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Designing helical peptide inhibitors of protein-protein interactions

Raheleh Rezaei Araghi[1] and **Amy E. Keating**[1,2,*]

[1]MIT Department of Biology, 77 Massachusetts Avenue, Cambridge, MA 02139, United States

[2]MIT Department of Biological Engineering, 77 Massachusetts Avenue, Cambridge, MA 02139, United States

Abstract

Short helical peptides combine characteristics of small molecules and large proteins and provide an exciting area of opportunity in protein design. A growing number of studies report novel helical peptide inhibitors of protein-protein interactions. New techniques have been developed for peptide design and for chemically stabilizing peptides in a helical conformation, which frequently improves protease resistance and cell permeability. We summarize advances in peptide crosslinking chemistry and give examples of peptide design studies targeting coiled-coil transcription factors, Bcl-2 family proteins, MDM2/MDMX, and HIV gp41, among other targets.

Keywords

Protein-protein interaction; peptide inhibitor; peptide design; peptide libraries; helix crosslinking; hydrocarbon staple

Introduction

Of the many protein functions that scientists would like to program and control, binding is among the most fundamental. Proteins or other molecules that bind selectively to desired targets can be used for investigative molecular biology, as tools to control molecular assembly, as diagnostics, or as therapeutics. Proteins or peptides that bind to a target in competition with an endogenous partner have high potential for therapeutic development because many protein-protein interactions (PPIs) are misregulated in disease.

Despite some successes, it is broadly recognized that PPIs are difficult to control using small molecules.[1] Small molecules may not find purchase in the sometimes-flat interfacial surfaces of proteins, and low-molecular weight compounds may not compete effectively with interactions that bury a large surface area. Developing protein-based PPI inhibitors also faces obstacles. For example, antibodies can be engineered to bind with high affinity to many targets, but these molecules are currently limited to extracellular applications. The large size of antibodies is also non-ideal for applications that require deep tumor penetration or fast clearance, and the immunoglobulin fold may not be optimal for binding narrow, hard-

*To whom correspondence should be addressed: keating@mit.edu.

to-access epitopes. Intermediate between large proteins and small molecules, peptides hold promise for inhibiting protein interactions both inside and outside living cells. The cell delivery problem is more tractable for peptides than for antibodies, and peptides have the flexibility to adopt diverse conformations and make a variety of chemical contacts. The diversity of synthetic peptides can be increased by incorporation of non-natural side chains or other chemical modifications.

Designing a short peptide to bind to a specific target-protein interaction surface poses a difficult problem. One approach is to design synthetic molecules that closely mimic a structural element found in a native interface.[2] Ideally, synthetic molecules would improve on native interactions to increase potency. The concept of mimicking naturally occurring interface structural elements has been explored for decades. Here, we provide an update on recent activity developing helical peptide inhibitors of protein-protein interactions. Several landmark protein targets of high biomedical importance use helices for binding, including the Bcl-2 family of proteins, MDM2/MDMX, and the HIV envelope protein gp41. All of these are discussed in this review; representative structures of helix-mediated complexes are shown in Fig. 1

The Arora group has surveyed the PDB to identify protein interfaces that include helices.[3] Bergey et al. published the Helical Interfaces in Protein-Protein Interactions (HiPP) database to compile examples of helix-mediated complexes.[4] HiPP includes information such as the length of the helix found at each interface, its contribution to the buried surface area of the complex, and an estimate of the energy change for mutation to alanine of each residue in the helix. The 2013 release compiles 7308 helices of minimum length 4 residues and average length ~13 residues that include at least two residues predicted to contribute at least 1 kcal/mol each to binding. Arora and colleagues recently moved on to consider interfaces that incorporate two interacting helices, and they have proposed and tested chemical linkage strategies for stabilizing short coiled-coil tertiary motifs that can potentially disrupt such complexes.[5,6]

Below we summarize studies that have used synthetic peptides to mimic and inhibit native helix-mediated interactions. The challenges addressed include: Can a native-sequence peptide partner be further optimized to improve binding affinity and/or specificity? Can synthetic peptides be modified to enhance cell permeability and protease resistance, both of which are critical for application of peptides as therapeutics? Can multiple peptide properties be optimized simultaneously? Much has been done in this field, and we highlight only recent advances. We also limit our treatment to inhibitors based on alpha-amino peptides, despite the exciting prospect of using other types of molecules, such as beta-peptides, as inhibitors.

Methods for improving native peptide sequence and scaffold structure

A short peptide corresponding to a native interfacial structural element can sometimes function as an interaction inhibitor. Improving on this simple approach can involve changing the sequence and/or altering the scaffold structure to improve peptide properties such as binding affinity and specificity, stability, solubility, protease resistance and cell permeability.

Peptide library screening and peptide chemical modification are essential tools for these tasks.

Library screening can be used to identify high-affinity peptide ligands, with in-cell screening presenting an attractive strategy for optimizing peptides for the environment in which they must function.[7] Particularly for longer peptides, an element of rational design can help focus the search on sequences likely to enhance function. In some design problems it is important to consider binding specificity and to improve the affinity of a peptide for one target but not for paralogous family members; this is a consideration in many of the case studies described below. In this situation, computational methods can be used to evaluate both desired and undesired interactions. Using computational optimization to design a library is a promising way to combine a rational approach with the power of high-throughput screening; Chen and Keating have reviewed advances in computationally guided library design.[8]

Short peptides are typically unstructured in solution, which makes them susceptible to proteolysis and means that the entropic price of folding must be paid upon binding. Furthermore, most unmodified peptides are poorly cell penetrating. Extensive research has led to diverse methods for stabilizing peptides in a helical conformation. One strategy is to incorporate a helical motif into a larger folded protein scaffold, which can also introduce additional favorable inhibitor-target contacts.[9,10] To promote cell entry, however, it is preferable to minimize peptide molecular weight. For this reason, many methods have been developed to stabilize short peptides in a helical conformation.

Crosslinking to enforce covalent *i, i+4* or *i, i+7* linkages is a common way to enhance helicity. Widely applied methods include lactam bridge formation between lysine and aspartate or glutamate, and the more recent and now widespread use of all-hydrocarbon stapling (Fig. 2a).[11] Hydrocarbon stapling can impart remarkable structural stability, extracellular and intracellular protease resistance, and cell entry via macropinocytosis.[12] The stapling modification can enhance or disrupt binding, and extensive optimization of linker length and position, as well as fine-tuning of the peptide sequence, is often required. Following the successful design of stapled peptides that engage high-profile biomedical targets, an increasing number of groups have reported new crosslinking and convenient chemistries. For example, Wang et al. demonstrated a facile synthetic platform for two-component thiol–ene coupling and applied this method to synthesize p53 mimetics that selectively induce cell death.[13]

Recent work on chemical crosslinking has included studies of how peptide modifications impact cell permeability. Pentelute and coworkers used a perfluorinated aryl linker, attached to cysteine residues, to make a peptide inhibitor of the HIV-1 capsid assembly polyprotein (Fig. 2b).[14] The lipophilicity of the perfluorinated moieties improved cell uptake. The chemistry of cysteine arylation that was used operates on unprotected peptides made of standard amino acids and does not require a metal catalyst.

Muppidi et al. used a series of distance-matched aryl and vinylaryl groups to link *i, i+ 7* cysteine residues by alkylation and found a correlation between cell permeability and

hydrophobicity (Fig. 2b).[15] This study highlighted the importance of factors beyond helicity and binding affinity. Spring and coworkers developed an *i, i+ 7* double-click stapling technique (Fig. 2c) and made a set of peptides with nanomolar affinity to MDM2.[16] However, the tightest-binding peptide showed poor uptake and was not active in a cellular p53 reporter assay. Modification of the dialkynyl linkers to add net positive charge resulted in improved cell entry.

New helix-stabilizing crosslinkers provide opportunities for further functional elaboration at the modification site. For example, Vasco et al. demonstrated that the Ugi reaction operating on modified side chains can both stabilize an α-turn and introduce exocyclic N-functionalization at the resulting lactam bridge.[17] In another example, the acetone-linked bridge reported by Assem et al. has been shown to enhance helical secondary structure when introduced between peptide side-chain nucleophiles such as thiols.[18] In addition to stabilizing helical structures, the ketone moiety in the linker can be modified with diverse molecular tags by oxime ligation. These chemistries provide additional opportunities to improve both peptide-target interactions and other peptide properties.

Switchable crosslinkers provide the ability to control peptide helicity with light or other inputs. The strategy of connecting cysteine residues with a crosslinker containing azobenzene has been used to provide photocontrol in several systems.[19,20] Martín-Quíros et al. recently developed a helical peptide inhibitor of the interaction between β-arrestin and β-adaptin for which the inhibition constant differs by ~12.6 fold in the presence vs. absence of light.[19] Interestingly, Belotto et al. have shown how peptides including azobenze crosslinkers can be selected for binding using phage display, which may lead to exciting applications in helical peptide design.[21] In an example of redox switching, Miller et al. developed a reversible stapling system in which peptide conformation can be controlled through thiol oxidation.[22] Another example of a reversible stapling strategy is the formation of oxime linkages through the chemoselective reaction between aminooxy- and aldehyde-functionalized amino acids (Fig. 2a).[23] Switchable peptide inhibitors are useful for probing the relation between protein structure and interaction, and potentially for turning interactions on and off in a complex environment.

Coiled-coil transcription factors

The simplest helix-mediated interaction is a coiled coil. No other structural elements are involved in these supercoiled bundles of 2 or more helices that can be aligned either parallel or antiparallel. Coiled coils are prevalent in the proteome and mediate many interactions important in disease. Designing a replacement for one of the native helices in a coiled coil is an attractive strategy for making an inhibitor, and this has been demonstrated, e.g. for bZIP and bHLHzip transcription factors (Fig. 1a) and recently for a coiled coil in the juxtamembrane region of epidermal growth factor receptor.[24–28]

The Keating lab designed binding partners for 19 human bZIP transcription factors that dimerize via a coiled coil (Fig. 1a),[28,29] using a computational strategy that simultaneously optimized interactions with the desired target while destabilizing interactions with related off-target proteins.[30] Chen et al. and Reinke et al. extended this approach to

viral bZIP targets.[31,32] More recently, Kaplan et al. further optimized first-generation designs that target human transcription factors including the disease-related FOS and XBP1 proteins.[33] The strategy was to improve affinity by altering the choice of surface residues in order to increase peptide helicity. This approach consistently gave tighter-binding peptides. But introducing charged residues on the surface altered the originally designed specificity patterns, sometimes leading to undesired off-target interactions and highlighting the difficulty of simultaneously optimizing affinity and specificity.

Computational design of coiled-coil partners requires a scoring function that can evaluate binding to desired targets and undesired off-targets. Grigoryan et al. used a hybrid function that blends residue-level interaction terms measured experimentally with other terms derived from molecular modeling.[34] Incorporating experimental terms was critical for good performance. Now that large data sets have been generated to describe some classes of PPIs, it has become possible to bias computational scoring functions even more using experimental observations. Potapov et al. recently reported the use of 4549 bZIP dimerization affinities to train a scoring function for bZIP interaction prediction.[35,36] The new scoring method showed significant improvement over other functions and was used to design selective helical peptide inhibitors of bZIP proteins JUN, XBP-1, ATF4 and ATF5.

Arndt and coworkers have developed and applied in-cell library screening methods to generate peptide inhibitors of coiled-coil interactions involving FOS/JUN and cMyc.[26,27] More recently this group reported the design of peptides that bind to (1) the coiled-coil domain of AF10, a transcription factor involved in a leukemia-inducing chromosomal translocation and (2) the coiled-coil domain of microphthalmia-associated transcription factor (MITF).[37,38] These peptides were engineered by screening libraries designed to introduce variation at interfacial sites known to be important for interaction. Using phage display or a “hitchhiker” translocation (HiT) complementation assay that made it possible to select simultaneously for expression, solubility and binding in *Escherichia coli*, the authors were able to identify peptides with high affinity for their targets. The structure of a designed peptide bound to the MITF coiled coil shows details of the design-target interaction (Fig. 1a).[37]

Rao et al. investigated the truncation and crosslinking of designed inhibitors of FOS.[39] Starting with a previously engineered peptide binder of FOS, $JunW_{CANDI}$, these authors showed that dissociation constants < 10 μM could be retained when peptides were shortened by 12 residues and stabilized by introduction of 2 or 3 lactam crosslinks. A crosslinked peptide of this type retained preferential binding to FOS over JUN, but the binding specificity with respect to other related bZIP proteins was not tested. It will be important to establish whether designed helical peptides retain broadly selective binding when shortened.

Bcl-2 family proteins

Members of the anti-apoptotic Bcl-2 family of proteins, which include paralogs Bcl-x_L, Bcl-2, Bcl-w, Mcl-1 and Bfl-1, are overexpressed in many cancers and can bind to and inhibit the function of pro-death proteins that include a helical Bcl-2 homology 3 (BH3) motif. Small molecules that mimic BH3 motifs can block protein interactions with Bcl-x_L

and Bcl-2 and induce tumor regression; such molecules are in clinical trials.[40] Inhibiting Bcl-x_L is toxic to platelets, however, and it has proven difficult to identify selective and bioactive antagonists of other Bcl-2 family members such as Mcl-1, which is frequently overexpressed in cancers.[41] Designed peptides hold promise for this application.

Walensky et al. showed in 2004 that a helically constrained 23-residue peptide corresponding to the BH3 region of pro-apoptotic BID binds tightly to Bcl-x_L and also activates apoptosis in cell culture and in mice.[42] Subsequent work led to additional Bcl-2 family interaction inhibitors made by crosslinking peptides corresponding to native BH3 sequences.[43,44] Examples include an inhibitor of Mcl-1 derived from the sequence of the Mcl-1 BH3 motif itself and an inhibitor of Mcl-1 made by bis-aryl linkage of cysteine residues introduced into the Noxa BH3 motif. Variants of the BH3 motif from BIM (Fig. 1b) have also been crosslinked for cell studies.[45–47]

Many crosslinked BH3 peptides have enhanced helicity, increased protease resistance, and an improved ability to enter cells. Some, but not all, helically stabilized peptides show improvements in binding affinity. Interestingly, closing of the crosslink is not always required for these enhancements.[48] Important lessons derived from work on crosslinked BH3-like peptides are that not all crosslinks are equivalently effective at increasing helicity or binding, increased helicity is not a guarantee of increased binding, the properties of peptides that determine their cell permeability are not yet thoroughly understood, and any kind of modification has to be considered as an additional design variable. Also, the precise length and sequence of a peptide is important. Changing the sequence by just a residue or two can have a large impact on binding, as might be expected for such short peptide binders.[46,48]

Native BH3 motif sequences may not be optimal as Bcl-2 family inhibitors. Notably, BIM and BID, on which many peptide inhibitors have been modeled, bind promiscuously to all members of the Bcl-2 family. More selective inhibitors may be required to avoid unwanted toxicity. In our lab, we have developed peptides that have high affinity for just one human family member, targeting Bcl-x_L, Mcl-1 or Bfl-1.[49,50] Foight and Keating reported the design of selective inhibitors of Bcl-2 family proteins from Epstein-Barr virus (BHRF1) and Kaposi's sarcoma virus (KS-Bcl-2).[51] The approach in these studies was to use experimental data for many mutations, and in some cases structure-based modeling, to design focused libraries of ~10^7 high priority binding candidates. These libraries were screened for interaction with the target in the presence of competing paralogs using either yeast-surface or bacterial-surface display. The resulting peptides have low nanomolar dissociation constants and bind preferentially to the intended target over related proteins. The design process was guided in some cases by a novel structure-based scoring model.[52,53]

Although BH3-like peptides do not typically enter cells without modification, their efficacy can be tested in a cellular context using the technique of BH3 profiling.[54] In this assay, permeabilized cells are treated with peptides, and inhibitory activity is assayed by monitoring the loss of mitochondrial outer membrane integrity that occurs when anti-apoptotic factors are blocked. BH3 profiling can be used as a diagnostic to determine the

Bcl-2 dependencies of primary patient cells that resist chemotherapy. It is encouraging that designed helical peptide inhibitors targeting Bcl-x_L and Mcl-1 show good potency and selectivity in BH3 profiling assays.[50,55] Peptides active in this assay are excellent candidates for further therapeutic development.

MDM2 and MDMX

MDM2 and MDMX bind to a short helical region of the tumor suppressor p53 and inhibit its function both directly, by blocking activation activity, and by promoting ubiquitination that leads to degradation. The inhibitory interaction of MDM2 and MDMX with p53 is a “holy grail” drug target and, excitingly, designed alpha helices that block this interaction show promising results in pre-clinical testing. Earlier work designing MDM2 and/or MDMX inhibitors either used library methods (e.g. phage display screening) to identify peptide sequences that bound with high affinity, or introduced helix modifications such as hydrocarbon staples to stabilize short segments of p53 itself. Not surprisingly, the most effective inhibitors so far discovered have come from combining these enhancements.

In parallel efforts, a team from Aileron Therapeutics and a group at the p53 Laboratory in Singapore took different phage-display optimized peptides,[56,57] introduced a hydrocarbon staple to stabilize helical conformations, and mutated the resulting molecules to improve pharmacologic properties while improving or retaining tight binding. In the Aileron effort, hydrocarbon stapling immediately improved peptide binding to both MDM2 and MDMX.[58] Further intensive optimization, which involved introducing a cyclobutylmethyl side chain, altering the peptide length, and tuning solubility, led to ATSP-7041. This molecule binds MDM2 and MDMX with dissociation constants < 5 nM, enters cells, and re-activates p53 signaling. A crystal structure shows how 2 large, hydrophobic side chains and one tyrosine make critical contacts with the p53-binding groove, and how the hydrophobic atoms of the stapled crosslink also engage the protein target MDMX (Fig. 1c). Pharmaceutical companies have invested heavily to advance ATSP-7041 to the clinic. Truncation and stapling of another MDM2-binding peptide by Brown et al. failed to improve affinity, but stapled peptides showed dramatically increased activity in p53 activation assays.[59] The intensive effort and many iterations of design required to arrive at biologically active stapled peptide inhibitors of MDM2 and MDMX provide a cautionary tale: Developing pharmaceutical quality modified peptides requires much of the same slow, steady optimization as does traditional small-molecule discovery, and multiple molecular properties must be simultaneously considered. Tan et al. recently published a cellular thermal shift assay that may make it easier to optimize modified peptides for both cell entry and tight binding in a complex environment.[60]

In another strategy for making helical MDM2-targeting peptides, Lu and coworkers published optimization of D-peptide inhibitors originally identified by mirror-image phage display.[61,62] In mirror-image display, phage that display L-peptides are panned against a version of MDM2 synthesized out of D-amino acids; the hits are then synthesized with L-amino acids to target the native protein. Structure-guided optimization led to introduction of fluorine and tri-fluoromethyl groups on aromatic residues that increased hydrophobic contacts, yielding a ligand with an affinity of 220 pM.

Other targets

Helical peptide inhibitors targeting many other biomedically important proteins have been reported. Prominent among these is the HIV envelope protein gp41, which includes two helical segments, N-terminal and C-terminal, each of which can potentially be used as a starting point for inhibitor design. Enfuvirtide is an approved peptide drug derived from the C-terminal helical segment. The drug blocks membrane fusion to inhibit viral entry.[63] The half-life of enfuvirtide is short, however, and the peptide is bound by patient antibodies. Also, some HIV strains are resistant to the drug. Recent design efforts to improve the properties of gp41-targeting inhibitors have focused on altering the peptide sequence or stabilizing the folded structure of inhibitors. Shi et al. used structure-based modeling to design peptides with low sequence identity to enfuvirtide,[64] and these were later improved to give molecule AP3. AP3 is effective against resistant strains and non-reactive with patient antibodies.[65] Other recent work on HIV gp41 inhibitor design focused on the N-terminal portion of the gp41 ectodomain and on stabilizing a helical trimer sub-structure. Crespillo et al. engineered a single-chain construct to mimic the native gp41 N-segment homotrimer.[66] The resulting protein, which is ~170 residues long, has an up-up-down arrangement of helices combined in a single chain to present a binding surface similar to the native homotrimer. The engineered pseudo-trimer is active as a fusion inhibitor. To accomplish a similar objective, Chu et al. reversed the connectivity of N- and C-terminal helical segments in gp41, making a trimer of ~80 residue peptides in which it was possible to adjust the amount of the N-terminal binding surface exposed. This strategy was used to make a low-nanomolar inhibitor of fusion.[67] Ebola and other enveloped viruses can potentially be targeted using similar design strategies.[68]

A large number of other proteins have been successfully targeted using short, helical peptides; it is impractical to list them all in this short review. Recent advances indicate that in many cases where there is structural or biochemical evidence for a helix-mediated protein-protein interaction, a native helical segment can be made as a constrained peptide that works as an inhibitor of function. Examples include peptides that bind GTPases Rab and KRAS, insulin receptor substrate 1 (important in phosphatidylinositol 3-kinase α signaling), epidermal growth factor receptor, and the *Plasmodium falciparum* myoA tail interacting protein.[25,69–72] Interestingly, myoA tail interacting protein surrounds its helical peptide ligand, but structures show how peptides including either hydrocarbon staples or a hydrogen bond surrogate can be accommodated in this constrained binding site (Fig. 1d).[72] Cell-permeable peptide inhibitors of the type-I regulatory domains of protein kinase A were recently designed by adding a hydrocarbon staple to a previously engineered peptidic inhibitor.[73]

Conclusions

The alpha helix is a simple structural motif, yet the number of possible short helical peptide sequences and conformations – particularly when one considers non-natural amino acids – is vast. Adding to this the possibility of modifying peptides with extra-structural elements such as crosslinkers increases the design space by many more orders of magnitude. In the face of such a massive search problem, it is fortunate that for many protein-protein interactions a

helical peptide derived from a native protein sequence provides an excellent starting point for inhibitor design. Furthermore, given that there is little reason to assume that native-sequence peptides have optimal binding affinity, binding selectivity, solubility, cell delivery or pharmacokinetic properties for a given purpose, the large space of possible modifications provides opportunities. The design challenge that confronts the field is to efficiently improve on native-sequence inhibitors to accelerate discovery.

We highlight three areas of importance for advancing helical peptide design and development. First, computational modeling can be more extensively exploited to guide rational improvement of inhibitors and informed selection of peptide crosslinking positions. Structural information is available for many of the targets discussed above, yet computationally aided structure analysis is infrequently used in inhibitor optimization. Modeling can be applied to identify and rank candidate affinity enhancing mutations and to evaluate potential crosslinkers.[74] The work of Joseph et al. provides precedent in this area. [75] These authors used molecular modeling to design candidate new stapled peptides for binding to Mcl-1. Lama et al. have also used simulation to guide design of helical peptide inhibitors of EIF4E.[76] We expect this type of structure-guided design to be an area of growth going forward.

Improvements in library screening are also needed. For example, computational methods can be used to design custom libraries enriched in candidate high-affinity or high-specificity binders. Such methods can incorporate data from structural analyses and from prior experimental work, as we have shown in our studies of Bcl-2 proteins.[50,51] On the experimental side, library screening has so far been done on unmodified peptides made of natural amino acids (when using genetic encoding) or mixtures of natural and non-natural amino acids (when constructing libraries using chemical synthesis). But it will be important to adapt library methods to screen peptides that include chemical modifications that enhance helicity and cell entry. Towards this end, we recently developed one-bead-one-compound library methods that can be used to optimize stapled peptides (Rezaei Araghi and Keating, submitted). Das et al. have synthesized and screened libraries of cyclized peptides.[77] Their techniques provide synthetic flexibility to incorporate non-natural amino acids, allowing for post-synthesis modification of ~2 million sequences using Cu-catalyzed azide–alkyne cycloaddition or ring-closing metathesis. Screening such libraries led to the discovery of high-affinity ligands for a wide variety of protein targets.[77] Further advances of this type are needed to enable high-throughput screening of large libraries constructed with diverse chemistries.

Finally, our understanding of basic sequence-structure-function relationships for constrained peptide inhibitors remains somewhat primitive. As larger amounts of data are accumulated, and more structures are solved, it will be important to critically evaluate the properties of peptides that enhance binding and cell uptake and use this analysis to drive improvements. In-depth biophysical analysis is often neglected in the understandable rush to generate inhibitors that can help patients. But a better understanding of the molecular mechanisms of binding for modified helices will ultimately benefit the field and improve our capabilities in design.

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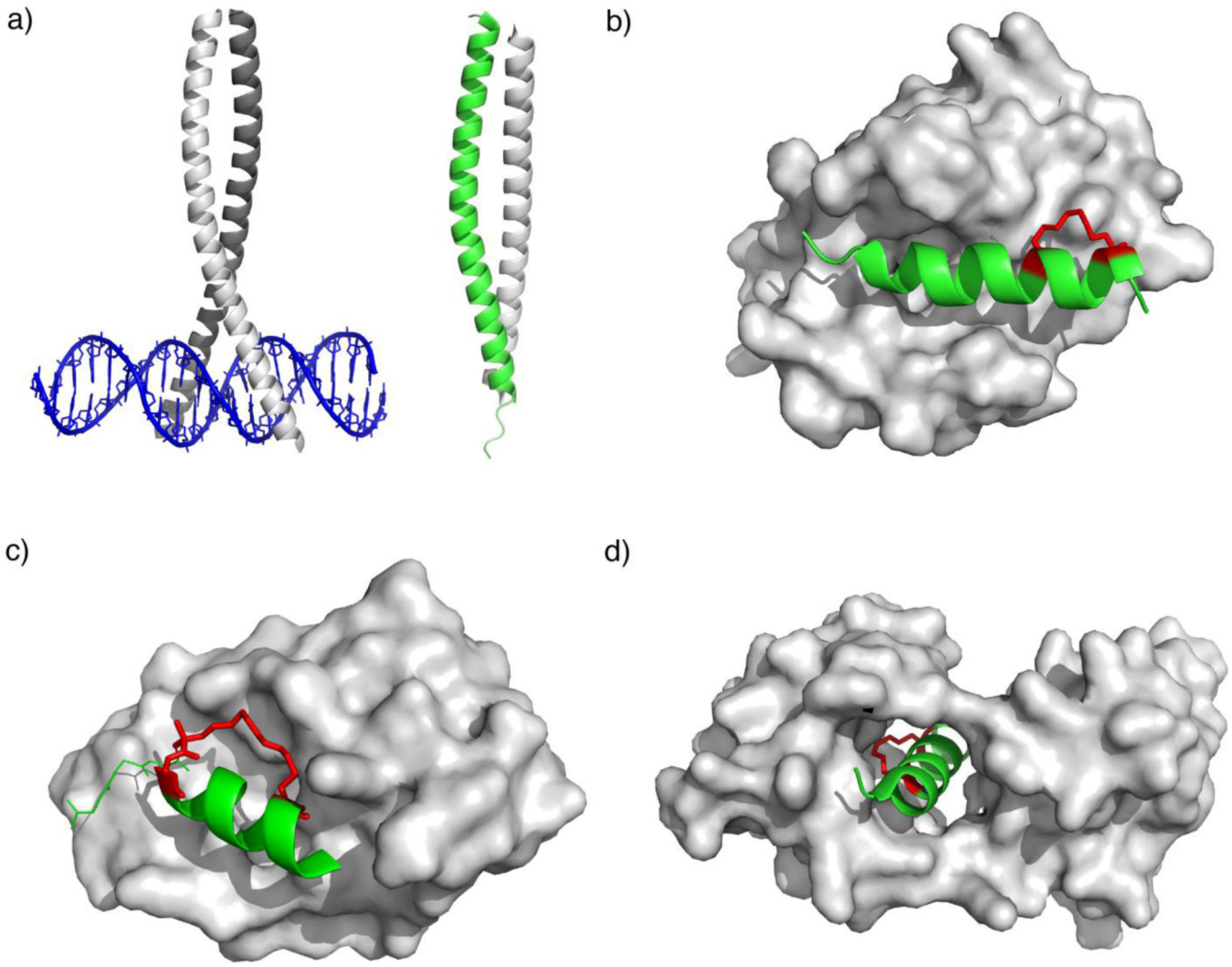

Figure 1.

Complexes including designed helical peptides. (a) At left, native bZIP complex FOS:JUN bound to DNA (1FOS),[28] which has been the target of many design studies.[24,26,30] At right, structure 4C7N shows a designed peptide binding to the coiled coil from bHLHzip transcription factor MITF. (b) Mcl-1 $SAHB_D$ engages Mcl-1 in the canonical BH3-binding groove. The hydrocarbon staple contributes additional contacts at the perimeter of the core interaction site (3MK8).[44] (c) Peptide-based ATSP-7041 bound to MDMX shows how the hydrocarbon staple contributes to the interaction (5N5T).[58] (d) A designed peptide corresponding to a segment of myoA is stabilized by a hydrocarbon staple and accommodated in the myoA tail interacting protein binding site (4MZK).[72] Target proteins are grey, with designed peptides in green and any helix modifying crosslinks highlighted in red.

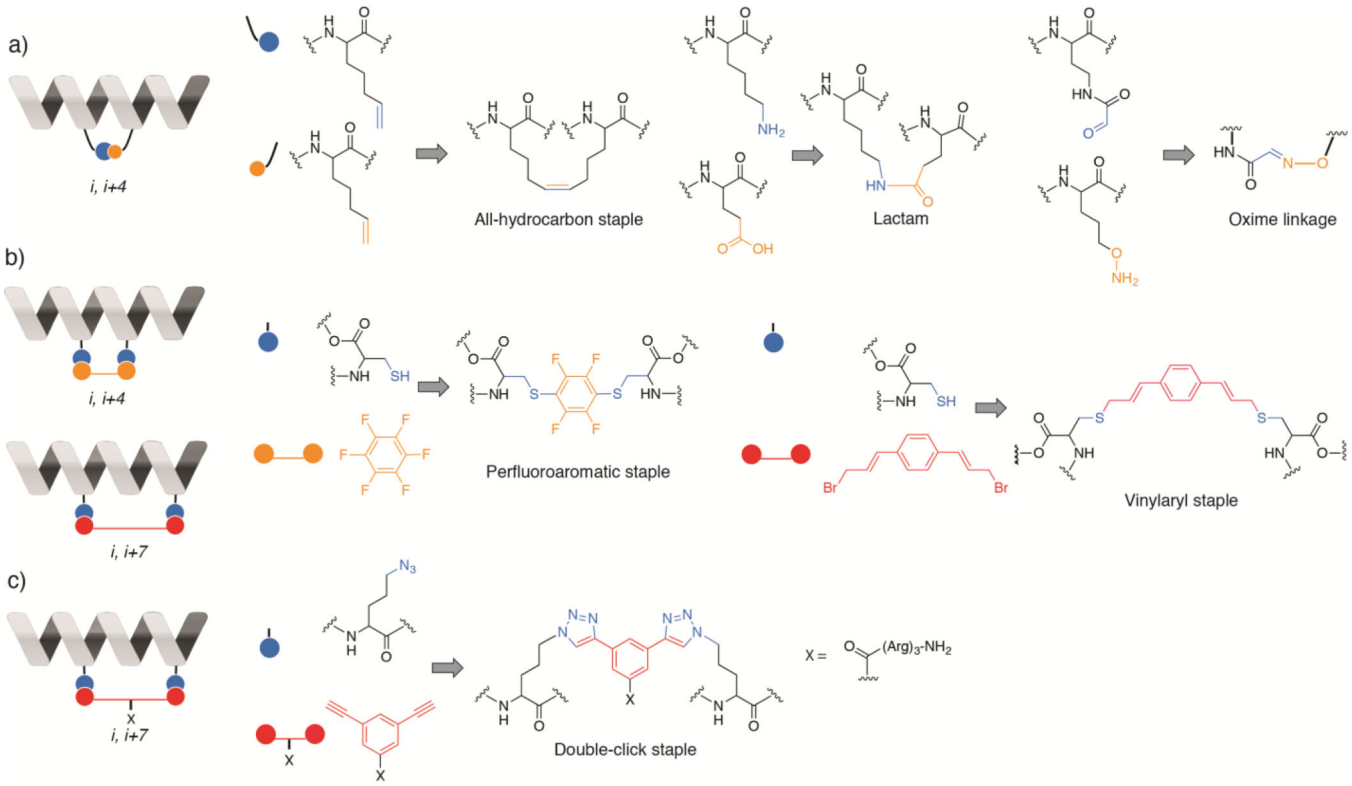

Figure 2.

Chemical modifications for stabilizing alpha helical structure in short peptides. a) Direct side chain stapling of amino acids at positions *i, i + 4*. All-hydrocarbon stapling using α,α-disubstituted amino acids bearing olefinic side-chains, lactamization of peptides containing lysine and glutamate or aspartate residues, and oxime stapling of peptides containing amino acids bearing aminooxy and aldehyde side chains. b) and c) Side chain stapling via functionalized linkers. b) Bis-arylation of two cysteine residues at positions *i, i + 4* with hexafluorobenzene and crosslinking of peptides containing cysteines at positions *i, i + 7* with vinylaryl linkage. c) Double-click stapling of peptides containing two azidoornithine residues at positions *i, i + 7* with a dialkynyl linker.