not always enough and, more often than not, the sequence of the peptide has to be subtly modified to optimise its net charge. The adjustment of overall charge to within the range of 0 to +2 has often been shown to be an effective strategy to enhance the cell permeability of hydrocarbon stapled peptides [70]. The importance of formal charge is borne out in a study of more than 200 hydrocarbon stapled peptides, in which peptides carrying a net negative charge displayed poor cellular uptake, whereas peptides with a net positive charge exhibited significantly higher cellular uptake [37]. This observation can be explained by experimental data reported in the same study, which indicated the importance of cell surface anionic sulphated proteoglycans in mediating cellular uptake of peptides via a clathrin- and caveolin-independent endocytosis pathway.

There are three ways to increase peptide net charge. Positively charged residues can be added to the N- or C-terminus of the peptide to increase the net charge directly [26,71]. Negative charges can be neutralised by mutating Asp and Glu to their respective uncharged analogues, Asn and Gln [39]. Lastly, negatively charged residues may be replaced by a strategically placed hydrocarbon staple [53]. However, charge alteration should be practised with caution, as peptides with high positive charges tend to cause cell membrane disruption, thus reducing target specificity [72].

Roles of computation
The development of stapled peptides tends to be an expensive process, in terms of labour, cost, and time. As discussed above, there are many variables in stapled peptide design, and the number of permutations is tremendous. Comprehensive staple scanning to determine the optimal staple positions is not always feasible, especially when the peptide sequence is long and the research group is constrained by limited funds. Computational methods can be used to develop *in silico* models, which may help in reducing the number of candidate stapled peptides to be tested experimentally and to rationalise certain experimental observations. Here, we discuss the roles that various computational techniques have played in stapled peptide design thus far (Fig. 5).

Energy minimisation
Energy minimisation is by far the simplest, fastest, and easiest computational method to use to obtain a model of a stapled peptide. It is useful for relieving any unfavourable interactions in the initial molecular model. Wang and coworkers used a commercial molecular modelling software suite to perform energy minimisation on several triazole stapled Bcl9 peptides to determine the optimal combination of linker length and stereochemistry for the staple [50]. They found that the linker that causes the least perturbation of the peptide backbone has an (S,R) configuration, with the triazole moiety sandwiched between five methylene units. The peptide stapled by this linker was later verified to be the most helical and tightest-binding peptide among those tested in the study. Energy minimisation has also been used to rationalise experimental observations. A hydrocarbon stapled HIV-1 integrase peptide was found to have high helicity but extremely low potency compared with the wild-type peptide [27]. The two peptides were energy minimised using a commercial software suite to obtain low-energy conformers. An essential binding residue in the stapled peptide was found to deviate conformationally from that in the wild-type peptide, and this was proposed as the reason for the loss in activity. However, energy minimisation is not the best method for obtaining accurate stapled peptide models, as it is only able to calculate local energy minima based on the initial conformation of the peptide, and will likely not be able to locate its lowest-energy conformation, which may be more relevant for the evaluation of different staple structures.

Monte Carlo simulations
Monte Carlo (MC) simulation is one method to sample different low-energy states of stapled peptides. It generates an ensemble of conformations by making random changes to the positions of the atoms at each step. The new configuration is accepted or rejected based on the Metropolis algorithm. Multiple local energy minima can then be sampled.

Kutchukian *et al.* observed that the *i*, *i* + 7 hydrocarbon staple is more helix stabilising than the *i*, *i* + 4 version in some cases, and vice versa in others [73]. RNAse A and BID BH3 peptides were selected as case studies. MC simulations of the unstapled, *i*, *i* + 4, and *i*, *i* + 7 stapled peptides were performed to obtain a conformational ensemble. Multiple runs were carried out until the average helicities converged. The simulations reproduced the helicity trends observed in experiments, showing that the *i*, *i* + 7 staple is indeed less effective than the *i*, *i* + 4 staple at enhancing the helicity of the BID peptide. This is attributed to the high occupancy of a partially helical decoy state by the *i*, *i* + 7 stapled peptide, which hinders optimal helix stabilisation. However, the authors did not address the question of what it is in the sequences of RNase A and BID that causes this disparity in helix stabilisation by different staples. The results also come with a caveat, as the hydrocarbon linkers in the simulations were saturated. It is not known whether the use of the unsaturated linkers will yield similar results.

MC simulations have also proven to be useful for the identification of optimal combinations of stereoisomers for new staple systems. In a study that described the development of the *i*, *i* + 3 hydrocarbon staple system, MC simulations identified the (R,S) configuration as the most helix-stabilising stereochemical combination, similar to what was determined from the experiments [61]. This configuration is the only one that avoids so-called 'syn-pentane' interactions between the α-methyl group and the hydrocarbon crosslink, which lead to deformation from the helical
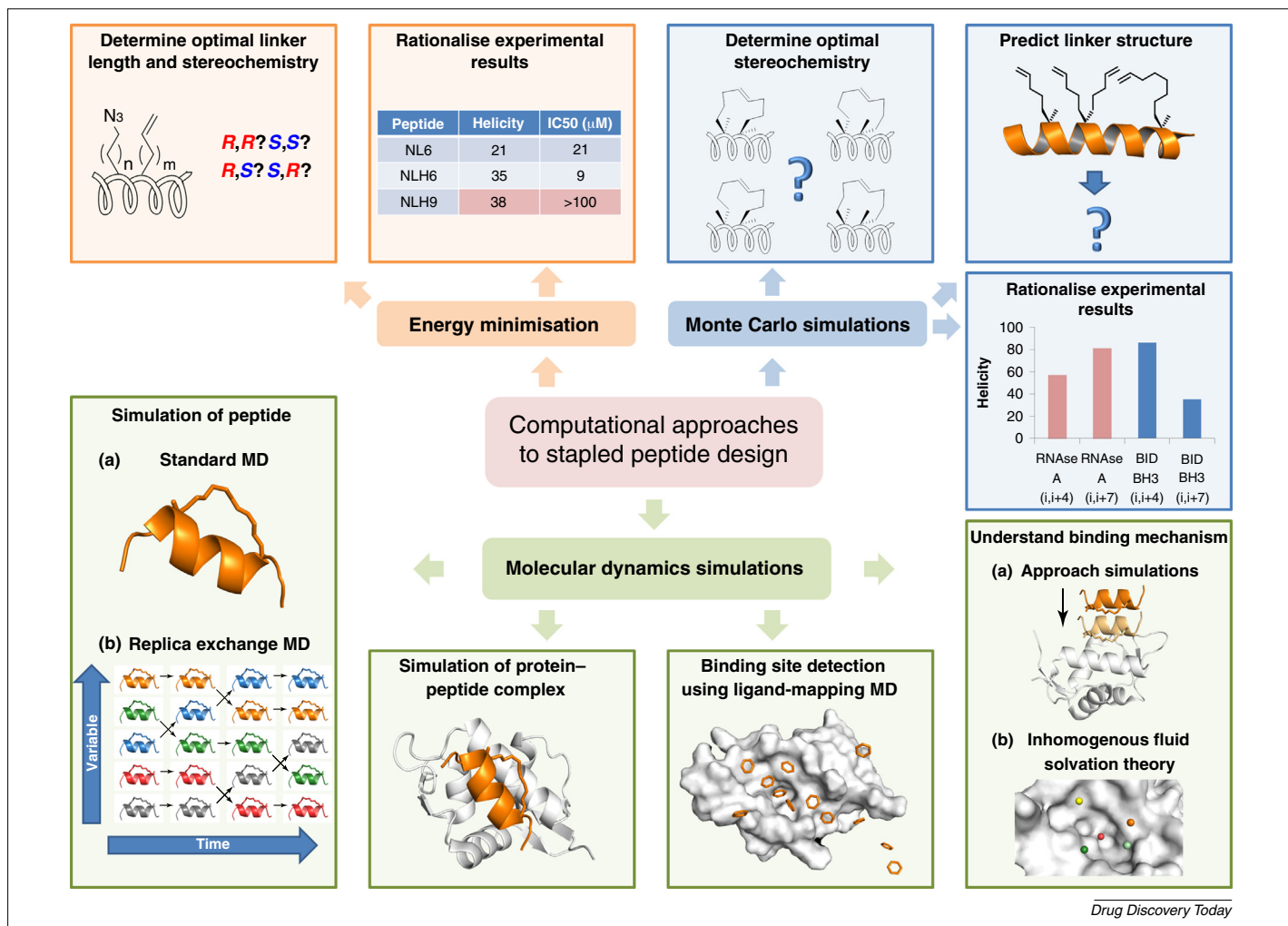
FIGURE 5

Overview of computational approaches in current use for stapled peptide design.

structure. MC simulations have also been used to predict the structure of the linker when multiple metathesis products are obtained in the synthesis of stitched peptides [69]. Here, syn-pentane interactions again have an important role in determining the structure of the major stitched product.

Molecular dynamics simulations

Molecular dynamics (MD) is a computer simulation method that generates new molecular conformations by the integration of Newton's equations of motion, resulting in a trajectory that describes the temporal evolution of a set of interacting atoms.

MD simulations of the unbound peptide of interest in solution may be performed to provide insight into its dynamics at the atomistic level. Helicity trends derived from such simulations were shown to correspond to those obtained by circular dichroism spectroscopy [69]. Within the same study, the authors also provided an atomistic explanation for the enhanced helicity of stitched peptides relative to singly stapled peptides. Being more extended, stitches are able to shield more peptide backbone hydrogen bonds from hydration compared with a single hydrocarbon staple, thus accounting for their greater helix stabilisation effect. In cases where multiple stapled products are obtained

during synthesis, MD simulations of the unbound peptide can be used to predict the structures of the major and minor products. In a study on *in situ* stapling, the double-click reaction that formed the triazole stapled peptide resulted in four possible regioisomers [22]. MD simulations of the isomers indicated that the most helical isomer corresponded to the major product, whose structure was subsequently confirmed by X-ray crystallography. This suggests a role for MD simulations in linker structure prediction, especially in cases of complex staple linkages formed by two-component stapling techniques [18].

Replica exchange MD (REMD) is a variation of the standard MD simulation method that enhances sampling efficiency by simulation at different temperatures. It was used to study a set of i, $i + 7$ hydrocarbon stapled p53 peptides that varied in their staple position and helix propensities [74]. The helicities obtained from the REMD simulations were in good qualitative agreement with the experimental values. Low helix propensity was linked to the population of decoy states, also observed by Kutchukian *et al.* in their MC simulations of BID stapled peptides [73]. Certain staple positions resulted in highly populated semihelical decoy states and lowly populated fully helical states, thus accounting for the difference in helicities within the set of stapled peptides. The MD

studies described so far indicate that MD simulations of unbound stapled peptides are able to provide good qualitative approximations of the experimental helicity trends, and can be used to whittle down the number of stapled peptides to be synthesised and tested in a project. REMD also proved useful for guiding the design of a set of stapled eIF4G peptides, for which binding affinity was found to correlate with the extent of conformational similarity between the unbound and bound peptides [56].

Besides analysing simulations of the unbound peptide, simulations of the protein–peptide complex can also yield valuable information that helps in stapled peptide design. A combination of such simulations can be a very useful tool for in silico staple scanning in situations where experimental staple scanning is not possible because of limited resources. A library of stapled peptides containing all possible permutations of staple positions and architecture can be easily created and then screened via MD simulations to identify the most helical and tightest-binding stapled peptide. A simulation study conducted by Joseph et al. on several MCL-1 BH3 hydrocarbon stapled peptides with varying staple positions helped to provide structural insights into the experimental binding trends [75]. The results also indicated that a new staple position that was untried in the experiments is likely to provide a bigger affinity boost than the current optimal position. Simulations of the protein–peptide complex were also used in conjunction with REMD simulations of the unbound peptides to design stapled eIF4G peptides with improved binding affinities [56]. As mentioned above, MD simulations of the protein–peptide complex were also instrumental in revealing the interaction of the hydrocarbon staple of a stapled p53 peptide with the surface of MDM2 [57], which was subsequently validated by X-ray crystallography [59]. This has contributed to the important realisation that the hydrocarbon staple itself is able to contribute to target binding and should be considered in future stapled peptide designs. In these studies, MD simulations were complemented by the molecular mechanics/generalised Born surface area (MM/GBSA) method [76] to estimate the free energy of binding, which is estimated as the sum of gas-phase molecular mechanics energies, solvation free energies, and configurational entropies. MM/GBSA and the closely related molecular mechanics/Poisson–Boltzmann surface area (MM/PBSA) [77] are computationally efficient free energy methods that also allow for the decomposition of the binding free energy into contributions from the side chain and backbone atoms of the protein and peptide on a per-residue basis [78]. They are usually preferred to the more rigorous, but expensive, free energy perturbation and thermodynamic integration methods [79], as well as the linear interaction energy method [80], which requires the use of empirical parameters. A major drawback of the MM/PB(GB)SA method relates to the calculation of the entropy term, which is computationally demanding and leads to large fluctuations arising from inadequate sampling [81].

A special type of MD simulation method called ligand-mapping molecular dynamics (LMMD) has been shown to be effective at locating the shallow hydrophobic binding sites occupied by these hydrocarbon staples [82]. In LMMD, benzene molecules are used at low concentrations in explicit-solvent MD simulations of the target protein to probe for hydrophobic binding sites. The positions sampled by the benzene probes during the simulations are then converted into occupancy maps that are represented by density grids. Such simulations have been successfully used to identify a novel ligand-binding mode at a cryptic binding pocket [83], enhance conformational sampling, and locate hydrophobic peptide binding sites [82,84]. The method was tested on five proteins that have known hydrocarbon staple interaction sites. All of them were successfully detected in the simulations by the benzene probes (Fig. 6). A novel staple binding site on one of the test proteins, MCL-1, was also predicted to be more druggable than the current known staple binding site. This is in agreement with the results of the computational study mentioned earlier by Joseph et al., who utilised staple-scanning MD simulations to study the MCL-1–peptide interaction [75], further lending support to the targeting of this alternative binding site. We eagerly await the testing of this prediction by experimentalists.

The temporal nature of MD simulations means that they can be useful for understanding binding events, such as peptide–protein binding. Multiple short approach simulations were performed for a range of peptide–protein distances on a set of wild-type, unstapled, and stapled p53 peptides, to study the effect of the hydrocarbon staple on the mechanism of peptide binding [85]. The peptide set featured those with staples that associate with the MDM2 protein surface in the complex. While the wild-type p53 peptide favours rebinding to MDM2 by tilting in the direction of Phe19, one of the key binding residues, the stapled peptide has no such preference and tilts initially towards either the Phe19 or Leu26 binding pockets. An ordered water network was also observed between the staple and protein surface as the peptide rebound to the protein in these approach simulations. This was conspicuously absent for the unstapled peptides. A subsequent detailed study on these interfacial water molecules restrained the position of the peptides instead of allowing rebinding [86]. The results suggest that the hydrophobic staple facilitates the formation of the interfacial water network, which helps to lower the energy barrier for binding due to enthalpic gain from the formation of hydrogen bonds between the structured water molecules.

The role of individual water molecules in facilitating hydrophobic interactions can be further elucidated by using inhomogeneous fluid solvation theory (IFST) [87], which quantifies solvation free energies by analysing the extent of perturbation of bulk water structure and thermodynamics by a solute. IFST is included in the commercial drug discovery software suite, Schrodinger, as the WaterMap module. The ring closing metathesis reaction that generates the hydrocarbon staples usually results in the formation of a mixture of cis (Z) and trans (E) isomers, which are challenging to separate. A computational study investigated the effects of geometric isomerism in i, i + 7 hydrocarbon staples using MD simulations [88]. Free energies obtained from WaterMap indicate that the trans linker packs more tightly than the cis linker against the hydrophobic binding pocket of the staple, even though REMD simulations show that both cis and trans configurations confer comparable helicities; however, these observations have yet to be experimentally verified. Nevertheless, the results suggest that geometric isomerism in hydrocarbon staples influences peptide potency. Recent advances in synthetic methodologies have allowed the stereoselective synthesis of the Z isomer [89]. It is hoped that further progress in catalyst design will enable E-selective olefin metathesis, which should facilitate experimental investigation of the role of geometric isomerism in stapled peptide helicity and activity.

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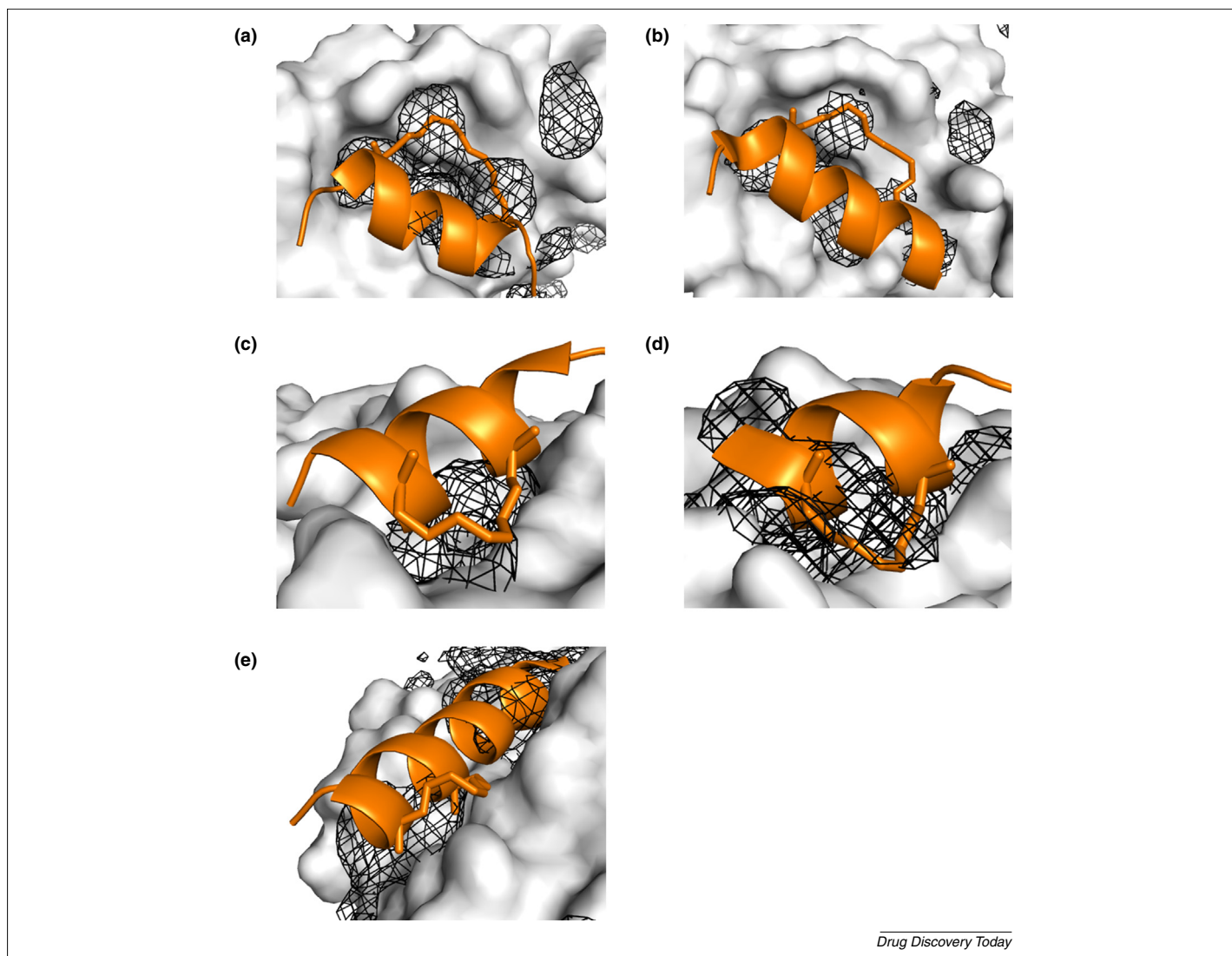
FIGURE 6

Benzene occupancy maps (black mesh) of **(a)** MDM2 (PDB 3V3B [59]), **(b)** MDMX (PDB 4N5T [41]), **(c)** ERα (PDB 2YJA [60]), **(d)** ERβ (PDB 2YJD [60]), and **(e)** MCL-1 (PDB 3MK8 [58]) with their respective stapled peptide binders (orange) superimposed. Adapted with permission from [82]. Copyright 2015 American Chemical Society.

A key consideration that might limit the application of MC and MD simulations in stapled peptide design is accurate parameterisation of the unnatural amino acid residues that form the staple linkages. Force fields have been developed separately to describe the common amino acid residues found in proteins [90] and small molecules [91,92], but there are none specifically developed for unnatural amino acid residues. Parameters for these non-standard residues could be obtained by using approaches based on quantum mechanical calculations [93,94]; however, their suitability for the parameterisation of residues with a third attachment point, such as those found in stapled peptides, has not been properly evaluated.

Future directions

The application of computational techniques to stapled peptide design is still at a nascent stage, and there are many questions that can potentially be addressed by the appropriate method. Of utmost concern are the relations between peptide helicity, *in vitro* binding affinity, cellular activity, and biological potency. Contrary to expectations, there appears to be weak correlations between these factors. For instance, in a study on the development of hydrocarbon stapled MCL-1 BH3 peptides, there was no discernible relation between helicity, binding affinity, and cellular activity [58]. Peptides that were less helical displayed better binding affinity for MCL-1 compared with a more helical peptide. The most helical stapled peptide was the second least active in cells, while the least helical stapled peptide was the second most cell active. No direct correlation was also found between helical content and cell activity in another study on the targeting of HIV-1 integrase by hydrocarbon stapled peptides [27]. Stapling also does not always improve the peptide's binding affinity, as observed in several independent studies [40,95–97]. Enthalpy–entropy compensation has recently been invoked to explain these perplexing observations [97]. It is suggested that, because of the presence of backbone hydrogen bonding prior to binding, the gain in favourable enthalpy upon binding by a stapled peptide is not as significant as that observed with the unstapled peptide. This could offset the entropic advantage that the stapled peptide has over the unstapled peptide. Atomistic MD simulations coupled with rigorous free energy

methods may be able to provide meaningful insights into this perplexing phenomenon.

Given the recent rapid advances in computational efficiency and hardware that allow MD simulations on timescales as long as milliseconds to be performed [98], it might be possible for these simulations to provide insights into key aspects of the mechanism of action of stapled peptides. As more is understood about the mechanism of cell entry by stapled peptides [37], appropriate models of peptide–membrane systems could be built and simulated to understand peptide cell penetration at the atomic level, thus aiding in the optimisation of cell-permeable stapled peptides. Longer simulation timescales can be attained by the use of coarse-grained models [99]. It has been shown that long MD simulations are able to reproduce the crystallographic binding mode of a small molecule that is initially randomly placed away from the target protein [100]. Similarly, it may not be long before such extended simulations are able to provide an atomistic understanding of the entire process of peptide binding. These could help researchers to reconcile the apparent discordance between peptide helicity, *in vitro* binding affinity, and cell activity trends.

The inhibitory effect of serum on the biological activity of hydrocarbon stapled peptides has been highlighted in recent reports [40,41,101]. As serum concentration increases, the activity of about one third of published stapled peptide constructs diminishes [70]. This effect might result from the direct binding of the stapled peptides to serum components, thus inhibiting their cell permeability. However, a recent study by Li *et al.* suggested that the cytotoxic activity of certain stapled peptides targeting the MDM2–p53 interaction may be due to target-independent membrane disruption rather than specific target binding, and that serum protects the cell membrane from stapled peptide-mediated membrane damage by an as-yet-unknown mechanism [72]. To exclude the possibility of direct interaction between serum proteins and stapled peptides, the authors performed experiments to show that the activities of the studied stapled peptides remain unchanged in cell lysates that have serum added. These results are contradictory to those reported by Chang *et al.* [41], who showed that one of the stapled peptides in the Li *et al.* study is highly associated (98%) with serum proteins in ultracentrifugation experiments. Thus, there is a need for more conclusive studies. One such possible study would be to screen a set of common serum proteins for direct association with stapled peptides in biophysical binding assays. These experimental efforts could be complemented by computational methods, such as molecular docking [102] and MD simulations, to develop and evaluate models of the putative serum protein–stapled peptide complexes.

Despite the plethora of published studies on stapled peptides, no one has managed to come up with a set of guiding principles for choosing a suitable staple architecture. More often than not, there is no detailed investigation into the use of alternative staple architectures, presumably because of limited resources. For example, in the study on the development of hydrocarbon stapled peptides to target the NOTCH transcription factor complex, the i, $i + 4$ staple was arbitrarily employed, even though the helical peptide sequence is long enough to accommodate an i, $i + 7$ staple [25]. The 2009 computational study by Kutchukian *et al.* proposed the concept of decoy states to explain the staple preference for a particular peptide sequence [73]. That study could have been furthered by the use of MD simulations to investigate the origins of the decoy states and provide useful insights into the decoy-forming propensity of different hydrocarbon staple architectures, thus contributing to our knowledge of stapled peptide design.

Concluding remarks

Peptide stapling to enhance helicity and biological activity has been practised since 1988, when the first lactam staples were formed between lysine and aspartic acid residues [103]. Stapling ensures that the peptide retains the α-helical conformation essential for binding at α-helix-mediated protein–protein interfaces, thus enhancing binding potency. However, the field only truly burst into life after the introduction of hydrocarbon staples by Verdine and coworkers in 2000 [30]. Since then, a range of studies have demonstrated the potential utility of hydrocarbon stapled peptides in the treatment of various human diseases. Two hydrocarbon stapled peptides have reached clinical trials, epitomising the remarkable progress made in this field within the past 15 years [49]. A paradigm for stapled peptide design is also gradually being developed based on the huge body of experimental data that has been collected so far.

While design principles for optimising affinity and cellular uptake are still being refined, computational methods are playing an increasingly important role in the design process. They can help to rationalise experimental observations and reduce the number of stapled peptide variants to be tested, saving time and money. Simulation techniques, such as MC and MD simulations, have been used to predict optimal positions for staple placement and stereochemical combinations of building blocks for staple formation, as well as to understand the binding mechanism. There is tremendous potential for computation to play an even bigger role in stapled peptide design. Pressing issues, such as the lack of correlation between helicity and cellular activity trends, the mechanism of stapled peptide cellular uptake, the role of serum in affecting membrane penetration, and choosing the most suitable staple architecture, could potentially be addressed with the help of computational models. We expect computational methods to assume expanded and even more prominent roles in stapled peptide design in the near future.

Acknowledgement

References

1 Wells, J.A. and McClendon, C.L. (2007) Reaching for high-hanging fruit in drug discovery at protein–protein interfaces. *Nature* 450, 1001–1009

2 Spencer, R.W. (1998) High-throughput screening of historic collections: observations on file size, biological targets, and file diversity. *Biotechnol. Bioeng.* 61, 61–67

3 Barker, A. *et al.* (2013) Expanding medicinal chemistry space. *Drug Discov. Today* 18, 298–304

4 Arkin Michelle, R. *et al.* (2014) Small-molecule inhibitors of protein–protein interactions: progressing toward the reality. *Chem. Biol.* 21, 1102–1114

5 Mullard, A. (2012) Protein–protein interaction inhibitors get into the groove. *Nat. Rev. Drug Discov.* 11, 173–175

6 Shangary, S. and Wang, S.M. (2009) Small-molecule inhibitors of the MDM2-p53 protein–protein interaction to reactivate p53 function: a novel approach for cancer therapy. *Annu. Rev. Pharmacol. Toxicol.* 49, 223–241

7 Vogler, M. *et al.* (2009) Bcl-2 inhibitors: small molecules with a big impact on cancer therapy. *Cell Death Differ.* 16, 360–367

8 Wilson, A.J. (2009) Inhibition of protein–protein interactions using designed molecules. *Chem. Soc. Rev.* 38, 3289–3300

9 Craik, D.J. *et al.* (2013) The future of peptide-based drugs. *Chem. Biol. Drug Des.* 81, 136–147

10 Vlieghe, P. *et al.* (2010) Synthetic therapeutic peptides: science and market. *Drug Discov. Today* 15, 40–56

11 Guharoy, M. and Chakrabarti, P. (2007) Secondary structure based analysis and classification of biological interfaces: identification of binding motifs in protein–protein interactions. *Bioinformatics* 23, 1909–1918

12 Jones, S. and Thornton, J.M. (1995) Protein–protein interactions: a review of protein dimer structures. *Prog. Biophys. Mol. Biol.* 63, 31–65

13 Bock, J.E. *et al.* (2013) Getting in shape: controlling peptide bioactivity and bioavailability using conformational constraints. *ACS Chem. Biol.* 8, 488–499

14 Balaram, P. (1992) Non-standard amino acids in peptide design and protein engineering. *Curr. Opin. Struct. Biol.* 2, 845–851

15 Karle, I.L. *et al.* (1990) Apolar peptide models for conformational heterogeneity, hydration, and packing of polypeptide helices: crystal structure of hepta- and octapeptides containing alpha-aminoisobutyric acid. *Proteins* 7, 62–73

16 Cabezas, E. and Satterthwait, A.C. (1999) The hydrogen bond mimic approach: solid-phase synthesis of a peptide stabilized as an α-helix with a hydrazone link. *J. Am. Chem. Soc.* 121, 3862–3875

17 Patgiri, A. *et al.* (2008) A hydrogen bond surrogate approach for stabilization of short peptide sequences in alpha-helical conformation. *Acc. Chem. Res.* 41, 1289–1300

18 Lau, Y.H. *et al.* (2015) Peptide stapling techniques based on different macrocyclisation chemistries. *Chem. Soc. Rev.* 44, 91–102

19 Verdine, G.L. and Hilinski, G.J. (2012) All-hydrocarbon stapled peptides as synthetic cell-accessible mini-proteins. *Drug Discov. Today Technol.* 9, e41–e47

20 Harrison, R.S. *et al.* (2010) Downsizing human, bacterial, and viral proteins to short water-stable alpha helices that maintain biological potency. *Proc. Natl. Acad. Sci. U. S. A.* 107, 11686–11691

21 Harrison, R.S. *et al.* (2010) Novel helix-constrained nociceptin derivatives are potent agonists and antagonists of ERK phosphorylation and thermal analgesia in mice. *J. Med. Chem.* 53, 8400–8408

22 Lau, Y.H. *et al.* (2015) Double strain-promoted macrocyclization for the rapid selection of cell-active stapled peptides. *Angew. Chem. Int. Ed.* 54, 15410–15413

23 Bullock, B.N. *et al.* (2011) Assessing helical protein interfaces for inhibitor design. *J. Am. Chem. Soc.* 133, 14220–14223

24 Watkins, A.M. *et al.* (2015) Protein–protein interactions mediated by helical tertiary structure motifs. *J. Am. Chem. Soc.* 137, 11622–11630

25 Moellering, R.E. *et al.* (2009) Direct inhibition of the NOTCH transcription factor complex. *Nature* 462, 182–188

26 Grossmann, T.N. *et al.* (2012) Inhibition of oncogenic Wnt signaling through direct targeting of β-catenin. *Proc. Natl. Acad. Sci. U. S. A.* 109, 17942–17947

27 Long, Y-Q. *et al.* (2013) Design of cell-permeable stapled peptides as HIV-1 integrase inhibitors. *J. Med. Chem.* 56, 5601–5612

28 Bellmann-Sickert, K. *et al.* (2015) Efflux by small multidrug resistance proteins is inhibited by membrane-interactive helix-stapled peptides. *J. Biol. Chem.* 290, 1752–1759

29 Sinclair, J.K.L. *et al.* (2014) Inhibiting epidermal growth factor receptor at a distance. *J. Am. Chem. Soc.* 136, 11232–11235

30 Schafmeister, C.E. *et al.* (2000) An all-hydrocarbon cross-linking system for enhancing the helicity and metabolic stability of peptides. *J. Am. Chem. Soc.* 122, 5891–5892

31 Scrima, M. *et al.* (2010) CuI-catalysed azide-alkyne intramolecular *i*-to-(*i* + 4) side-chain-to-side-chain cyclization promotes the formation of helix-like secondary structures. *Eur. J. Org. Chem.* 2010, 446–457

32 Taylor, J.W. (2002) The synthesis and study of side-chain lactam-bridged peptides. *Pept. Sci.* 66, 49–75

33 Jackson, D.Y. *et al.* (1991) General approach to the synthesis of short α-helical peptides. *J. Am. Chem. Soc.* 113, 9391–9392

34 Brunel, F.M. and Dawson, P.E. (2005) Synthesis of constrained helical peptides by thioether ligation: application to analogs of gp41. *Chem. Commun.* 2552–2554

35 Kumita, J.R. *et al.* (2000) Photo-control of helix content in a short peptide. *Proc. Natl. Acad. Sci. U. S. A.* 97, 3803–3808

36 Henchey, L.K. *et al.* (2008) Contemporary strategies for the stabilization of peptides in the α-helical conformation. *Curr. Opin. Chem. Biol.* 12, 692–697

37 Chu, Q. *et al.* (2015) Towards understanding cell penetration by stapled peptides. *MedChemComm* 6, 111–119

38 Blackwell, H.E. and Grubbs, R.H. (1998) Highly efficient synthesis of covalently cross-linked peptide helices by ring-closing metathesis. *Angew. Chem. Int. Ed.* 37, 3281–3284

39 Bernal, F. *et al.* (2007) Reactivation of the p53 tumor suppressor pathway by a stapled p53 peptide. *J. Am. Chem. Soc.* 129, 2456–2457

40 Brown, C.J. *et al.* (2013) Stapled peptides with improved potency and specificity that activate p53. *ACS Chem. Biol.* 8, 506–512

41 Chang, Y.S. *et al.* (2013) Stapled α-helical peptide drug development: a potent dual inhibitor of MDM2 and MDMX for p53-dependent cancer therapy. *Proc. Natl. Acad. Sci. U. S. A.* 110, E3445–E3454

42 Chen, X. *et al.* (2015) A stapled peptide antagonist of MDM2 carried by polymeric micelles sensitizes glioblastoma to temozolomide treatment through p53 activation. *J. Control. Release* 218, 29–35

43 Walensky, L.D. *et al.* (2004) Activation of apoptosis *in vivo* by a hydrocarbon-stapled BH3 helix. *Science* 305, 1466–1470

44 Bhattacharya, S. *et al.* (2008) Solution structure of a hydrocarbon stapled peptide inhibitor in complex with monomeric C-terminal domain of HIV-1 capsid. *J. Biol. Chem.* 283, 16274–16278

45 Zhang, H. *et al.* (2008) A cell-penetrating helical peptide as a potential HIV-1 inhibitor. *J. Mol. Biol.* 378, 565–580

46 Danial, N.N. *et al.* (2008) Dual role of proapoptotic BAD in insulin secretion and beta cell survival. *Nat. Med.* 14, 144–153

47 Sviridov, D.O. *et al.* (2011) Helix stabilization of amphipathic peptides by hydrocarbon stapling increases cholesterol efflux by the ABCA1 transporter. *Biochem. Biophys. Res. Commun.* 410, 446–451

48 Bird, G.H. *et al.* (2014) Mucosal delivery of a double-stapled RSV peptide prevents nasopulmonary infection. *J. Clin. Invest.* 124, 2113–2124

49 Cromm, P.M. *et al.* (2015) Hydrocarbon stapled peptides as modulators of biological function. *ACS Chem. Biol.* 10, 1362–1375

50 Kawamoto, S.A. *et al.* (2012) Design of triazole-stapled BCL9 α-helical peptides to target the β-catenin/B-cell CLL/lymphoma 9 (BCL9) protein–protein interaction. *J. Med. Chem.* 55, 1137–1146

51 Torres, O. *et al.* (2008) Peptide tertiary structure nucleation by sidechain crosslinking with metal complexation and double 'click' cycloaddition. *ChemBioChem* 9, 1701–1705

52 Lau, Y.H. *et al.* (2014) Functionalised staple linkages for modulating the cellular activity of stapled peptides. *Chem. Sci.* 5, 1804–1809

53 Frank, A.O. *et al.* (2014) Discovery of a potent stapled helix peptide that binds to the 70N domain of replication protein A. *J. Med. Chem.* 57, 2455–2461

54 Cunningham, B. and Wells, J. (1989) High-resolution epitope mapping of hGH-receptor interactions by alanine-scanning mutagenesis. *Science* 244, 1081–1085

55 Gavathiotis, E. *et al.* (2008) BAX activation is initiated at a novel interaction site. *Nature* 455, 1076–1081

56 Lama, D. *et al.* (2013) Rational optimization of conformational effects induced by hydrocarbon staples in peptides and their binding interfaces. *Sci. Rep.* 3, 3451

57 Joseph, T.L. *et al.* (2010) Stapled peptides in the p53 pathway: computer simulations reveal novel interactions of the staples with the target protein. *Cell Cycle* 9, 4560–4568

58 Stewart, M.L. *et al.* (2010) The MCL-1 BH3 helix is an exclusive MCL-1 inhibitor and apoptosis sensitizer. *Nat. Chem. Biol.* 6, 595–601

59 Baek, S. *et al.* (2012) Structure of the stapled p53 peptide bound to Mdm2. *J. Am. Chem. Soc.* 134, 103–106

60 Phillips, C. *et al.* (2011) Design and structure of stapled peptides binding to estrogen receptors. *J. Am. Chem. Soc.* 133, 9696–9699

61 Kim, Y-W. *et al.* (2010) Introduction of all-hydrocarbon *i*, *i* + 3 staples into alpha-helices via ring-closing olefin metathesis. *Org. Lett.* 12, 3046–3049

62 Kim, Y-W. and Verdine, G.L. (2009) Stereochemical effects of all-hydrocarbon tethers in *i*, *i* + 4 stapled peptides. *Bioorg. Med. Chem. Lett.* 19, 2533–2536

63 Ran, X. *et al.* (2016) Design of high-affinity stapled peptides to target the repressor activator protein 1 (RAP1)/telomeric repeat-binding factor 2 (TRF2) protein–protein interaction in the shelterin complex. *J. Med. Chem.* 59, 328–334

64 Yeo, D.J. *et al.* (2013) Monosubstituted alkenyl amino acids for peptide 'stapling'. *Chem. Commun.* 49, 9131–9133

65 Shim, S.Y. *et al.* (2013) A new *i*, *i* + 3 peptide stapling system for α-helix stabilization. *Chem. Biol. Drug Des.* 82, 635–642

66 Bird, G.H. *et al.* (2014) Stapled HIV-1 peptides recapitulate antigenic structures and engage broadly neutralizing antibodies. *Nat. Struct. Mol. Biol.* 21, 1058–1067

67 Giordanetto, F. *et al.* (2013) Stapled vasoactive intestinal peptide (VIP) derivatives improve VPAC2 agonism and glucose-dependent insulin secretion. *ACS Med. Chem. Lett.* 4, 1163–1168

68 Bird, G.H. *et al.* (2010) Hydrocarbon double-stapling remedies the proteolytic instability of a lengthy peptide therapeutic. *Proc. Natl. Acad. Sci. U. S. A.* 107, 14093–14098

69 Hilinski, G.J. *et al.* (2014) Stitched α-helical peptides via bis ring-closing metathesis. *J. Am. Chem. Soc.* 136, 12314–12322

70 Walensky, L.D. and Bird, G.H. (2014) Hydrocarbon-stapled peptides: principles, practice, and progress. *J. Med. Chem.* 57, 6275–6288

71 Leshchiner, E.S. *et al.* (2015) Direct inhibition of oncogenic KRAS by hydrocarbon-stapled SOS1 helices. *Proc. Natl. Acad. Sci. U. S. A.* 112, 1761–1766

72 Li, Y.-C. *et al.* (2014) A versatile platform to analyse low-affinity and transient protein–protein interactions in living cells in real time. *Cell Rep.* 9, 1946–1958

73 Kutchukian, P.S. *et al.* (2009) All-atom model for stabilization of alpha-helical structure in peptides by hydrocarbon staples. *J. Am. Chem. Soc.* 131, 4622–4627

74 Guo, Z. *et al.* (2010) Probing the α-helical structural stability of stapled p53 peptides: molecular dynamics simulations and analysis. *Chem. Biol. Drug Des.* 75, 348–359

75 Joseph, T.L. *et al.* (2012) Stapled BH3 peptides against MCL-1: mechanism and design using atomistic simulations. *PLoS ONE* 7, e43985

76 Srinivasan, J. *et al.* (1998) Continuum solvent studies of the stability of DNA, RNA, and phosphoramidate–DNA helices. *J. Am. Chem. Soc.* 120, 9401–9409

77 Kollman, P.A. *et al.* (2000) Calculating structures and free energies of complex molecules: combining molecular mechanics and continuum models. *Acc. Chem. Res.* 33, 889–897

78 Gohlke, H. *et al.* (2003) Insights into protein–protein binding by binding free energy calculation and free energy decomposition for the Ras-Raf and Ras-RaIGDS complexes. *J. Mol. Biol.* 330, 891–913

79 Beveridge, D.L. and DiCapua, F.M. (1989) Free energy via molecular simulation: applications to chemical and biomolecular systems. *Annu. Rev. Biophys. Biophys. Chem.* 18, 431–492

80 Åqvist, J. *et al.* (1994) A new method for predicting binding affinity in computer-aided drug design. *Protein Eng.* 7, 385–391

81 Hou, T. *et al.* (2011) Assessing the performance of the MM/PBSA and MM/GBSA methods. 1. The accuracy of binding free energy calculations based on molecular dynamics simulations. *J. Chem. Inf. Model.* 51, 69–82

82 Tan, Y.S. *et al.* (2015) The application of ligand-mapping molecular dynamics simulations to the rational design of peptidic modulators of protein–protein interactions. *J. Chem. Theory Comput.* 11, 3199–3210

83 Tan, Y.S. *et al.* (2012) Using ligand-mapping simulations to design a ligand selectively targeting a cryptic surface pocket of polo-like kinase 1. *Angew. Chem. Int. Ed.* 51, 10078–10081

84 Tan, Y.S. *et al.* (2014) The use of chlorobenzene as a probe molecule in molecular dynamics simulations. *J. Chem. Inf. Model.* 54, 1821–1827

85 Sim, A.Y.L. *et al.* (2014) Mechanism of stapled peptide binding to MDM2: possible consequences for peptide design. *J. Chem. Theory Comput.* 10, 1753–1761

86 Sim, A.Y.L. and Verma, C. (2015) How does a hydrocarbon staple affect peptide hydrophobicity? *J. Comput. Chem.* 36, 773–784

87 Lazaridis, T. (1998) Inhomogeneous fluid approach to solvation thermodynamics. 1. Theory. *J. Phys. Chem. B* 102, 3531–3541

88 Guo, Z. *et al.* (2014) Probing the origin of structural stability of single and double stapled p53 peptide analogs bound to MDM2. *Chem. Biol. Drug Des.* 83, 631–642

89 Mangold, S.L. *et al.* (2014) Z-Selective olefin metathesis on peptides: investigation of side-chain influence, preorganization, and guidelines in substrate selection. *J. Am. Chem. Soc.* 136, 12469–12478

90 Ponder, J.W. and Case, D.A. (2003) Force fields for protein simulations. *Adv. Protein Chem.* 66, 27–85

91 Wang, J.M. *et al.* (2004) Development and testing of a general amber force field. *J. Comput. Chem.* 25, 1157–1174

92 Vanommeslaeghe, K. *et al.* (2010) CHARMM general force field: a force field for drug-like molecules compatible with the CHARMM all-atom additive biological force fields. *J. Comput. Chem.* 31, 671–690

93 Vanquelef, E. *et al.* (2011) R.E.D. Server: a web service for deriving RESP and ESP charges and building force field libraries for new molecules and molecular fragments. *Nucleic Acids Res.* 39, W511–W517

94 Huang, L. and Roux, B. (2013) Automated force field parameterization for nonpolarizable and polarizable atomic models based on *ab initio* target data. *J. Chem. Theory Comput.* 9, 3543–3556

95 Okamoto, T. *et al.* (2013) Stabilizing the pro-apoptotic BimBH3 helix (BimSAHB) does not necessarily enhance affinity or biological activity. *ACS Chem. Biol.* 8, 297–302

96 Okamoto, T. *et al.* (2014) Further insights into the effects of pre-organizing the BimBH3 helix. *ACS Chem. Biol.* 9, 838–839

97 Miles, J.A. *et al.* (2016) Hydrocarbon constrained peptides – understanding preorganisation and binding affinity. *Chem. Sci.* 7, 3694–3702

98 Dror, R.O. *et al.* (2012) Biomolecular simulation: a computational microscope for molecular biology. *Annu. Rev. Biophys.* 41, 429–452

99 Saiz, L. and Klein, M.L. (2002) Computer simulation studies of model biological membranes. *Acc. Chem. Res.* 35, 482–489

100 Shan, Y. *et al.* (2011) How does a drug molecule find its target binding site? *J. Am. Chem. Soc.* 133, 9181–9183

101 Edwards, A.L. *et al.* (2013) Multimodal interaction with BCL-2 family proteins underlies the proapoptotic activity of PUMA BH3. *Chem. Biol.* 20, 888–902

102 London, N. *et al.* (2013) Peptide docking and structure-based characterization of peptide binding: from knowledge to know-how. *Curr. Opin. Struct. Biol.* 23, 894–902

103 Felix, A.M. *et al.* (1988) Synthesis, biological activity and conformational analysis of cyclic GRF analogs. *Int. J. Pept. Protein Res.* 32, 441–454