Spring 5-1-2013

# The Use of p53-Derived Stapled Peptides as Affinity Isolation Reagents

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# The Use of p53-Derived Stapled Peptides as Affinity Isolation Reagents

A Capstone Project Submitted in Partial Fulfillment of the
Requirements of the Renée Crown University Honors Program at
Syracuse University

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May 2013

Honors Capstone Project in Biochemistry

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**ABSTRACT**

The transcription factor p53 can induce apoptosis and cell cycle arrest in response to cellular distress. Cancer cells often display increased cell survival. In most cases, this is due to a p53-related defect, such as mutation, deletion, degradation, or sequestration. HDM2 and HDMX are homologous proteins that regulate the function of p53, and their over-expression can lead to an ineffective p53 response. Various inhibitors, including hydrocarbon stabilized alpha-helices of p53 (SAH-p53s), have been developed to target HDM2 and HDMX and restore functionality to the p53 pathway. It has been recently found that SAH-p53 factors also elicit cell death responses in the absence of transcriptionally active p53. In order to investigate other potential targets of SAH-p53, a protocol was developed based on a paper by Craig Braun [11] which discusses a mechanism utilizing photoactivatable amino substitutions to capture binding partners covalently. Using this protocol, p53-derived stapled peptides were found to bind selectively to p53's known target, HDM2 as well as other unknown proteins. The various p53-derived stapled peptides were utilized to obtain further insight into the p53-derived stapled peptide targets.

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**Acknowledgements**

Thank you to Bethanie Morisson, PhD for your help and guidance throughout my internship at the National Cancer Institute. Thank you to Kevin Murray for determining the binding affinity of the p53-derived peptides for HDMX and HDM2 polarization assay as well as purifying HDMX and HDM2 for my experiments. Thank you to Joeseph Mitala, PhD for your help preparing FPLC buffers and using the FPLC. Thank you to Elizabeth Russel for your guidance throughout my internship. Thank you to Federico Bernal, PhD, for your help and guidance throughout my internship as well as hosting me for the summer in your laboratory where I learned an extraordinary amount about p53-derived stapled peptide.

Thank you to James Dabrowiak, PhD for your help on crafting and preparing my honors capstone. Thank you to Surabhi Raina, PhD for your ideas and thoughts on my honors capstone.

**Introduction**

The "guardian of the genome", p53, is a transcription factor involved in the cell cycle and is an important protein in various cell cycle processes, such as cell cycle arrest, senescence, apoptosis, DNA repair, and angiogenesis.[1, 2] Additionally, p53 is believed to act as a regulator in determining the responses to cellular stresses. These stresses include DNA damage due to UV radiation, carcinogens, cytotoxic drugs, hypoxia, nucleotide depletion, oncogene activation, microtubual disruption, and loss of normal cell contacts.[1] Therefore, p53 is believed to determine the future repair or death of the cell. Loss of p53 or mutations to p53 have been shown to increase the likelihood of tumor growth as the cell loses the capability to regulate the cell cycle, particularly, cell death mechanisms. [3, 1]

The p53 transcriptional activities are enormously important to cell response, specifically to its development of cancerous qualities. Intriguingly, however, p53 levels vary according to the level of stress. During normal conditions of the cell, p53 levels are often at undetectable levels of expression due to its short half-life, high activities of its inhibitors, including HDM2, and a melting temperature a slightly above body temperature. In contrast, during stressful conditions, the level and activity of p53 increase, increasing the likelihood of apoptosis and facilitating cell death mechanisms in normal cells. Often, this mechanism occurs via post-translational modifications which affects the p53 binding of various targets.[1] Notably, cellular stressors activate p53 modifications that inhibit its binding to specific DNA targets and determine,

altering cellular transcription. The up-regulated transcripts ultimately lead to a induction of apoptosis or growth arrest.

There are a multitude of p53 pathways known to interact with over 100 genes and proteins [1] through p53's various domains and functions. In a micro-array analysis,[1] it was determined that p53 up-regulates 500 genes, and down-regulates 260 genes, induces 107 genes, and represses 54 genes, demonstrating that p53 is involved with many biochemical processes.[4, 1] Furthermore, the level and activity of p53 have been found to help predict tumor response.[3] It is therefore important to both study p53 for its involvement in cellular processes and for target for cancer therapies, since increased levels of p53 levels and activity reinstates cell death processes. [1]

The interaction of p53 with many different molecules determines the cell response through these multiple signaling cascades. The protein, p53 is defined by three main regions: (1) an acidic N-terminal region. (2) a central DNA-binding core region. (3) a basic C-terminal Region. [1] The major post translational modifications of p53 include phosphorylation/dephosphorylation, cis/trans isomerization, acetylation/deacetylation, ubiquitination/deubiquitination, sumolation, methylation, neddylation, ADP-ribosylation, and O-glycosylation. [1]

Notably, the N-terminal region has two domains: (1) a transactivation domain where HDM2 and HDMX bind[1] and (2) a proline-rich domain. HDM2 and HDMX bind p53 and signals for its degradation by ubiquitinilating p53, and initiates a pathway is involved in increasing the life of the cell by inhibiting p53.[1] In the transactivation domain of the N-terminal region, there also is a LXXLL-

type co-activator recognition motif that interacts with histone acetyltransferase and effects P300 binding.[1, 5] The transactivation domain also contains a secondary nuclear export signal (NES) involved with transporting p53 out of the cell. [1] Phosphorylation inhibits of the NES signal and acts as a pathway to inhibit p53 export. [1] The second region of the N-terminal region, the DNA-binding region, recognizes promoter regions on DNA domain and interacts with the DNA to up-regulate or down-regulate the transcription of various signal proteins. [1] In contrast, the C-terminal region, has three regions: (1) three nuclear localization signals for import of p53 into the nucleus, (2) a tetramerization domain involved in binding stability of p53 to DNA, and (3) a negative regulatory region preventing unwanted DNA binding.[6, 1]

The two known inhibitors of p53, HDMX and HDM2, are used in this paper to demonstrate known p53 binding partners. In the cell, HDMX sequesters p53 upon binding to the transactivation domain of p53, while HDM2 targets p53 for degradation through ubiquitination. These processes lower the activity and levels of p53 within the cell, lowering the likelihood of their proceeding to cell death pathways. Often, it is found that HDM2 and HDMX levels are higher in cancer cells. Not surprisingly, by increasing the inhibitors of p53, the cancer cell bypasses pathways that lead to cell death. Synthesized p53-derived stapled peptides can be introduced into cancerous cells and can bind to HDMX and HDM2, limiting the amount of active p53 inhibitors. Frequently, such treatments have been shown to reinstate a normal cell death mechanism by artificially increasing p53 levels in cancer cells.

Notably, in a typical cell, HDM2 activity is significantly lowered through the ribosomal proteins RPL5, RPL11 and RPL23, which prevent p53 ubiquitinilation and thereby increase p53 levels and activity. [1] Furthermore, it has been found that following DNA damage, the ribosomal protein RPL26 binds the 5' untranslated region of p53 mRNA and acts as an enhancer by the recruiting polysomes, which increase p53 translation and subseqeuntly induces G1 cell-cycle arrest and irradiation-induced apoptosis. This indicates that ribsome biogenesis is a regulatory factor for p53 pathways. [7]

HDM2 is also inhibited/activated through post-translational modifications. For example, AKT or protein kinase B (RKB) is activated through phosphatidyl inositol triphosphate (PIP3), phosphoinositide 3-kinase (PI-3K), signaling a cascade through various activators, namely growth factors and hormones. [1] Upon AKT activation, AKT phosphosphorylates HDM2 and prevents HDM2 from self-ubiquitination, or targeting itself for degradation. This, in turn, increases cell survival by increasing the level of the major inhibitor of p53. [1]

Another two proteins that interact with HDM2 are $P19^{ARF}$ (a cell cycle regulating transcription factor) and $P16^{INK4A}$ a tumor suppressor and cyclin-dependent kinase inhibitor. Mutations of these two proteins are common to cancer cell lines.[1] Notably, in MCF-7 breast cancer lines both $P19^{ARF}$and $P16^{INK4A}$ are co-deleted. Other important examples of p53 HDM2 activation/inhibition include: Gankyrin(a cell cycle regulating protein), KAP1 (a nuclear co-repressor protein), and retinoblastoma 1 (a tumor supressor protein). [1]

An important interaction of p53, is the interaction between p53 with p63

and p73. The proteins, p63 and p73 are similar to p53 with similar structures and gene sequences. [1] In contrast to p53, mutations to p63 and p73 are not known to hinder their functions nor do they gain new functions. However, their functions are vital to the cell, as demonstrated by p63 and p73 deletions, which result in developmental defects. It is believed that p53, p63 and p73 interact to facilitate p53 cell-cycle pathways through isoforms. [1] Interestingly, long isoforms have similar targets that activate p53 pathways while short isoforms tend to have negative regulatory mechanisms against p53 activity and level. [1]

These various protein interactions demonstrate how p53 is vital to normal cell processes. Mutations of p53 in cancer cell lines can promote metastasis and resilience in cancer since nonfunctional p53 is incapable of initiating cell death pathways and many p53 mutants are unable to act as a transcription factor for DNA targets. Furthermore, mutant p53 has been shown not only to lose normal p53 function, but also to have increased activity as a transcription factor, and this results in increased migration, proliferation, survival, and metabolism within the cell. This phenomenon is demonstrated experimentally when mutant p53 is added to normal cell lines, resulting in cancerous properties in the cells.[1] Interestingly, p53 mutations have been found to be the most common mutation present in cancers with an occurrence in 50% of human cancers.[2, 8, 1]

An example of the effect of mutations in p53 is the disease Li-Fraumeni Syndrome, caused by a Tp53 mutant allele.[1] The resulting autosomal disorder has a high likelihood of yielding multiple cancers, particularly breast tumors.[1]

Many mutant p53 proteins contain mutations within the transactivation

domain, and mutant p53 is believed to function through inhibition of the cellular cycle processes by binding to p53 target protein promoters, thus preventing functional p53 from binding.[9, 1] Typically, most mutant p53 peptides also have higher stability in the chemical environment of the cell and have present in high levels within the nucleus of neo-plastic (abnormally growing) cells. Furthermore, the loss of p53 the function of activating the expression of its inhibitor HDM2 contributes to a high level of mutant p53, since the mutant p53 is not ubiquitinilated for degredation.[1]

Mutant p53 can also have some unexpected activity within the cells. For example, some mutant p53 can trans-activate CDKN1A promoters, which is a promoter for the growth arrest pathway, but not BAX or Tp53I3/PIG promoters, which are apoptosis pathway promoters. Additionally, a few types of mutant p53 have been found to generate higher apoptotic activity than p53, these are called super p53 and have been shown to have higher resistance to characteristic cancer qualitites.[10] Because of this, super-apoptotic mutants of p53 are not typically observed in cancer cases.

There are many proteins that are influenced by p53. Therefore, p53 is a major target for targeted therapies for manipulating the cell cycle and cell death mechanisms. It is of particular importance to learn more of these p53 cell death pathways and what proteins are involved and interacting so researchers can learn how to regulate them.

Recently, it was found that a cell-death mechanism initiated by addition of rationally designed p53-derived stapled peptides to mutant p53 cell lines. Since

mutant p53 causes characteristic cancer effects, it is surprising that the addition of a peptide that inhibits HDMX and HDM2, freeing mutant p53 into the cell, results in cellular death. To investigate this curious phenomenon, it was hypothesized that the p53-derived stapled peptides may bind to proteins other than HDM2 and HDMX, eliciting a cell-death response.

There are hundreds of proteins that interact, bind, and are regulated by p53. To learn more about these pathways, a method was developed by Braun et al. to use p53-derived stapled peptides to bind targets, which can elucidate the

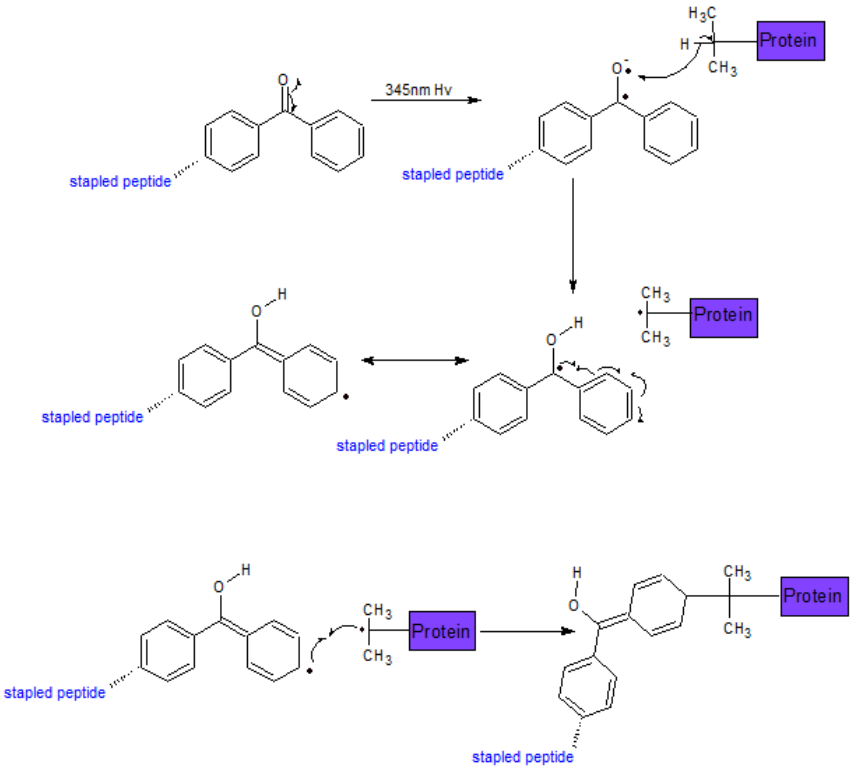


***Figure 1:*** *Reaction scheme for photo-coupling a stapled peptide-benzophenone conjugate to a protein.*

mechanism of cell death upon addition of p53-derived stapled peptides to mutant-p53 cell lines (MCF7-2-231). In this method, the locations of protein-protein

interactions are determined by binding to sequences derived from a protein with a reactive benzophenone (BPA) molecule within the binding domain of the stapled peptide.[11] Upon irradiation at a wavelength of 365 nm, ketyl radical formation initiates a cascade that culminates in the covalent-capture of a protein-protein binding interaction. (Figure 1). To test the ability of a photo-activatable stapled alpha helix of p53 (pSAH-p53) to reach its target, a compound based on the sequence of SAH-p53-4 was synthesized with the requisite substitution of an aromatic amino acid for BPA. *N*-terminal FITC tagging was used to detect reactivity by visual inspection of the gel.

The p53-derived stapled peptide, pSAH-p53-8, was engineered to bind HDM2 by utilizing hydrogen bonding of L54 to W23, and hydrophobic contacts with Leu54, Phe55, Gly58, and Met62. The photo-activatable peptide, pSAH-p53-8 L26, covalently cross-linked the peptide to HDM2 at the L26 position (Figure 2). In this paper, it is demonstrated that, following exposure to UV light to a mixture of pSAH-p53-derived peptide and target protein, the peptide binds selectively to its target, HDM2. It was furthermore demonstrated, that upon exposure to of UV light to a pSAH-p53-8 L26 and MDA-MB-231 lysate (breast cancer cells) mixture. The pSAH-p53-8 L26 binds to multiple proteins and may indicate the presence of another p-53 pathway eliciting the cell-death mechanism.

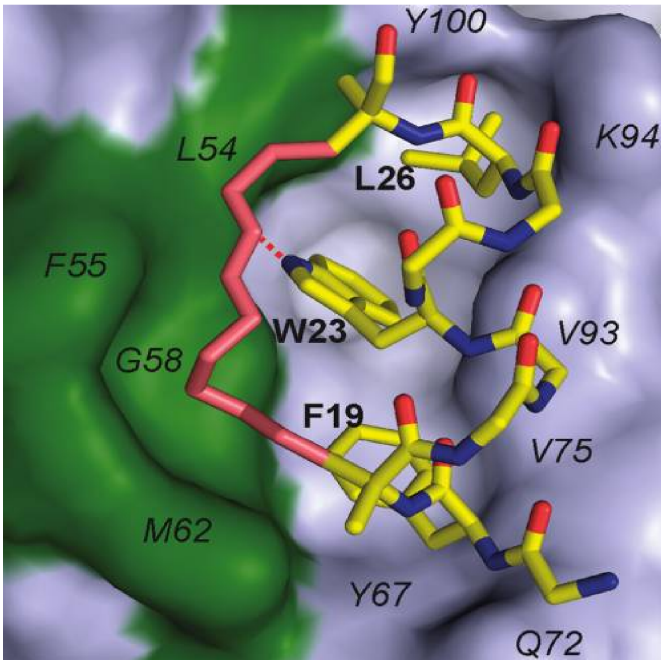


***Figure 2:*** *Crystal structure of a stapled p53 peptide bound to Mdm2. The photoactivatable peptide has three main interactions sites with HDM2, F19, W23, and L26. The staple (in red) holds the peptide in position for optimal HDM2 binding; including hydrogen bonding of L54 to W23 as well as hydrophobic contacts with Leu54, Phe55, Gly58, and Met62.The pSAH-p53-8 L26 benzophenone covalently crosslinks at the L26 position.*[PPP]

HDMX is also known to bind p63 (described above), a protein with a significant homology to p53, specifically in the transactivation domain. It is hypothesized that the cell-death mechanism present in mutant p53 cell-lines may occur because the p53-derived stapled peptides are interfering with a p63 pathway. To learn more about p63 protein targets, a photo-activatable p-63-derived stapled peptide, pSAH-p63-4, was also added to a cell lysate and exposed to UV light. This experiment demonstrated that p-63 binds to multiple protein targets and provided the first steps towards elucidating p63 biochemical pathways.

**PROCEDURE:**

**PART A)**

**Evaluation of Specificity of FITC-SAH-p53-4Bpa to target protein, HDM2**

**Table 1: Volumes of FITC-pSAH-p53-4, HDM2, BSA (negative control), and Buffer added to eight 1.5 mL eppendorf tubes.**

| Sample | FITC-pSAH-p53-4 (1mM) | HDM2 (72.4mM) | BSA (1mM) | Buffer (1XPBS) |
|---|---|---|---|---|
| 1 | 6 µL | - | - | 94 µL |
| 2 | - | 28 µL | - | 72 µL |
| 3 | - | - | 2 µL | 98 µL |
| 4 | 6 µL | 28 µL | - | 66 µL |
| 5 | 6 µL | - | 2 µL | 92 µL |
| 6 | 6 µL | 28 µL | 2 µL | 64 µL |
| 7 | 6 µL | 28 µL | 6 µL | 60 µL |
| 8 | - | - | - | 100 µL |

This experiment utilizes a fluorescently tagged photoactivatable peptide and provides insight into the specificity of the peptide and consequently applicability for use in a Lysate.

The above were added to eight 1.5 mL eppendorf tubes (table 1). The samples were exposed to UV radiation at 365 nm for 8 hours. 10µL samples were taken after 2 hours, 4 hours, 6 hours, and 8 hours of UV radiation. The samples were run on a 4-12% SDS-PAGE gel MES buffer at 120V and stained with Coomassie Blue.

**PART B)**

**Experiment to corroborate the presence of polymers in a FITC-SAH-p53-4Bpa and HDM2 reaction.**

To two 1.5ml eppendorf tubes, 15µL of FITC-pSAH-p53 (1mM) 70µL of HDM2 (72.4µM) and 165 µL of 1XPBS buffer were added. One tube was placed under 365nm UV radiation for 8 hours at room temperature. The other was kept in a light free environment at room temperature. The samples were run through an FPLC size exclusion column using HDM2 FPLC buffer (with 2-mercaptoethanol and with no EDTA)

**PART C)**

**Evaluation of Specificity of Photoactivatable Peptide, Bt-pSAH-p53-4, to known target protein, HDM2.**

**Table 2: Volumes of Bt-pSAH-p53-4, HDM2, BSA (negative control), and Buffer added to eight 1.5 mL eppendorf tubes.**

| Sample | Bt-pSAH-p53-4 (10mM) | HDM2 (72.4mM) | BSA (1mM) | Buffer (1XPBS) |
|---|---|---|---|---|
| 1 | 0.6 µL | - | - | 99.4 µL |
| 2 | - | 28 µL | - | 72 µL |
| 3 | - | - | 2 µL | 98 µL |
| 4 | 0.6 µL | 28 µL | - | 71.4 µL |
| 5 | 0.6 µL | - | 2 µL | 92 µL |
| 6 | 0.6 µL | 28 µL | 2 µL | 69.4 µL |
| 7 | 0.6 µL | 28 µL | 6 µL | 65.4 µL |
| 8 | - | - | - | 100µL |

This experiment utilizes a fluorescently tagged photoactivatable peptide

and provides insight into the specificity of the peptide and consequently applicability for use in a Lysate.

The Bt-SAH-p53-4, HDM2, and BSA were added to eight 1.5mL eppendorf tubes (Table 2). The samples were exposed to 365 nm UV radiation for 8 hours. From the eight eppendorfs, 10µL samples were taken after 2 hours, 4 hours, 6 hours, and 8 hours of UV radiation. The samples were run on an 4-12% SDS-PAGE gel MES buffer at 120V, transferred for 2 hours, washed in TBS-T, blocked with LI-COR blocking buffer for 2 hours, and blotted for anti-biotin (rabbit mAb; Cell Signaling Technology D54A7; 1:1000) overnight at 10 degrees Celsius. The sample was then allowed to incubate for 2 hours at room temperature, and then washed once with Phosphate Buffered Saline (1XPBS) (137 mM Nacl, 2.7 mM KCl, 10 mM Ma2HPO4, 1.8 mM KH2PO4 pH 7.4),three times with TBS-T (50 mM Tris, 150 mM NaCl, 0.05% Tween 20 pH 7.6), and once with 1XPBS again. An image was taken using a LI-COR Odyssey Imaging System.

**PART D)**

**Evaluation of Specificity of Photoactivatable Peptide, Bt-pSAH-p53-8 W23, to known target protein, HDM2.**

| Table 3: Volumes of Bt-pSAH-p53-8 W23, HDM2, BSA (negative control), and Buffer added to eight 1.5 mL eppendorf tubes. | | | | |
|---|---|---|---|---|
| Sample | Bt-pSAH-p53-8 W23 (10 mM) | HDM2 (72.4mM) | BSA (1mM) | Buffer (1XPBS) |
| 1 | 0.6 µL | - | - | 99.4 µL |
| 2 | - | 28 µL | - | 72 µL |
| 3 | - | - | 2 µL | 98 µL |
| 4 | 0.6 µL | 28 µL | - | 71.4 µL |
| 5 | 0.6 µL | - | 2 µL | 92 µL |
| 6 | 0.6 µL | 28 µL | 2 µL | 69.4 µL |
| 7 | 0.6 µL | 28 µL | 6 µL | 65.4 µL |
| 8 | - | - | - | 100 µL |

This experiment utilizes a fluorescently tagged photoactivatable peptide and provides insight into the specificity of the peptide and consequently applicability for use in a Lysate.

The above were added to eight 1.5mL e12endorf tubes. The samples were exposed to 365 nm UV radiation for 8 hours. From the eight eppendorfs, 10µL samples were taken after 2 hours, 4 hours, 6 hours, and 8 hours of UV radiation. The samples were run on a 4-12% SDS-PAGE gel MES buffer at 120V, transferred for 2 hours, washed in TBS-T, blocked with LI-COR blocking buffer for 2 hours, and blotted for anti-biotin (Rabbit mAb; Cell Signaling Technology D54A7;1:1000) overnight at 4 degrees Celsius. The sample was then allowed to incubate for 2 hours at room temperature, and was washed once with 1XPBS,

three times with TBS-T, and once more 1XPBS. The image was taken using a LI-COR Odyssey Imaging System.

**PART E)**

**Evaluation of Specificity of Photoactivatable Peptide, Bt-pSAH-p53-8 L26, to known target protein, HDM2.**

**Table 4: Volumes of Bt-pSAH-p53-8 L26, HDM2, BSA (negative control), and Buffer added to eight 1.5 mL eppendorf tubes.**

| Sample | Bt-pSAH-p53-L26bpa (10 mM) | HDM2 (72.4mM) | BSA (1mM) | Buffer (1XPBS) |
|---|---|---|---|---|
| 1 | 0.6 µL | - | - | 99.4 µL |
| 2 | - | 28 µL | - | 72 µL |
| 3 | - | - | 2 µL | 98 µL |
| 4 | 0.6 µL | 28 µL | - | 71.4 µL |
| 5 | 0.6 µL | - | 2 µL | 92 µL |
| 6 | 0.6 µL | 28 µL | 2 µL | 69.4 µL |
| 7 | 0.6 µL | 28 µL | 6 µL | 65.4 µL |
| 8 | - | - | - | 100 µL |

This experiment utilizes a fluorescently tagged photoactivatable peptide and provides insight into the specificity of the peptide and consequently applicability for use in a Lysate.

The Bt-SAH-p53-L26bpa, HDM2, and BSA were added to eight 1.5mL eppendorf tubes (Table 4). The samples were exposed to 365 nm UV radiation for 8 hours. 10µL samples were taken after 2 hours, 4 hours, 6 hours, and 8 hours of UV radiation. The samples were run on a 4-12% SDS-PAGE gel MES buffer at 120V, transferred for 2 hours, washed in TBS-T, blocked with LI-COR blocking

buffer for 2 hours, and blotted for anti-biotin (Rabbit mAb; Cell Signaling Technology D54A7;1:1000) overnight at 4 degrees Celsius. The sample was then allowed to incubate for 2 hours at room temperature, and was washed once with 1XPBS, three times with TBS-T, and once more 1XPBS. An image was taken using a LI-COR Odyssey Imaging System.

**PART F)**

**Experiment to identify unknown target proteins of Bt-pSAH-p53-8 L26 in a Breast Cancer Cell Lysate (MDA-MB-231)**

*Cell Culture and Prep of a Lysate*

Four 75cm$^2$ flasks were seeded with 231, breast cancer mutant p53 cells until a 60% confluence was achieved. The media were aspirated off, washed with 3-5ml of DPBS, aspirated off DPBS again, trysinized with 3ml 0.25% trypsin, and incubated at 37$^o$C for 30 seconds. All trypsinized mixtures were combined to give one 50 ml tube. An RPMI mixture was added to achieve a 1:1 ratio of media-to-trypsin. The reaction was mixed gently by repeated inversion. Then, 10µL of the 1:1 trypsin/media mixture was added to a hemocycler to determine that there was a total of 50x10$^4$cell/ml in the 1:1 ratio of media to trysin mixture. To achieve a 4.5mg/ml sample, 7.4x10$^6$ cells were added to two 15ml conical tubes. The samples were pelleted for 5 minutes at 1500 rpm. The media was aspirated off, and the pellet was resuspended in 3 mL 1XPBS. The cells were pelleted for 5 minutes at 1500 rpm, and the 1XPBS was aspirated off. This step was repeated three times.

To each pellet, 150µL of lysis buffer (Lysis Buffer: 50 mM Tris [pH 7.4], 150 mM NaCl, 0.1% Triton, 1mM EDTA, and 1.5 mM $MgCl_2$ with 0.5 µL Benzonase Nuclease, 5 µL [PMSF]=1M, and ¼ of a Roche EDTA free inhibitor tablet) was added.

Sample was transferred to a 1.5ml eppendorf tube and subsequently, centrifuged 10 seconds and left on ice for 5 minutes. The sample was then mixed to resuspend the cells. The sample was left on ice for 15-30 minutes, every 5-10 minutes the sample was lightly vortexed. The sample was centrifuged in the 5430R centrifuge at 14000 rpm at $4^{o}C$ for 15 minutes. The supernatant lysate was added to a new eppendorf tube.

To an eppendorf tube, 75µL of streptavidin were added. The tube was then centrifuged for 30 seconds at room temperature. The supernatant buffer was removed, and 100µL of lysis buffer was added to the beads, vortexed lightly and centrifuged for 30 seconds; this was repeated 3 times. The supernatant lysate was added to the washed streptavidin beads and allowed to mix on the rotisserie for 2 hours in the cold room. The lysate/bead mix was then centrifuged on the microcentrifuge at room temperature for 30 seconds. The lysate was removed. A bio-rad was taken of the protein to determine the protein concentration. It was determined that the protein concentration was 5.14 mg/ml. The lysate was stored in a $4^{o}C$ freezer after fast-freezing in liquid nitrogen

| **Table 5: Volumes of Bt-pSAH-p53-8 L26, HDM2, BSA (negative control), and Buffer added to eight 1.5 mL eppendorf tubes.** | | | |
|---|---|---|---|
| Sample | Bt-SAH-p53-8-L26 Bpa (200μM) | Lysate (2.5-4.5 mg/ml) | Lysis buffer (50 mM Tris [pH 7.4], 150 mM NaCl, 0.1% Triton, 1mM EDTA, and 1.5 mM $MgCl_2$. With 0.5μL Benzonase Nuclease, 5μL [PMSF]=1M, ¼ Roche EDTA free inhibitor tablet. ) |
| 1 | 10 μL | - | 90 μL |
| 1b | | | |
| 2 | 10 μL | 90 μL | - |
| 2b | | | |
| 3 | - | 100 μL | - |
| 3b | | | |
| 4 | - | - | 100 μL |
| 4b | | | |

The Bt-SAH-p53-8-L26 Bpa and cell lysate was added to eight 1.5mL eppendorf tubes. Eppendorfs 1, 2, 3, and 4 were placed under the UV lamp at 365nm at room temperature. To the side of the UV lamp chamber, eppendorfs, 1b, 2b, 3b, and 4b were placed in aluminum foil so that it would not be exposed to ultraviolet light. After 2 hours of UV exposure, 10μL samples were taken from each eppendorf (for Western blotting). The samples were run on a 4-12% Bis-Tris gel with MOPS buffer at 50-120V. A prestained Seeblue plus2 protein standard ladder was added to first lane. The gel was transferred onto a membrane for two hours. The membrane was washed with TBS-T and blocked for 1 hour with 5 ml of LI-COR blocking buffer. The membrane was then blotted for anti-biotin (Rabbit mAb; Cell Signaling Technology D54A7;1:1000) overnight at 4 degrees Celsius. The sample was then allowed to incubate for 2 hours at room

temperature, and then was washed once with 1XPBS, three times with TBS-T, and once more 1XPBS. An image was taken using a LI-COR Odyssey Imaging System.

The membrane was then stripped using LI-COR stripping buffer for 1 hour, washed with TBS-T and blocked for 1 hour with 5 ml of LI-COR blocking buffer. The membrane was then blotted for Anti-mdm2 (mouse mAb; IF2 Calbiochem; 1:100) overnight at 4 degrees Celsius. The sample was then allowed to incubate for 2 hours at room temperature, and then washed once with 1XPBS, three times with TBS-T, and once more 1XPBS. An image was taken using a LI-COR Odyssey Imaging System.

**PART G)**

**Bt-pSAH-p63-4 and Bt-pSAH-p53-8 L26 with MDA-MB-231 Cell Lysate**

Four 75cm$^2$ flasks were seeded with breast cancer cells until a 60% confluence was achieved. The media were aspirated off, washed with 3-5ml of DPBS, aspirated off DPBS again, trypsinized with 3ml of 0.25% trypsin, and incubated at 37$^o$C for 30 seconds. All trypsinized mixtures were combined in one 50 ml tube. RPMI mixture was added to achieve a 1:1 ratio of media-to-trypsin. The mixture was mixed gently with repeated inversion, and 10µL of the mixture was added to a hemocytometer, to determine that there was a total of 40x10$^4$cell/ml in the 1:1 ratio of media to trypsin mixture. To achieve a 4.5mg/ml sample: 7.4x10$^6$ cells were added to two 50ml conical tubes. The samples were pelleted for 5 minutes at 1500 rpm. The medium was aspirated off, and the pellet was resuspended in 3 mL of 1XPBS. The cells were pelleted for 5 minutes at 1500

rpm, and the 1XPBS was aspirated off. The centrifugation followed by washing with 1XPBS was repeated three times. To each pellet, 150µL of lysis buffer (lysis buffer: 50 mM Tris [pH 7.4], 150 mM NaCl, 0.1% Triton, 1mM EDTA, and 1.5 mM $MgCl_2$ with 0.5 µL Benzonase Nuclease, 5 µL [PMSF]=1M, and ¼ of a Roche EDTA free inhibitor tablet) was added.

The sample was transferred to a 1.5ml eppendorf tube, centrifuged for 10 seconds and left on ice for 5 minutes. The cells were then re-suspended. Next, the sample was left on ice for 15-30 minutes, and every 5-10 minutes it was lightly vortexed. Then it was centrifuged in the 5430R centrifuge at 14,000 rpm at 4$^{o}$C for 15 minutes. The supernatant lysate was added to a new eppendorf tube.

To preclear the sample from biotin, 75µL of streptavidin were added to an 1.5 mL eppendorf tube. The tube was then centrifuged for 30 seconds at room temperature. The supernatant buffer was removed, and 100µL of lysis buffer was added to the beads, vortexed lightly and centrifuged for 30 seconds; this was repeated 3 times. The supernatant lysate was added to the washed streptavidin beads and allowed to mix again on a rotisserie for 2 hours in the cold room. The lysate/bead mix was then centrifuged on the microcentrifuge at room temperature for 30 seconds. The supernatant lysate was then added to another washed streptavidin beads and allowed to mix on the rotisserie for 2 hours in the cold room. The lysate/bead mix was then centrifuged on the microcentrifuge at room temperature for 30 seconds. A Bio-rad was taken of the protein to determine the protein concentration. It was determined that the protein concentration was 2.8 mg/ml. The lysate was then diluted to 2.5 mg/ml with lysis buffer. The lysate was

stored in a 4$^{o}$C freezer after fast-freezing in liquid nitrogen

| **Table 6: Volumes of Bt-pSAH-p53-8 L26, HDM2, BSA (negative control), and Buffer added to four 1.5 mL eppendorf tubes.** | | | |
|---|---|---|---|
| Sample | Bt-SAH-p53-8-L26 Bpa (200*μM)* | Lysis (2.5 mg/ml) | Lysis buffer (50 mM Tris [pH 7.4], 150 mM NaCl, 0.1% Triton, 1mM EDTA, and 1.5 mM $MgCl_2$. With 0.5*μL Benzonase Nuclease, 5μL [PMSF]=1M, ¼ Roche EDTA free inhibitor tablet. )* |
| 1 | 15*μL* pSAH-p53-8 L26 | 150*μL* | |
| 1b | | | |
| 2 | 10*μL*pSAH-p63-4 | *150μL* | |
| 2b | | | |

Eppendorfs 1 and 1b were placed under the 365nm UV lamp at room temperature. To the side of the UV lamp chamber, eppendorfs, 1b, 2b were wrapped in aluminum foil to shield from ultraviolet light to serve as a control. After 2 hours of UV exposure, 10μL samples were taken from each eppendorf for western blotting. The samples were run on two separate 4-12% Bis-Tris gels with MOPS buffer at 50-120V. A prestained Seeblue plus2 protein standard ladder was added to the first lane of each gel. The gels were transferred onto membranes for two hours. The membranes were washed with TBS-T and blocked for 1 hour with 5 ml of LI-COR blocking buffer. The membranes were then blotted for anti-biotin ( Rabbit mAb; Cell Signaling Technology D54A7;1:1000) overnight at 4 degrees Celsius. The sample was then allowed to incubate for 2 hours at room temperature, and then was washed once with 1XPBS, three times with TBS-T, and once more 1XPBS. Images were taken using a LI-COR Odyssey Imaging System.

The Bt-pSAH-p63-4 membrane was stripped using LI-COR stripping

buffer for 1 hour, washed with TBS-T and blocked for 1 hour with 5 ml of LI-COR blocking buffer. The membrane was then blotted for Anti-p53 (DO-1) overnight at 4 degrees Celsius. The sample was then allowed to incubate for 2 hours at room temperature, and was washed once with 1XPBS, three times with TBS-T, and once more 1XPBS. An image was taken using a LI-COR Odyssey Imaging System.

**Results/Discussion:**

**Photoactivatable Peptide, SAH-p53-4Bpa binds specifically to HDM2 (Results of Part A-D)**

To determine whether the SAH-p53-4Bpa peptide could specifically target HDM2 within a protein mixture, FITC-pSAH-p53-4 and recombinant HDM2 were mixed and exposed to UV light at 365 nm. BSA was used as a control. The results show the exclusive formation of adducts with recombinant HDM2. More importantly, FITC-pSAH-p53-4 showed a preference for its target HDM2, even in the presence of superstoichiometric amounts of BSA, highlighting the specificity of the reaction (Figure 3). However, interestingly, the FITC-pSAH-p53-4 peptide displayed a further reaction with the peptide and target HDM2, showing a polymeric reaction (Figure 3). To investigate further the gel pattern displayed by the HDM2 and FITC-pSAH-p53-4 peptide, the reaction was separated into 30 fractions with fast protein liquid chromatography. Unfortunately, the fractions did not indicate the presence of HDM2 polymers (Figure 4). Further confirmation of a specific reaction with peptide was obtained by using a biotinylated version of pSAH-p53-4, followed by an anti-biotin Western blot analysis (Figure 5).

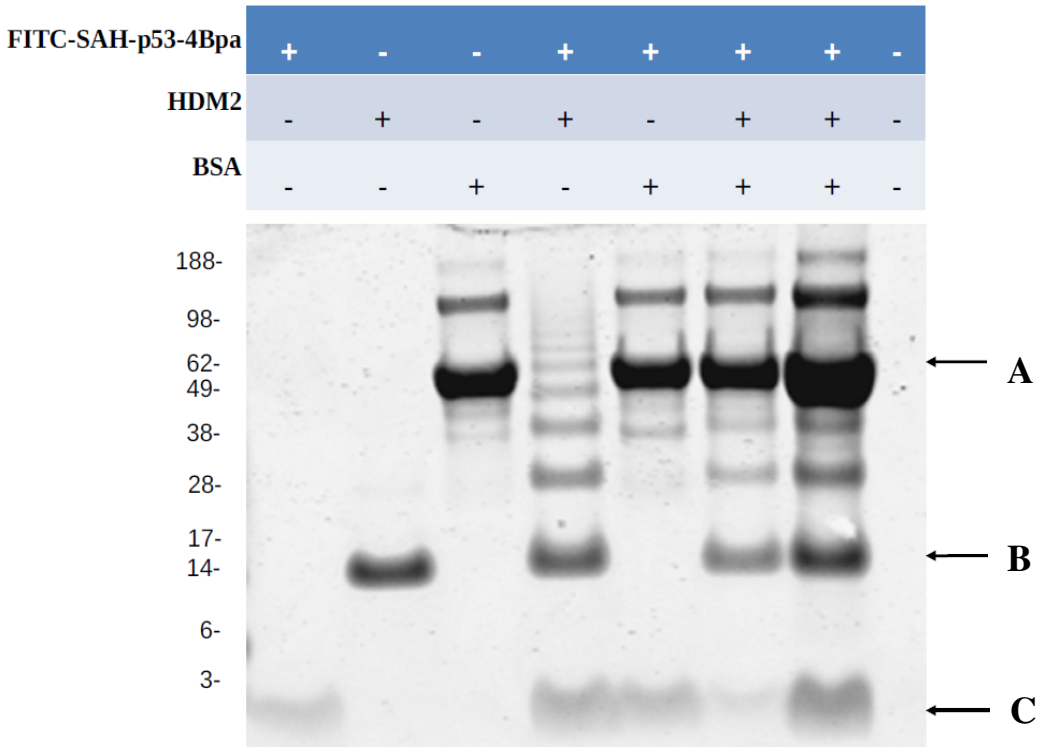


***Figure 3**: Coomassie Blue stained 4-12% Bis-Tris SDS-protein gel of FITC-pSAH-p53-4 in the presence or absence of HDM2 and BSA where, HDM2/peptide = 3 and BSA/peptide = 1 or 3 Samples were irradiated with 365 nm radiation for 8 h prior to gel analysis. Band A is BSA, molecular weight: 62 kDA. Band B is HDM2, molecular weight 14.8kDa. Band C is FITC-SAH-p53-4bpa, molecular weight 2kDa.*

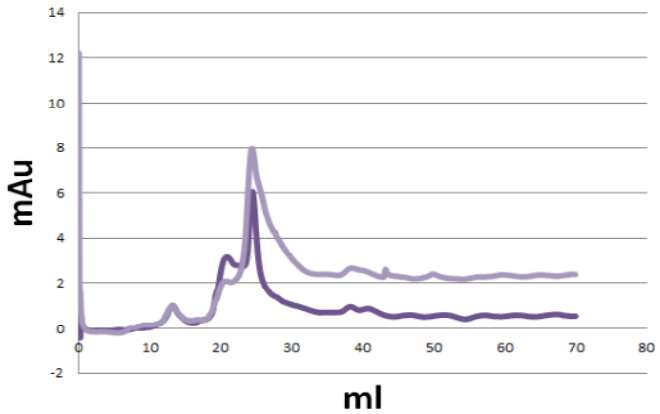


***Figure 4**: Fast Protein Liquid Chromatography of a mixture of FITC-SAH-p53-4bpa peptide and HDM2 that was previously exposed (or not exposed) to UV radiation, where, mAu is—milli absorbance units and ml is the elution volume in milliliters.*

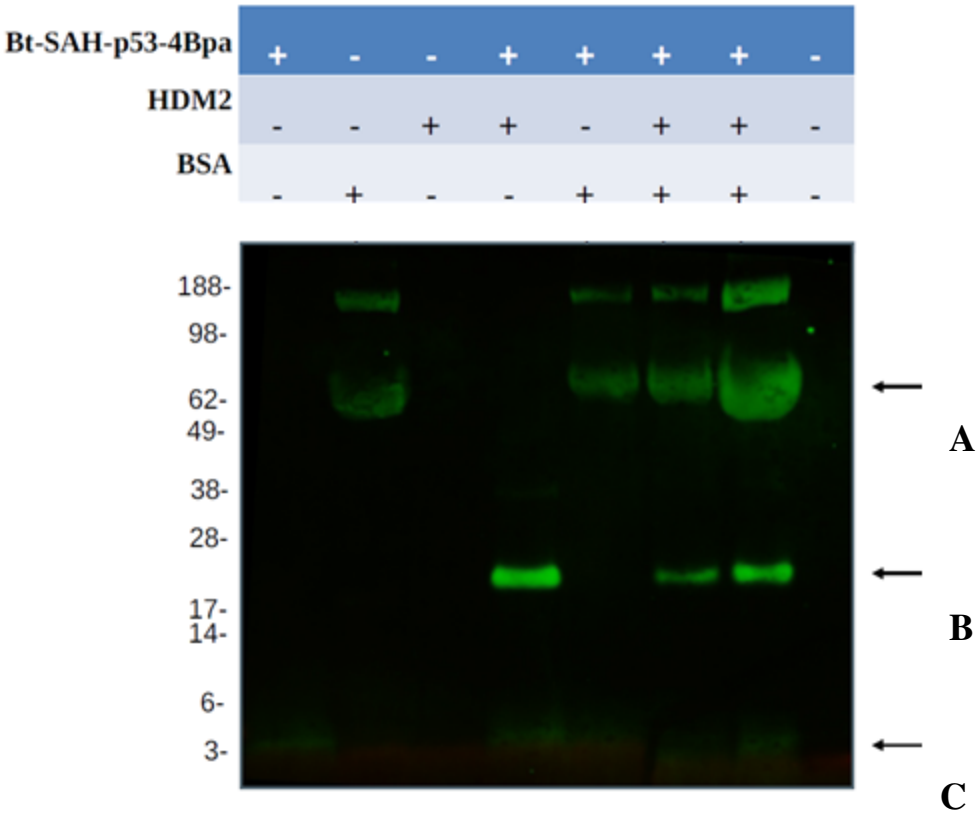


***Figure 5:*** *Western blot showing the anti-biotin antibody, D54A7, detected products of reaction of Bt-SAH-p53-4bpa with HDM2. 4-12% Bis-Tris SDS-protein gel of Bt-SAH-p53-4bpa in the presence or absence of HDM2 and BSA where, HDM2/peptide = 3 and BSA/peptide = 1 or 3. Samples were irradiated with 365 nm radiation for 8 h prior to gel analysis. Band A is BSA, molecular weight: 62 kDA. Band B is HDM2, molecular weight 14.8kDa. Band C is Bt-SAH-p53-4bpa, molecular weight 2kDa.*

**Photoactivatable Peptides, Bt-pSAH-p53-8 L26 and Bt-pSAH-p53- W23 Crosslink specifically with HDM2 after UV Radiation**

**(Results Parts D and E)**

To determine whether Bt-p53-8 L26 could target its binding partner from a mixture of proteins, Bt-pSAH-p53-8 L26, HDM2, and BSA were mixed and exposed to UV light. Electrophoresis, followed by Western blotting and detection

with anti-biotin antibody, showed that Bt-pSAH-p53-8 L26 crosslinks to HDM2 (Figure 6). Similarly, photo-activatable peptide, Bt-SAH-p53-8 W23 also displayed specific binding to HDM2 (Figure 7).

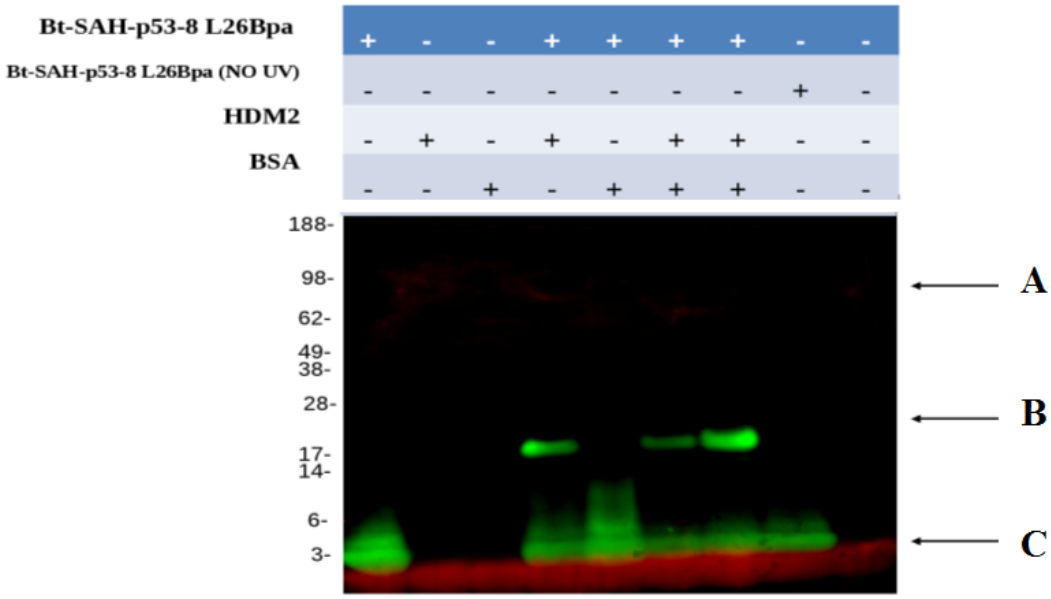


***Figure 6:*** *Western blot showing the anti-biotin antibody, D54A7, detected products of reaction of Bt-SAH-p53-8 L26Bpa with HDM2. 4-12% Bis-Tris SDS-protein gel of Bt-SAH-p53-8 W23bpa in the presence or absence of HDM2 and BSA where, HDM2/peptide = 3 and BSA/peptide = 1 or 3. Samples were irradiated with 365 nm radiation for 6 h prior to gel analysis.*

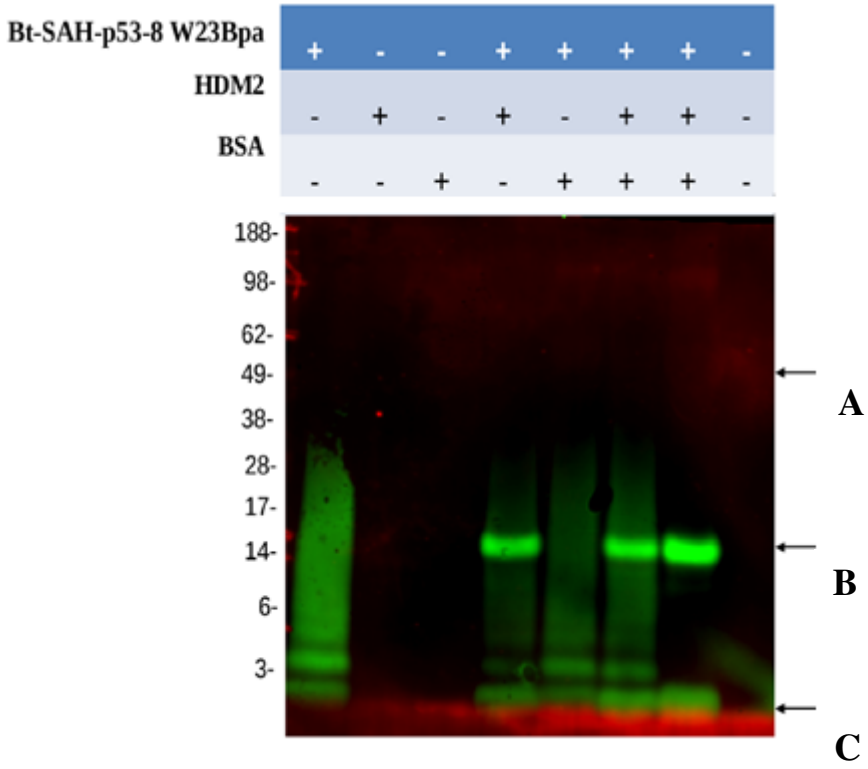


***Figure 7:*** *Western blot showing the anti-biotin antibody, D54A7, detected products of reaction of Bt-SAH-p53-8 W23bpa with HDM2. 4-12% Bis-Tris SDS-protein gel of Bt-SAH-p53-8 W23bpa in the presence or absence of HDM2 and BSA where, HDM2/peptide = 3 and BSA/peptide = 1 or 3. Samples were irradiated with 365 nm radiation for 4 h prior to gel analysis. Band A is BSA, molecular weight: 62 kDA. Band B is HDM2, molecular weight 14.8kDa. Band C is Bt-SAH-p53-8 W23bpa, molecular weight 2kDa.*

**Photoactivatable Peptides, Bt-pSAH-p53-8 L26 Crosslinks with Proteins other than HDM2 after UV Radiation**

To test whether pSAH-p53-8 L26 could identify proteins within a lysate, Bt-pSAH-p53-8 L26 and a lysate derived from a mutant p53 cell line (MDA-MB-231 cells) were mixed and exposed to UV light. Gel electrophoresis and Western blotting for both biotin (Figure 8) and HDM2 (Figure 9) show that Bt-pSAH-p53-8 crosslinks to HDM2, as well as to additional unknown binding partners. The

reaction was upscaled and repeated, and it showed similar results for blotting with anti-biotin (Figure 10).

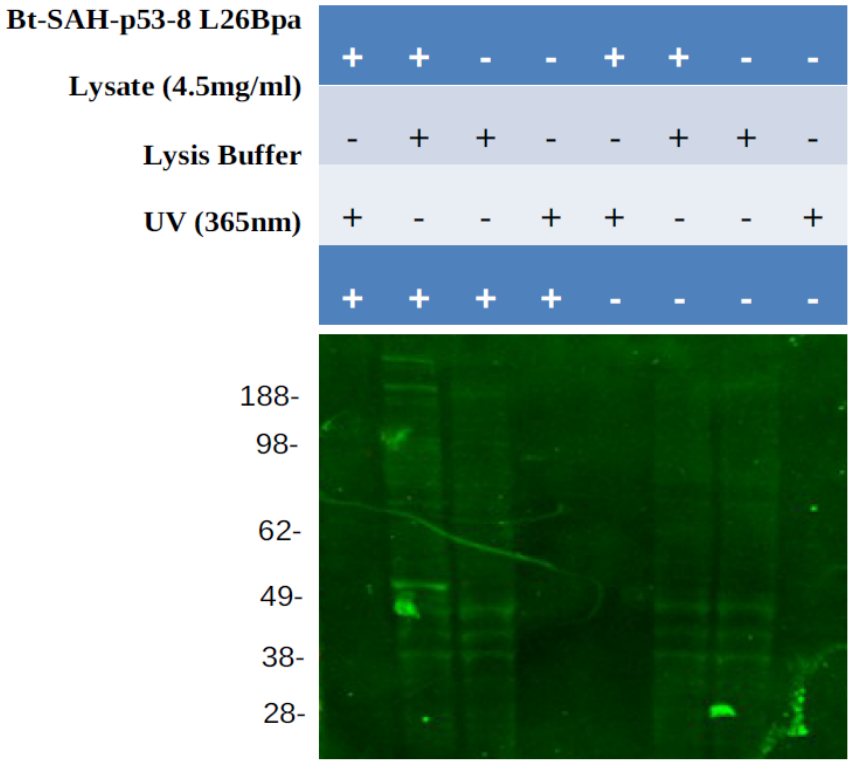


***Figure 8:*** *Western blot showing the anti-biotin antibody, D54A7, detected products of reaction of Bt-SAH-p53-8 L26Bpa with and without a protein lysate (4.5mg/ml). 4-12% Bis-Tris SDS-protein gel of Bt-SAH-p53-8 L26Bpa in the presence or absence of lysate or UV exposure. Samples were irradiated with 365 nm radiation for 2 h prior to gel analysis.*

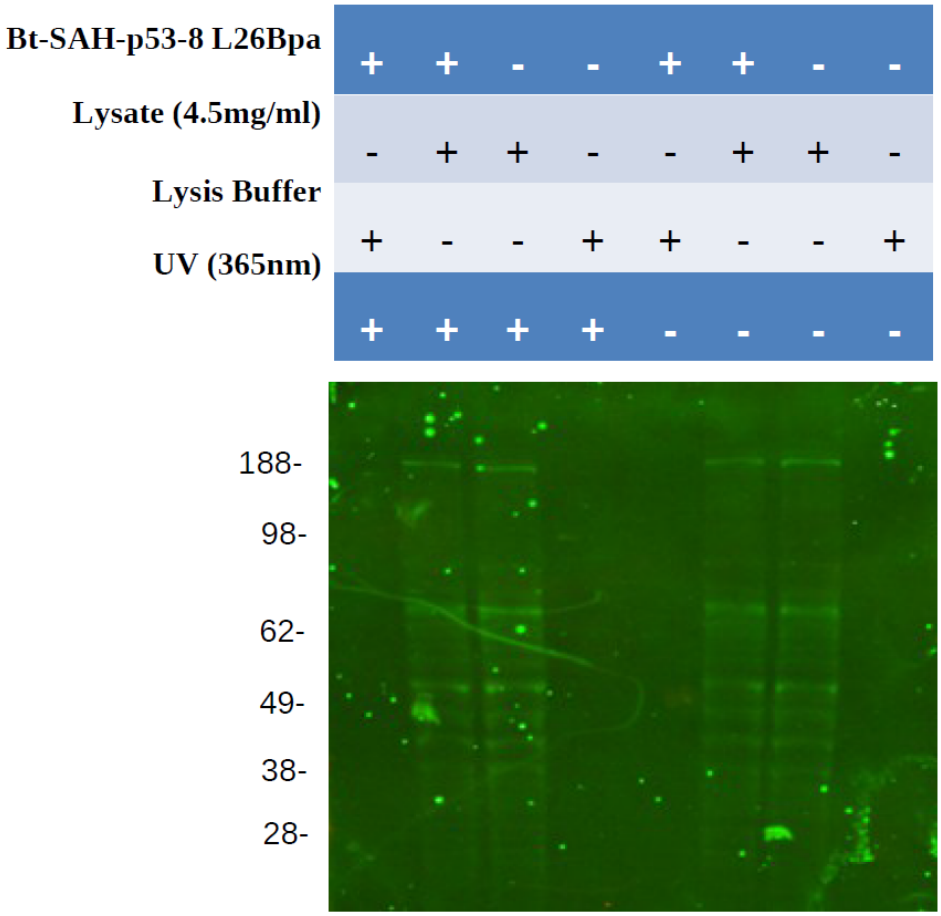


***Figure 9:*** *Western blot showing the anti-mdm2 antibody, IF2, detected products of reaction of Bt-SAH-p53-8 L26Bpa with and without a protein lysate (4.5mg/ml). 4-12% Bis-Tris SDS-protein gel of Bt-SAH-p53-8 L26Bpa in the presence or absence of lysate or UV exposure. Samples were irradiated with 365 nm radiation for 2 h prior to gel analysis.*

**Photoactivatable Peptides, Bt-pSAH-p53-8 L26 Crosslinks with Proteins other than HDM2 after UV Radiation**

To test whether if pSAH-p53-8 L26 could identify proteins within a lysate, Bt-pSAH-p53-8 L26 and a lysate derived from a mutant p53 cell line ( MDA-MB-231 cells) were mixed and exposed to UV light for 2 hours. Gel electrophoresis and Western blotting for both biotin (Figure 8) and HDM2 (Figure

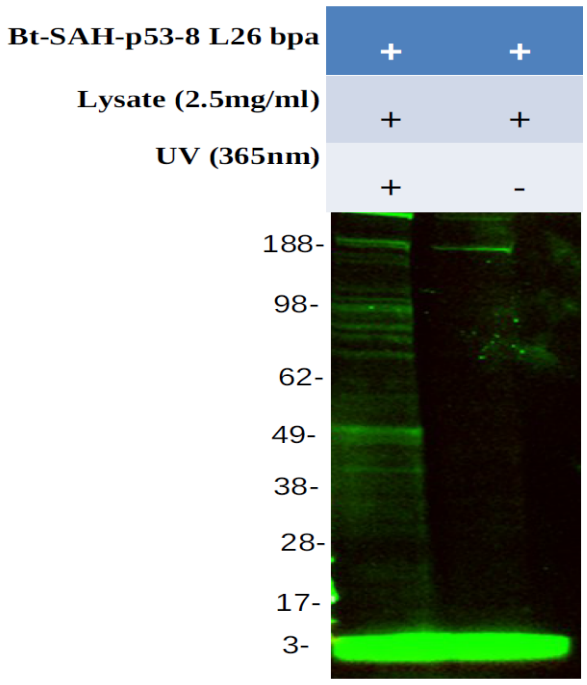


***Figure 10:*** *Western blot showing the anti-biotin antibody, D54A7, detected products of reaction of Bt-SAH-p53-8 L26Bpa with a protein lysate (2.5mg/ml) . 4-12% Bis-Tris SDS-protein gel of Bt-SAH-p53-8 L26Bpa in the presence of lysate with or without UV exposure (365 nm radiation for 0.75 h prior to gel analysis).*

9) showed that Bt-pSAH-p53-8 crosslinks to HDM2, as well as to additional unknown binding partners. The reaction was upscaled and repeated, and it showed similar results for blotting with anti-biotin SAH-p53-L26 and HDM2 (Figure 10). The reaction was also upscaled and exposed to UV light for 30 minute, 45 minute, and 1 hour increments. At 30 minutes exposure to Ultra Violet Light, no reaction was visible. At, 45 minutes and 1 hour exposure to UV light a similar banding pattern to the western blots at 2 hours of exposure.

**Bt-SAH-p63-4 bpa crosslinks to protein within a cell lysate.**

To learn more about the protein pathway of p63, a peptide derived from the transactivation domain of p63 was constructed. Remarkably, the peptide showed a reaction pattern similar to that of Bt-SAH-p53-8 L26 bpa. This may be because there is a significant homology in the transactivation domain of p53, compared to p63. A Western blot anti-p53 showed that the band at 49 kDa is not

p53, as the bands do not match making a possible p63 to p53 interaction unlikely to be the cause of the cell-death mechanism.

## CONCLUSION

It was determined that the photo-activatable peptide, Bt-pSAH-p53-L26, binds selectively to its target protein, HDM2. This peptide then was further to identify proteins within a cell lysate. The Western blot of the reaction showed that the peptide had indeed bound to proteins other than HDMX and HDM2. Additional research will lead to the identification of the bands revealed by the SDS-PAGE of the photoactivatable peptide and lysate reaction.

## FUTURE DIRECTIONS:

Targeting p53 pathways has become one of the most important areas of cancer research aims to reinstate normal apoptotic pathways and other normal cell death mechanism could be the key to treating cancer.[2] Because of the importance of p53, many avenues of investigation find a means by which to activate and inhibit p53 have been tested, including chemoradiation, chemotherapy, gene therapy, and small molecule targeting .

Chemoradiation methods are effective in treating cancer as they induce DNA damage which subsequently activates p53 and triggers apoptosis mechanisms. Understandably, these methods are most effective in patients with a wild type p53 and therefore a functional apoptosis mechanism .[2] Patients with a mutant p53 responded better to chemotherapy treatments, which utilize chemicals to produce an apoptotic response.[2] This suggests that genetics may be an

important factor to determining treatment methods. Similarly, gene therapy has been proven an effective method of treatment in clinical studies.

A replication defective adenovirus (Ad-p53) was utilized to restore p53 function within the cells and has been highly successful in reintroducing p53 function, including growth arrest and apoptosis in cancer patients. This technique is currently in clinical trials in the United States and China under the name Gendicine or Advexin. One promising study also utilizing adenovirus as a tool for targeting ineffective cells with ONXY-015, an E1B deleted adenovirus that replicates selectively in p53 deficient cells. [2] The adenovirus then kills the cells by lysing the cell membrane. The compound is currently in clinical trials and has been found to be most effective with the addition of chemotherapy agents. [2]

Finally, a synthetic peptide, similar to those described in this paper, derived from p53-binding protein 2 (54BP2 or ASPP) has been found to up regulate p53 transactivation by binding only to the properly folded, bioactive protein confirmation. This is believed to shift the equilibrium of folded proteins to unfolded proteins, to more folded proteins. [2] Unfortunately, these peptides do not have applicability towards treatment testing due to their lack of bioavailability in biological systems. However, the peptide serves as a testament to the utility of peptide treatments for regulating p53 levels using peptides derived from binding sequences. [2]

The research presented in this paper demonstrates that p53-derived stapled peptides may be useful in identify proteins involved in cell death pathways, providing a means by which to develop targeted therapies for cancer treatments.

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## Capstone Summary

The protein p53 is involved in regulating cell cycle processes within the cell, particularly it regulates when the cell should live or when the cell should die. This decision between life and death is p53's response to various cell stressors such as damage to genetic information, cell aging, and cell signaling. This important cycle filters for properly functioning cells by maintaining functioning cells and purposefully killing damaged cells through cell death mechanisms. Often disruption of this cycle, particularly the p53 pathway, can often result in cancer, the inability to control or stop cell growth. In cancer, cells continue to live and grow despite being unable to fulfill their function for the body. Cancer cells furthermore can metastasize into other tissues disrupting further function, and also consume essential nutrients from the body through an increased metabolism. A common disruption to this cycle is mutations in the p53 protein that hinder p53's ability to signal cell living and death pathways. Mutations in p53 are found is 50% of all cancer cases. It is therefore imperative to determine binding partners of p53 and discover drugs that will target this interaction and reinstate cell death mechanisms in cancer cells.

Recently, it was determined that the addition of p53-derived stapled peptides to cell lines will induce a cell death mechanism. This process works by having p53-derived stapled peptides binding and sequestering the targets of p53. Specifically, the peptide binds two major inhibitors of p53, HDM2 and HDMX. HDM2 inhibits p53 by ubiquitinilating p53 and targeting it for degradation while

HDMX binds p53 and sequesters p53. p53-derived stapled peptides are known to inhibit these inhibitors by binding HDMX and HDM2 making them unable to sequester p53 or target it for degradation, this in turn, increases p53 levels and therefore reinstates normal p53 cell cycle regulating pathways.

Mutant p53, with a mutation in the transactivation domain of p53, not only causes loss in p53 function but is also believed to have increased functions that cause characteristic cancer qualities. One of these pathways include the activation of HDM2, p53's inhibitor. Often this results in a build up of mutant p53 with activity that increasingly promotes increased characteristic cancer qualities such as increased cell survival. However, the addition of p53-derived stapled peptides to mutant p53 cell lines displayed a similar result to normal cell lines, showing a cell death mechanism. This result does not make logical sense considering that mutant p53 cell lines have increased functions that lead to characteristic cancer properties. Inhibition of HDMX and HDM2 with p53-derived stapled peptides increase mutant p53 and should lead to increased cancer qualities. This illogical result of reinstatement of a cell death mechanism to mutant p53 cancer cell lines by p53-derived stapled peptides, may indicate that these peptides are binding to other targets other than HDMX and HDM2.

To determine new binding partners of p53-derived stapled peptides, p53-derived stapled peptides were modified to contain a benzophenone at various essential binding residues following a method by Braun et al. In this method, upon the addition of UV light, the benzophenone will form a reactive keto radical

intermediate and will facilitate the binding of the peptide to its target. Using this method you can control the reaction utilizing UV light. Before and after UV exposure pictures of gel electrophoresis with Coomassie staining or Western blotting of reaction mixtures show if a reaction is occurring.

In this paper it is shown that p53-derived stapled peptides with bpa additions using the Braun et al method react specifically with its HDM2 targets (Figure 3, Figure 5, Figure 6, and Figure 7). It is furthermore shown that the p53-derived stapled peptide, SAH-p53-L26bpa binds to not just HDM2 but to other unknown proteins. (Figure 8, Figure 9, and Figure 20)

Future studies will look towards identifying what proteins specifically bind SAH-p53-L26bpa and from there determine if this reaction could be reinstating a cell death mechanism in both p53 cell lines and mutant p53 cell lines.