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Hydrocarbon-Stapled Helices: A Novel Approach for Blocking Protein-Protein Interactions

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Modulating protein–protein interfaces (PPIs) for therapeutic intervention has long been a vision of the research and pharmaceutical community, and approaches taken for inhibiting the Hdm2–p53 interaction illustrates the possibilities and problems associated with targeting PPIs. Normally, the tumor suppressor protein p53 functions as a molecular sentinel, which upon DNA damage, regulates genes that control cell-cycle arrest, apoptosis, senescence, differentiation, and DNA repair. The elimination or mutation of the p53 protein causes increased tumor formation, which is observed in p53 homozygous knockout mice and in Li-Fraumeni disease where germline mutations in p53 increases patients predisposition for cancer.[1] Understandably, p53 is itself tightly regulated to allow normal cell growth and differentiation. To this end, p53 induces the expression of its own negative regulators including the ubiquitin E3 ligase, Hdm2, which binds p53 directly, inhibiting its intrinsic activities, and promoting its ubiquitin-dependent degradation by the proteosome. However, if Hdm2 is overexpressed, as is found in 7% of all cancers,[2] then excessive p53 degradation occurs and its tumor suppression function is lost.

While p53 is deleted or mutated in ~50% of all tumors, there are many tumors that have wild-type p53.[1] In several wild-type p53 cancers, the inactivation of p53 occurs because of the overexpression or the aberrant regulation of Hdm2, which is thought to contribute significantly to the disease. Therefore, inhibiting the Hdm2–p53 protein–protein interaction is now a widely accepted therapeutic strategy to restore p53 protein levels and correspondingly its function as a tumor suppressor in such cancers.[3] This therapeutic approach was first validated in cellular assays by microinjection of antibodies that blocked the Hdm2–p53 interaction in mutant Ras transformed rat thyroid ephithelial cells[4] and by the use of anti-Hdm2 siRNA to knockdown Hdm2 in breast carcinoma cells (MCF7) and osteosarcoma cells (JAR).[5] In such validation experiments, p53 protein levels increased, and p53-dependent apoptosis activity was restored as predicted. As a result, numerous groups began making synthetic inhibitors of the Hdm2–p53 interaction, including peptidomimetics and small-molecule inhibitors with the goal of engineering a new cancer drug. Unfortunately, there were varying degrees of success in restoring functional wild-type p53 protein in tumor cells with small molecule inhibitors. As with other PPIs, it was thought that specifically targeting the Hdm2–p53 interaction with small molecules was difficult because of the large binding surface area at the protein–protein interface with multiple contacts involved.[6] Whereas peptidomimetics have overcome some of these issues, others arise such as their instability, low affinity, and lack of cell permeability in many cases. However, the Verdine Lab at the Harvard Medical School has provided an elegant and novel peptidomimetic strategy for inhibiting the Hdm2–p53 and other protein–protein interactions, which has overcome many of the problems associated with the previously available small molecule and peptidomimetic approaches.

In a recent paper published in Journal of American Chemical Society, the Verdine group developed and implemented a new technique termed "peptide stapling" and have applied it to inhibiting the Hdm2–p53 interaction.[7] Structural data revealed that the p53 peptide forms an amphipathic α-helix upon binding a hydrophobic cleft in Hdm2, with three residues on the same face of the α-helical peptide (F19, W23, and L26) interacting directly with Hdm2. Taking advantage of the fact that the region of p53 bound to Hdm2 is helical, the Verdine group strategically incorporates non-natural α,α-disubstituted amino acids containing olefinic side chains into the peptide and then cross-links the alkyl side chains using ruthenium-catalyzed ring-closing olefin metathesis. This reaction in effect "staples" the p53 peptide, which would otherwise adopt numerous conformations in solution, into one that nicely adopts a single α-helical secondary structure (Figure 1). In such a design, it is critically important that the modification does not occlude binding, as judged by examining 3D structures of the peptide–protein complex a priori. Thus, the affect of the hydrocarbon staple was examined by synthesizing a series of modified p53 peptides with varied positioning of the stereospecific modified amino acid. In addition, *R* or *S* stereochemistry at either one (i, $i+4$) or two (i, $i+7$) turns of the α-helix were used as were varied lengths of the linkers. After such molecules were synthesized, the various peptides were cross-linked (that is, stapled) and tested for

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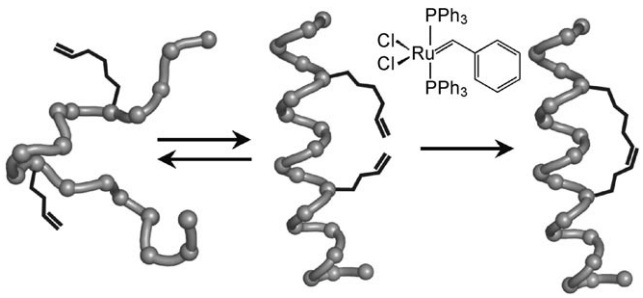

Figure 1. Stabilization of α-helical secondary structure in a p53 peptide was accomplished by hydrocarbon-stapling of olefinic side chains on non-natural amino acids introduced at either one (*i*, *i*+4) or two (*i*, *i*+7; as shown above) turns of the α-helix using ruthenium-catalyzed olefin metathesis. This method produces a stabilized, protease resistant, cell permeable peptide that effectively blocks Hdm2 degradation of p53 in cancer cells.[7]

their ability to bind Hdm2 until an optimal configuration was obtained.

Another part of the design was to engineer a stable molecule that could pass through a cell membrane and localize to the nucleus. With the exception of positively charged residues in select peptides,[8] it is generally recognized that a charge on a peptide will impede their transport across cell membranes. Therefore, as the wild-type p53 peptide has a net negative charge, the aspartic and glutamic acids were sequentially replaced with asparagines and glutamines, respectively. Additional engineering of the p53 peptide sequence was done to increases it nuclear localization and intracellular stability. Specifically, an amino acid involved in a nuclear export signal in p53 was mutated (L14Q) as was a putative ubiquitination site to prevent degradation (K24R).[7] The final optimized stapled p53 peptide was 85% α-helical as compared to 11% for unmodified peptide as judged by circular dichroism and its affinity was approximately eightfold higher ($K_d = 55 \pm 11$ nM) than the unmodified peptide,[7] and is well within the range of the well-characterized small molecule inhibitor of Hdm2, namely Nutlin-3 ($K_d = 36 \pm 9$ nM).[9]

The next important question is whether such a peptide could indeed enter cells and have the desired effect of p53 restoration. In this regard, fluorescently labeled derivatives of the stapled peptide were found to enter the cell cytoplasm within 30 min and confocal microscopy revealed localization of stapled p53 peptide with pinosomes suggesting it enters the cells using an active pinocytosis mechanism similar to what is seen for the cell permeable TAT and Antp peptides.[8] Once in the cells, the stapled p53 peptide was found to directly interact with Hdm2 by co-immunoprecipitation experiments and an increase in p53, p21, and Hdm2 protein levels were observed suggesting the peptide inhibits Hdm2 and restores active p53 as designed. Furthermore, cultured tumor cells were killed by the hydrocarbon-stapled p53 peptide and an increase in the proapoptotic caspase-3 activity was observed. Thus, the stapled peptide is both cell permeable and biologically active based on these criteria.[7]

The hydrocarbon-stapling of peptides overcomes several problems associated with peptidomimetics, specifically the Hdm2–p53 inhibitor is cell-permeable, protease resistant, and retains high affinity and selectivity for the target. It is often the case that chemical modifications that stabilize α-helical peptides result in polar residues making the peptide unable to penetrate cell membranes; however, this was not an issue with the hydrocarbon-stapled peptides. A second problem overcome by the Verdine approach is that the cross-linkers are not vulnerable to degradation, which was often a problem with some of the earlier peptidomimetics produced. As most proteases act on unstructured regions of peptides, locking the peptide into an α-helical conformation was predicted to not only increase its binding affinity by better mimicking its bound state, but to also protect it from degradation by peptidases. This was found to be the case as the stapled peptide survived nearly four times longer in fresh mouse serum as compared to the unmodified p53 peptide (half-life of 10.5 h versus 38.9 h).[7] Lastly, the generality of hydrocarbon-stapling technology will allow it to be applied to numerous PPIs composed of various amino acids with the only limitation being that the linking chemistry is not compatible with sulfur containing amino acids.[10]

The ability to produce biologically active hydrocarbon-stapled peptides will certainly be a powerful tool in dissecting intracellular protein pathways and will aid in the development of small-molecule therapies for manipulating protein–protein interactions. The stability and bioavailability of hydrocarbon-stapled peptides makes them viable therapeutics in their own right. For example, the Verdine group has also developed a hydrocarbon-stapled BID BH3 peptide that was not only shown to activate the proapoptotic BAX protein in cultured leukemia cells, but it was able to suppress the growth of leukemia cell growth and activate apoptotic pathways of human leukemia cells when given intravenously to a mouse xenograft model.[10,11] Whereas animal studies have not been done with the hydrocarbon-stapled p53 peptide, its properties in cellular assays are promising for future development. In summary, the Verdine group has overcome some of the major issues of the use of peptidomimics using elegant chemistry, which should be generally applicable to developing other PPIs rapidly as is necessary to examine/inhibit a multitude of cellular disorders and pathways involving protein–protein interactions.

Keywords: antitumor agents · drug design · Hdm2 · p53 · peptidomimetics

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