ABSTRACT

Changes to wild-type p53 that make it partially or completely lose or alter its function, to enable the progression of tumors, occur in ~ 50% cancers, highlighting the role of p53 as a significant tumor suppressor. Usually p53 is inactivated through the mutation of the gene in somatic cells and at times, through the disruption of pathways that are vital for p53 activation. Because of the prevalence of loss-of-p53 in most cancers, interventions to target the reactivation of defective p53 pathways are in progress. Since peptides are easier to synthesize, administer and are safer than most cancer drugs in the market, there is an interest in considering peptide derivatives in drug design. By synthesizing a small peptide from the C-terminal domain of p53 we tested if p53 would interact with this small peptide. This interaction is significant since the C-terminal domain of p53 is involved in the self-activation of p53 and the goal of peptide therapy in this case would be to restore p53 self-activation. The peptide was synthesized by solid-phase peptide synthesis and its affinity for p53 was tested using microarray printing technology. Based on the results, the C1 peptide, a small peptide with 15 amino acids, binds to p53. Our findings suggest that C1 should be investigated further, in order to develop it as an anti-cancer drug.

INTRODUCTION

In 2009, there were 10.9 million new cases of cancer in the world, 6.7 million people died from cancer and 24.6 million people were living with cancer. Lung, breast and colorectal cancers are the most frequently diagnosed cancers (Stevenson, 2009). The multistep genesis of tumors is a result of the progressive accumulation of mutations in significant oncogene and tumor pathways (Feldser et al., 2010). p53 is involved in one of these significant oncogene pathways (Feldser et al., 2010; Xu, 2008).

Normal Function of p53in human cells

Changes to the p53 protein that make it non-functional or alter its function occur in ~ 50% cancers, making p53 the most significant tumor suppressor (Feldser et al., 2010; Xu, 2008). p53, so-called because of its 53 kDa size, is a transcriptional enhancer phosphoprotein made of 393 amino acids (Sakaguchi et al, 1997). The normal protein functions as a tetramer; multimerization is affected by lysine methylation, which is a significant posttranslational modification that ensures the function of p53 (Cui et al., 2009; Pan et al., 2004; Pan et al., 2006). 122 target genes known to be involved in cell cycle arrest, apoptosis and other regulatory functions can potentially be activated by p53 in normal human cells (Riley et al., 2007). p53 DNA binding is sequence specific and regulates transcription of gene products involved in growth arrest, DNA repair, apoptosis and the inhibition of angiogenesis (see figure 1) (Stewart and Pietenpol, 2001; Oren, 2003). In unstressed cells, p53 is maintained in low steady-state levels and it survives in the cell for ~ 20 minutes before it is degraded through ubiquitin-dependent degradation (Stewart and Pietenpol, 2001). Mdm2 is the protein that targets p53 for ubiquitin-

mediated degradation (see figure 1). Mdm2 regulates the levels of p53 in cells by binding to p53 to block the self-transcriptional activation of the p53 protein (Ma et al., 2006). This interaction occurs through the binding of the N-terminus of p53 to the N-terminal domain of mdm2 while the acidic domain of mdm2 binds to the core domain of p53 (Ma et al., 2006).

p53 in human cancers

Even though the endogenous levels of Mdm2 are important for the regulation of the stability of p53, the inappropriate overexpression of *Mdm2* usually results in the reduction of p53 levels and this is a pathway by which some tumors eliminate p53 (Ben-Tal and Kalid, 2008; Ghosh et al, 2003; Oren, 2003).

Although affecting mdm2 activity is one way that p53 can be inactivated, it is more common for p53 to be inactivated through mutation in somatic cells and the tumor is derived from these mutated cells. (Xu, 2008). For instance, in human carcinoma cells, the disruption of the function of p53 enhances the cells' sensitivity to DNA-damaging agents while increasing their resistance to antimetabolic drugs (Stewart and Pietenpol, 2001). Restoring the function of p53 results in an increase in tumor suppressor signaling and this is a vital intervention to suppress tumors (Feldser et al., 2010)

The p53 tumor suppressor plays a critical role in the prevention of tumors in humans (Lacopetta and Royds, 2006; Oren, 2003). In unstressed cells p53 is present in low levels and its activation, change to a form that allows p53 to bind to DNA, is important for prevention of cell oncogenic activities (Oren, 2003; Stros et al., 2004; Xu, 2008). One way in which p53 can be reactivated is through the enhancement of the binding to DNA

by modification of the C-terminal negative regulatory domain (Cui et al., 2009). The reactivation of p53 pathways, the restoration of nonfunctional or mutated p53 to a functional p53, has the potential to result in tumor regression and thus is important for anti-cancer therapies (Feldser et al., 2010).

Most of the p53 mutations that occur in cancer involve the abrogation of protein-DNA interactions in p53 or the disruption of the folding of the p53 protein (Bouchet et al., 2006; Pan et al., 2006). Misfolding also interferes with DNA binding. The predominant mutations in cancer cells are missense mutations that lead to the expression of the full-length mutant p53; most of these mutations promote tumorigenesis and drug resistance since they develop novel oncogenic activities (Feldser et al., 2010). These novel activities include specific regulation of cancer invasion through the inhibition of apoptosis and the abolition of a signaling pathway that is involved in the control of the cell response to broken strands of double stranded DNA (Kastan & Berkovich, 2007; Roger et al., 2010).

Anti-cancer treatments that affect p53 function

Even though most of the p53 mutants completely lose wild-type p53 activity, some mutants do not irreversibly lose wild-type activity (Bouchet et al., 2006; Pan et al., 2006). For this reason, interventions to target the reactivation, defined as an altered p53 protein regaining tumor suppression activity, of defective p53 pathways are in progress (Feldser et al., 2010). Most of these interventions are drugs that target p53. These drugs in clinical trials either stimulate the function of p53 or reactivate the wild-type p53 function in p53 mutant proteins (Bouchet et al., 2006). For instance, PRIMA-1 and Ellepticine are drugs that are able to restore the DNA-binding function of mutant p53 proteins by covalently

modifying p53 mutant proteins (Bouchet et al., 2006; Lambert et al., 2009). Liver and prostate are the most prevalent peptide treated cancers (Stevenson, 2009).

Since recent targets of p53 reactivation are peptides and subsequently peptides can be custom synthesized, they are useful for specific targeting of intracellular proteins or receptors (Cho and Li, 2010). Through phage display that increases the rate of screening for peptides that bind to a certain target, these pharmacologically active peptides target proliferation, apoptosis, invasion, migration and angiogenesis required by cancer cells (Cho and Li, 2010). Since these peptides are useful, a number of technologies have been developed to modify and improve peptides so that they are viable therapeutic molecules.

These modifications usually involve efforts to make peptides more stable, soluble, with an adjusted lipophilicity and decreased degradation in the cell. Polyethylene glycol, through PEGylation, is usually used to make peptide derivatives have a bigger molecular mass, high water solubility, high mobility in solution, ready clearance from the body, protection from exopeptidases and lack of toxicity in cells (Cho and Li, 2010). In order to enable peptides to bind to cell membranes and not to be cleared to the kidneys, it is important to increase the hydrophobicity of peptide derivatives (Nestar, 2009). Since peptides are easier to synthesize, administer and are safer than cancer drugs on the market, there is an interest to consider peptide derivatives in vaccine design (Dudek et al., 2010).

Furthermore, there is increased interest in therapeutic drugs and therapeutic peptides are displaying their potential value in therapeutics by demonstrating significant therapeutic index in preclinical and clinical trials (Audie and Boyd, 2010; Cho and Li, 2010). Peptide

drugs are being used in the treatment of diabetes, osteoporosis, tumors, gastroenteritis, heart disease, immunosuppression, acromegaly, enuresis, bacteria, fungi (Stevenson, 2009).

Bortezomib, Liraglutinide, Primovax, Stimuvax, Goserelin are examples of prescribed peptide drugs. For instance, Bortezomib is a proteasome inhibitor useful in the treatment of cancer while Liraglutinide is a glucagon-like peptide receptor agonist being used in the treatment of diabetes. Goserelin is a gonadotrophin releasing hormone superagonist used in the treatment of prostate and breast cancer while Stimuvax induces an immune response to cancer cells that express the glycoprotein antigen mucin 1 (MUC-1) (this antigen is prevalent in cancer cells; Audie and Boyd, 2010; Cho and Li, 2010).

As a result of this therapeutic potential, peptide drugs stand a good chance of targeting p53 since inactived p53 can be activated for specific DNA binding in cells by manipulation of its C-terminal domain (Hupp et al., 1995; Selivanova et al., 1999). Since the status of p53 in cells is the difference between healthy cells and cancerous cells, the outcome of therapy relies on specific interactions of p53 and drugs that can reactivate it (Friedlander et al., 1996). Conventional drug constitution is being extended by the emergence of small peptide molecules (through the use of phage-display) that can be designed, through the use of biology and chemistry, to interact in a specific way with a particular protein (Audie and Boyd, 2010). This focus on sequence specificity is not only essential for the reactivation of p53 but is also important since p53 itself is a protein that identifies its targets based on sequence specificity.

There have been studies of peptide drugs that interact with p53 and some are currently on clinical trials (Bouchet et al., 2006). CDB3, RITA and APR are peptides that are under development for action on p53. APR is a more efficient analog of the p53 drug PRIMA-1, CBD3 triggers apoptosis by inducing oxidative stress and RITA functions by weakening the interaction of p53 with mdm2 (Bouchet et al., 2006). As of 2010, these drugs were still under clinical trials and the success of p53 mutant reactivating anticancer drugs should be a major contribution in anti-cancer treatments (Wiman, 2010).

The knowledge that peptide drugs are often superior to protein based therapeutics and that peptides can be synthesized to have specific interaction with p53, and to ultimately reactivate its function was useful. It led us to the hypothesis that a small peptide synthesized from the C-terminal domain of p53 would interact with p53 and would potentially be a drug for the reactivation of altered p53 that no longer binds to DNA like the normal protein does. This would be possible because p53 self-activates trimerization which enables it to bind to DNA. In the work presented here, we synthesized a peptide termed C1 and then tested its ability to bind to p53. Our findings suggest that C1 should be investigated further, in order to develop it as an anti-cancer drug.

MATERIALS AND METHODS

Synthesis of the peptide

To test the hypothesis that the C1 peptide could bind to p53 and thus might serve as the basis for a peptide-based anti-cancer drug, we first synthesized the peptide using solid-phase synthesis (figure 2). The sequence of the peptide was: N- leucine- lysine-serine-lysine-lysine-glycine-glutamine-serine-threonine-serine-arginine-histidine-lysice-lysine-

leucine-C. The C-terminal linker, gamma-aminobutyric acid (GABA) enables "capping" of the peptide via a free primary amine. Capping is the addition of non-peptide chemical groups that can enhance the pharmacological properties of peptides (such as stability or their ability to enter cells) or allow additional biochemical experiments to be performed. C1 peptides were variously capped with fluorescein for fluorescence polarization, N-Furfurylamine for printing to microarray, or an acetyl group for competitive binding experiments.

Rink Amide AM beads (Acros Organics, Fair Lawn, New Jersey, USA) were used as the resin for the solid-phase synthesis of the peptide. 280mg Rink Amide AM were weighed into a 5 ml syringe. These beads contain Fmoc protection with a carbamate group, termed "Fmoc" protection (Figure 2). The beads were swelled in dimethylformamide (DMF) for 30 minutes on a shaker. A ninhydrin test was done on a sample of the beads to determine if they were negative. A negative ninhydrin test indicates proper Fmoc protection. The beads were deprotected using 20% piperidine in DMF (10 ml piperidine/ 40 ml DMF) for 20 minutes on the shaker. This step was repeated. The beads were washed with DMF, dichloromethane (DCM) and DMF. This step was repeated three times. Beads were left in the third DMF wash so that they did not dry. A ninhydrin test was done on a sample of the beads to ascertain that the Fmoc protection on the resin had been removed (Sigma Aldrich, St. Louis, Missouri).

To synthesize the peptide a peptide synthesizer was used (Protein Technologies, Tucson, Arizona). The starting materials (amino acids and coupling agents) were weighed out in the appropriate equivalents as shown below in Table 1 with the Rink Amide AM beads as the limiting reagent. 260 mg Gamma-Aminobutyric Acid (GABA) was added to the Rink

Amide AM beads as a linker. O-Benzotriazole-N, N, N', N'-tetramethyl-uronium-hexafluoro-phosphate (HBTU) and N-Hydroxybenzotriazole (HoBt) were used as coupling agents. The coupling agents activate the protected amino acid before it is coupled to the sequence. During the coupling of each amino acid residue, 303.4mg HBTU and 122.4 mg HoBt would be added at the same time as the amino acid.

The solid-phase synthesis method was used to synthesize the desired peptide, termed the C-1 peptide. During this process, the amino group of one amino acid is linked to the carboxyl group of another and the synthesis goes from the carboxyl group to the amino group direction. This reaction can be controlled by using blocking groups, like t-Boc, to control the sequence of the resulting peptide. The carboxyl terminal of the last amino acid in the sequence is first anchored to an insoluble matrix, usually polystyrene beads. The amino group does not react with this matrix because it is t-Boc protected before the reaction. The t-Boc protecting group of this amino acid is then removed to avail the amino group of that amino acid for reaction with carboxyl group of the second to last amino acid, forming a peptide bond. The carboxyl group is activated with dicyclohexylcarbodiimide (DCC) and after the formation of the peptide bond; dicyclohexylurea is released (Berg et al., 2007).

Capping of the peptides

The synthesized peptide was divided into four vessels. One of the vessels was stored at -20 °C for future use and the other three were capped with Fluorescein, N-Furfurylamine or an Acetyl cap, respectively.

To conjugate the fluorescein, advantage was taken of the free primary amine at the N-terminus of the peptide (resulting from Fmoc deprotection of GABA). Primary amines readily react with N-Hydroxysuccinimide (NHS) esters. So we used NHS-Fluorescein to react with the primary amine. 5 equivalents of NHS-Fluorescein in anhydrous DMF were used and the reaction went overnight at 4°C.

To cap the peptide with an acetyl group, as seen in figure 8, 20% piperidine in DMF was added for 20 minutes to deprotect the Fmoc protection on the peptide. After 20 minutes, the solvent was decanted and this step was repeated. To complete the conjugation, 500µl 10% Acetic Anhydride and 250µl 5% Pyridine in DMF were added for 30 minutes at room temperature on shaker.

To cap the peptide with N- Furfurylamine (NFFA), Bromoacetic Acid (BAA) and Diisopropylcarbodiimide (DIC) were coupled to the primary amine by heating in the microwave twice for 10 seconds at Power Level 1.The beads were washed with DMF, DCM, and DMF. A 2M solution of furfurylamine in DMF was added to the beads and the vessel was heated in the microwave twice for 10 seconds at Power Level 1 to displace the bromine.

Successful capping was verified by mass spectrometry of the capped peptide. Mass spectrometry fragments the sample inside the instrument and analyses the resulting products generated. To interpret the structure and the sequencing of the peptides, mass spectrometry was useful.

Cleaving and purification of the peptides

The peptides were cleaved from the beads (Rink Amide resin) using a solution of 95% trifluoro acetic acid (TFA), 1.67% water, 1.67% thioanisole, 1.67% TIPS (Triisopropylsilane) two hours at room temp. This mixture causes the peptide-resin bond to hydrolyze, freeing the peptide from the resin) and removes side-chain protecting groups.To purify the peptides, High Performance Liquid Chromatography (HPLC) was used. Fractions containing the peptide were pooled and the concentrated peptide was identified using the timing of the peaks on the HPLC spectra. To verify the masses and purity of the wanted peptide, Matrix- Assisted Laser Desorption Ionization (MALDI) Mass Spectromentry was used.

High Performance Liquid Chromatography

The peptides, dissolved in 1:1 solution of Acetonitrile: H_2O were applied to C18 HPLC the column. Next, a gradient from 100% acetonitrile to 100% H_2O was applied over 40 minutes to separate the mixture. The presence of peptides in each fraction was detected using the spectra from the experiment. Peaks from the spectra were used to identify the tube where the purified peptide came out. This was possible because one fraction was collected per minute into a separate tube so using the spectra directed to the time and therefore the tube with the peptide.

Purification of His Tagged p53

The p53 protein containing a poly-histidine tag at its N terminus was over-expressed in *Escherichia coli* BL21 (DE3) Star cells. The culture was grown to an optical density of 0.7 and then induced with 0.5mM Isopropyl β-D-1-thiogalactopyranoside (IPTG) and incubated overnight at room temperature. The cells were harvested by centrifugation. We

purified His-tagged p53 for the fluorescence polarization experiments. There was no Flag tag on this protein. For the microarray we used Flag-tagged protein that was present in an *E. coli* lysate.

When tagged with 6 residues of histidine at its N terminus, the p53 protein is termed "His Tagged p53." The histidine tag is attached to the protein though a linker that includes a target sequence for Tobacco Etch Virus (TEV). 15 ml lysis buffer (1× Tris Buffered Saline (TBS), Protease inhibitor cocktail tablet and 5mM dithiolthreitol (DTT)) was used to the resuspend the cell pellet. This resuspended pellet was then sonicated three times on ice by slowly turning the knob to setting 7. After sonication, the total cell lysate was spun down at 20, 000 rpm for 20 minutes. A nickel column was used to purify the protein in the supernatant. The supernatant was passed through a nickel column at 1 ml/minute using a Peristaltic pump, allowing the poly-histidine tag to bind to the immobilized nickel. After the protein had passed through the column, 0mmol, 50 mmol, 100mmol imidazole buffers were passed through the column to remove any proteins bound non-specifically. The column was put on ice in preparation for collection of fractions. 5ml 300 mmol imidazole buffer was used to elute His Tagged p53 protein. The protein was collected and placed on ice. 5ml 300 mmol imidazole buffer was added again and the second fraction was collected, then the third.

The fractions were mixed and poured into desalting tubes to separate the imidazole from the protein. The desalting tubes were used to separate the imidazole from the his-tagged p53. They separate according to size; the p53 passes through the resin quickly, while the imidazole is trapped in the small pores of the resin. The desalting tubes were PD-10 desalting columns that contained 8.3ml of sephadex G-25 medium from GE Healthcare

(Waukesha, Wisconsin). They were equilibrated with 25ml of TBS; 2.5ml of protein sample were applied to the column, then 3.5ml of the desired buffer TBS was used to elute the protein from the column. The His Tag was cleaved from the protein with his-tagged TEV protease, previously purified from *E. coli*, at 4°C overnight. The his-taggedTEV protease, and any uncleaved his-tagged p53, was removed from the p53 sample by passing the solution through the nickel column again. After cleaving the his-tag from p53, it no longer binds to the nickel column and passes through, thereby separating the native protein from the solution.

SDS Polyacrylamide Gel Electrophoresis (SDS-PAGE) was used to check the results of the protein purification. The 12% gel was run at 200V for 45 minutes, stained with coomasssie and visualized.

Testing for binding to p53

The purified his-tagged p53 protein was concentrated by centrifuging for 20 minutes at 3,500rpm using Amicon Ultra centrifugal filter device filter units (Millipore, Billerica, Massachusetts). The filter units carried 15 ml sample size at a time and the molecular cut-off was 3000. Beer's Law was used to find the concentration of the Fluorescein conjugated peptide that would enable binding with p53. The equation for Beer's Law is A= ebc where 'e' is the molar absorbtivity with units of L mol^{-1} cm^{-1}, 'b' is the path length of the cuvette and 'c' is the concentration of the compound in solution in mol L^{-1} (Beer's law, 2011). The absorbence of the C1 peptide with a fluorescein cap was measured using a NanoDrop spectrophotometer using A492 absorbance readings (Thermo Scientific, Rockford, Illinois). The absorbance reading and the width of the cuvette used

(1 cm) were applied into the Beer's Law equation. Bovine Serum Albumin (BSA) assays were used to find the concentration of the protein. These are protein concentration standards that are used to form a standard curve that can be used to deduce the concentration of a sample (see figure 12). The p53 protein was concentrated until it went from 15 ml to 3ml using filter units.

Serial dilutions of p53 protein were done for use in Fluorescence Polarization (FP). Samples were transferred to long thin glass tubes used in FP. Samples of p53 and the fluorescein conjugated peptide were incubated for 1 hour at 4°C wrapped in aluminium foil. Fluorescence polarization readings were done on the serial dilutions of p53 and the Fluorescein capped C1 peptide.

Fluorescence Polarization

The fluorescent C1 peptide was diluted to a concentration of 20nM (much lower than the expected K_d). The purified p53 protein was serial-diluted to generate protein solutions from 10nM to 30µM. The mixture of fluorescent C1 peptide and p53 protein was excited at 492nm and the fluorescence intensity parallel and perpendicular to the excitation plane were measured at 532nm and used to calculate the polarization value.

Microarray Printing

The C1 peptide containing a C-terminal furfurylamine was printed onto maleimide-coated glass slides with a NanoPrint LM360 (TeleChem International Inc., Sunnyvale, CA) with MP946 Micro Spotting Pins (figure 4). Slides were left in 50% humidity for 12 hour before and after printing. The free maleimide groups were blocked with 2% 2-mercaptoethanol in DMF for 1 hour followed by washing with DMF, THF, DMF, ACN,

isopropanol, then TBST. The washed slides were dried in a centrifuge for 5 min at 2000 rpm. The dried slides were blocked with StartingBlock (Fisher, Waltham, Massachusetts) for 1 hour at room temperature, and then washed with TBST. *E. coli* lysate containing 100µM flag-tagged p53 was incubated with the slide overnight at 4°C on an orbital shaker. The slide was washed 3 times with TBST before adding 1ng/µl Anti-flag M2 antibody produced in mouse (Sigma, St Louis, Missouri) diluted in 1:1 TBST: StartingBlock for 1 hour at 4°C. The slide was washed 3 times with TBST before adding 5ng/µl Alexa647 goat anti-mouse IgG secondary antibodies diluted in 1:1 TBST: StartingBlock for 1 hour at 4°C. The slide was washed 3 times with TBST, then once with 0.1 TBST, spun dry at 2000 rpm, then scanned using a GenePix Autoloader 4200AL Scanner (Molecular Devices, Sunnyvale, CA). Slides were scanned with an excitation wavelength of 490nm and an emission wavelength of 532nm at a power of 100x and photomultiplier tube setting of 500x-600x.

After fluorescence polarization did not produce the desired results, microarray was used to test binding of the C-1 peptide to p53. Binding of the peptide to p53 can be tested using a method that is grounded on hybridization. To create a microarray, the C1 peptide was covalently linked to the maleimide coated glass microscope slide through a Diels-Alder reaction with the furfurylamine cap (shown in Figure 4). Hybridization between the flag-tagged p53 protein and the C1 peptide is visualized by fluorescence from a secondary antibody (shown in Figure 11). First, *E. coli* lysate containing flag-tagged p53 is incubated with the microarray (green protein with black tag indicating the flag epitope in Figure 11). The microarray is then washed to remove unbound proteins and the primary anti-Flag antibody produced in mouse is incubated with the microarray (black

antibody shown in Figure 11). If the flag-tagged p53 remains bound to the C1-peptide on the slide, then the anti-flag antibody will bind to the Flag epitope on the p53 protein. The microarray is washed again to remove any unbound anti-flag antibody, and then Alexaflour 647-conjugated Goat-Anti-Mouse-IgG antibody (Red antibody shown in Figure 11) is incubated with the slide and will bind to the anti-Flag antibody to produce a fluorescent signal. The brighter the fluorescent light emitted, the more hybridization between the C-1 peptide and p53 (Berg et al, p152).

A few printing controls were included on the microscope slide in addition to the C1 peptide. To ensure that the compounds were successfully printed onto the microscope slide, two fluorescent control peptoids were included. The first is a peptoid previously synthesized in the lab that contains furfurylamine at the C-terminus, a short peptoid sequence, and a fluroescein cap (black circle, Figure 11). The second peptoid is identical, but contains a red fluorescent dye at the C-terminus, DyLight 655 (green circle, Figure 11). These controls were included so that immediately after printing, the slide could be scanned at wavelengths 532 and 655, and a signal should be observed from these peptoids to ensure that the printing was successful. A third control was included that is a known peptoid ligand for p53 (yellow circle, Figure 11) with a furfurylamine at the C-terminus. This peptoid was previously synthesized in the lab and was used as a positive control for functional p53 protein in the microarray. This peptoid should result in a strong fluorescent signal if the flag-tagged p53 protein used for this experiment is functional. Lastly, the Flag peptide was synthesized with a C-terminal furfurylamine residue (purple circle, Figure 11) and was used as a positive control for functional antibodies. After hybridization with the anti-flag antibody and the secondary anti-mouse antibody, these

spots should result in a very strong fluorescent signal if both antibodies are functioning properly.

RESULTS

Solid-Phase Protein Synthesis

To test the hypothesis that the C1 peptide could bind to p53 and thus might someday serve as the basis for a peptide-based anti-cancer drug, we first synthesized the peptide using solid-phase synthesis (figure 2, see Materials and Methods). The sequence of the peptide synthesized was: N- leucine- lysine-serine-lysine-lysine-glycine-glutamine-serine-threonine-serine-arginine-histidine-lysice-lysine-leucine-C. This sequence of the C1 peptide was derived from long peptides that are known to bind to the C-terminus of p53 (Hupp et al., 1995; Friedlander et al., 1996; Selivanova et al., 1999).

Purification of the Fluorescence Peptide

After solid-phase synthesis, the peptides must be released from the solid matrix and purified to eliminate the small proportion of impurities. We used High Performance Liquid Chromatography (HPLC) to purity the peptide for use in future experiments. As an example, the HPLC chromatogram (Figure 5) shows the times when compounds in the fluorescein peptide sample eluted during the analytical HPLC. Each peak represents a specific compound and the ones that are merged represent compounds that eluted simultaneously. The arrow points to the time when the fluorescein peptide eluted during the analytical HPLC. Each peak was subjected to mass spectrometry to identify the peak containing the desired fluorescein peptide. Based on this analysis, we estimate that the

peptide was at least 95% pure. We did not purify the acetyl capped peptoid, and for the Nffa cap we did an ether precipitation that probably removed most of the impurities.

Fluorescence Peptide

The peak at 11 minutes in Figure 5 was identified by Mass Spectrophotometry to contain the fluorescein peptide. The calculated mass of the peptide capped with fluorescein was 2167.15 g/mol and the mass in the dominant peak was 2169 g/mol instead of 2167.15 g/mol because the mass shows a plus two addition to the desired mass as a result of the positive charge around the molecule (figure 6). The solution corresponding to this peak was frozen in liquid nitrogen and lyophilized to dry the fluorescent peptide. For use in further experiments, the peptide was dissolved in water and the concentration was determined using the known extinction coefficient for fluorescein and the absorbance at 492nm.

N-furfurylamine

We also capped the C-1 peptide with N-furfurylamine (Nffa; Figure 7); the NFFA ring enables printing of the peptide on a microarray. The calculated mass of the capped peptide was 2033.18 g/mol, while the measured mass was 2034.1 g/mol because of extra delocalized charges around the peptide. The peptide was precipitated using ice-cold ether, and pelleted in a microcentrifuge. The supernatant solution was removed with a pipet. This ether precipitation was repeated three times and finally the peptide was dried using the speed-vacuum. It is generally estimated that after ether precipitation the peptide is at least 80% pure. Using the molecular weight of the peptide, enough dry peptide was

weighed out and dissolved in DMSO (dimethylsulfoxide) to give a 20mM stock solution for printing.

Purification of p53

In order to study the interactions of the C-1 peptide (or its capped derivatives) and p53, we purified p53 protein. The procedure used to clone the p53 gene results in addition of a poly-histidine tag, suitable for nickel column purification, to the protein through an amino acid linkage that can be cleaved by a viral protease, resulting in native p53 not containing any non-native amino acids. To monitor the purification process, we used SDS-PAGE (Figure 9).

Use of purified p53 to test binding to C1 using fluorescence polarization

The purified p53 protein was used in combination with the fluorescent C1 peptide to test binding between the two molecules using fluorescence polarization. Unfortunately, even the highest concentration of p53 protein used did not result in saturation of binding as observed by polarization increase and plateau. We therefore turned to an alternative procedure, use of microarrays, to investigate binding of C1 peptide to p53.

Use of Microarrays to test C-1 binding to p53

After adding varying concentration of flag-tagged p53 to the microarray, ranging from 0-100μM, incubating it under appropriate conditions, and washing away unbound protein, we used an immunoassay to detect p53 binding. We tested the ability of C-1 to bind to p53 by monitoring the fluorescence caused by the CI peptide- p53-Flag-primaryantibody-secondary antibody moiety. Binding by p53 increases fluorescence because the ultimate

secondary antibody has a fluorescent molecule conjugated to it. Therefore we tested a dilution series of p53 to find out if p53 binds to the C1 peptide. The microarray results indicate that p53 does bind to C-1 (Figure 10).

DISCUSSION

There is focus on p53 mutations since they are the most common events in the progression of human cancers (Bouchet et al., 2006; Murata et al., 2006). To test if a peptide synthesized from the C-terminal domain of p53 would bind to p53, the C1 peptide was synthesized and its affinity for p53 was tested using microarray printing technology. Based on the results, the C1 peptide, a small peptide with 15 amino acids, binds to p53. The size is important because a shorter peptide has more chances of bioavailability and less chance of being degraded by enzymes that target peptides. Mobility and induction of a smaller peptide into cells would be easier in the body than of a longer peptide. These data contribute to efforts to develop drugs that can be used to target and reactivate mutant p53 in cancerous cells.

The focus on p53 mutations stems from the understanding of the alteration or loss of function of mutated p53 cells in tumors. The tumor suppressor protein, p53, is a transcription factor that plays a critical role in protecting the cell in response to various stress signals by regulating the expression of genes related to cell cycle arrest, apoptosis or senescence. p53 either quarantines damaged cells or directs their death and interferes with the proliferation of cancerous cells (Bullock and Fersht, 2001). Loss or mutation of p53 increases susceptibility to cancer development (Vousden and Lane, 2007) and mutations are found in the Tumor Protein (TP) 53 gene of 50% of tumors (Cheok et al.,

2011). Cancers with inactive mutant p53 have been shown to be aggressive and to resist ionizing radiation and chemotherapy (Bullock and Fersht, 2001). In addition, the inactive p53 mutants evade MDM2 dependent degradation (Bullock and Fersht, 2001) enabling the mutants to facilitate the proliferation of tumors. Altered p53 is not only inactive, but it also reduces the transcriptional activity of p67 and p73, the other p53 family members (Brown et al, 2009). Altered p53 is also the dominant-negative inhibitor of wild-type p53 in tumors since wild-type p53 is down-regulated in the presence of altered p53 (Vousden and Lane, 2007).

Since the functionality of p53 is vital for the protection of the genome of an organism, small molecules capable of restoring the DNA binding ability of p53 have enormous potential as broadly applicable cancer therapeutics. Studies have shown that the binding of small peptides can reactivate abnormal p53 proteins that no longer bind to DNA because of a loss-of-function mutation (Lane, 1995; Selivanova, 1999). One such peptide derived from the amino acid sequence of the C-terminal domain of p53, named the C1 peptide, targets mutated p53 directly, and is nearly small enough that it could be suitable for development into a drug that rescues the functionality of p53 in most tumors.

The data procured in this study is vital since tumors are known to regress and to clear *in vivo* in the presence of active p53 (Meek, 2009; Kastan, 2007). Showing that the C1 peptide has potential to rescue the function of mutant p53 would suggest that the C1 peptide could potentially provide an opportunity for the regression of some tumors. We could use the microarray technology to find out if C1 restores activity to altered p53 proteins because we already have the C1 peptide with the Nffa cap. We could print the C1 peptide to the microarray; incubate the slide with mutant p53 protein (such as the

R175H mutant). If C1 did bind to the altered p53 proteins, Next, we would use a fluorescently labeled DNA sequence (known to bind to p53 because it is found in the promoter region of many of the genes activated by p53) to determine if the mutant p53 is able to bind its DNA sequence in the presence of the C1 peptide. Figure 13 shows this scheme.

With all the data in mind, peptides do not make effective drugs because of cell penetration and stability problems (Brown et al, 2009). For this reason, peptides are usually "stapled" into a conformation that improves their pharmacodynamics (Brown et al., 2009). So a peptide whose structure is more stable would have a greater chance of being a better drug. Manipulation of the structure of some peptides that are being used in trials has led to development of receptor ligands with longer action (Edwards, 1999). An increasing number of active pharmaceuticals need protection of enzymatic and acid-catalyzed degradation in the cell (Svenson, 2004). The next step in this study would be to modify the structure of the C1 peptide to make it rigid and less hydrophilic, contributing to the work to make peptides more bioavailable and less prone to degradation. Methylation at the hydrogen on the amine group is an example of a modification that would potentially improve the pharmacological properties of C1. Similar to experiments presented here, we could employ microarray technology to print a library of methylated compounds on a microscope slide, incubate the slide with mutant p53, followed by fluorescently labeled DNA, and scan the slide for any spots that show fluorescence. The fluorescent spots would indicate that the library compound binds to the mutant and reactivates its ability to bind to DNA. The compounds that give positive results would

then need to be identified by tandem mass spectrometry and used for additional biochemical experiments.

This structural rigidity would aid in the elucidation of the mode of action of the peptide. Since the structure of traditional peptides is flexible, it is hard to predict the specific interaction between the protein target and its peptide ligand. A more rigid ligand may enable predictability in the mechanism of its binding (Brown et al, 2009). In addition to structural rigidity, a good drug must be transported to the target cells, and be taken in by the cell membrane, once inside the body (Svenson, 2004). Furthermore, it also has to effectively mimic the interactions needed for activation yet still maintain good drug-like properties (Brown et al, 2009). A less hydrophilic peptide would be an improved drug in that it would be polar enough to be transported by blood but still hydrophobic enough to pass through the phospholipid bilayer (Svenson, 2004). This modification is important since peptide structural rigidity facilitates induction (Lattig- Tunnemann, 2011). The work presented in this study focuses on the synthesis of a Peptide Tertiary Amine library (PTA) version of the C1 peptide, which is a modification to the structure of the C1 peptide (as shown in figure14).

Interventions that act on tumor microenvironment have had some successes but their efficacy is limited to a small percentage of patients (Cheok et al., 2011) which is why the development of drugs that target more patients is necessary. There are also clinical trials using immunization with long peptides derived from p53 (Cheok et al, 2011).These peptides are supposed to invoke a natural immune response to p53 (Cheok et al, 2011).

The C1 peptide is shorter and this could be strategic since longer peptides could be more susceptible to enzymatic degradation.

Next Steps Needed to Show that C-1 Can Rescue mutant p53

Since these results show that the C1 peptide interacts with the p53 protein, the next step in the project is to determine if the binding of the C1 peptide reactivates the mutant p53 proteins. We could use the microarray technology to determine this because we already have the C1 peptide with the Nffa cap. We could print the C1 peptide to the microarray; incubate the slide with mutant p53 protein (such as the R175H mutant). Next, we plan to use a fluorescently labeled DNA sequence (known to bind to p53 because it is found in the promoter region of many of the genes activated by p53) to determine if the mutant p53 is able to bind its DNA sequence in the presence of the C1 peptide. Figure 13 shows this scheme. Ideally, if we confirm that the C1 peptide is able to reactivate the mutant p53 protein, then we could begin to test methyl substituted peptide tertiary amine (PTA) versions of the C1 peptide for their ability to reactivate mutated p53 (figure 14). PTA chemistry uses PTA residues that differ from traditional amino acid residues in that bromine is substituted for $-NH_2$ (the amine group) allowing for the subsequent displacement with methylamine. PTA chemistry is useful for substituting methyl groups for hydrogens bonded to nitrogens in the peptide, thereby replacing a polar bond with a non-polar one. The peptide could be altered by replacing increasing numbers of the hydrogens normally bonded to the N of the peptide backbone with methyl groups; the peptide with all possible substitutions of this nature is shown in Figure 14. Again, we could employ microarray technology to print all the methylated library compounds on a microscope slide, incubate the slide with mutant p53, followed by fluorescently labeled

DNA, and scan the slide for any spots that show fluorescence. The fluorescent spots would indicate that the library compound binds to the mutant and reactivates its ability to bind to DNA. The compounds that give positive results would then need to be identified by tandem mass spectrometry and used for additional biochemical experiments.

p53 has been implicated in cancer for about 30 years, and drugs that attempt to restore its function to tumors lacking functional p53, or that attempt to interfere with the function of dominant-negative p53 proteins, have been tested in trials. Modified peptides remain attractive anti-cancer drugs because they have a longer half-life and their more rigid chemical structure allows for predictability in their function. The C1 peptide we studied is a promising candidate and future work should follow up on the observations reported here.

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First and foremost, I would like to thank Professor Phoebe Lostroh for her guidance and support throughout the writing of this thesis and in college in general. I would also like to thank Professor Darrell Killian for his thorough guidance during the writing of this work. I would also like to recognize the Biology Department, especially Donna Sison and my academic advisor Professor Ralph Bertrand. I am grateful to the Scripps Research Institute for funding my research, especially Dr Thomas Kodadek. I also appreciate Dr Kimberly's strict yet friendly instruction in the lab. Finally, I would like to thank my friends and family, especially Lee Pedzisa, for their continuous support and encouragement.

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TABLES

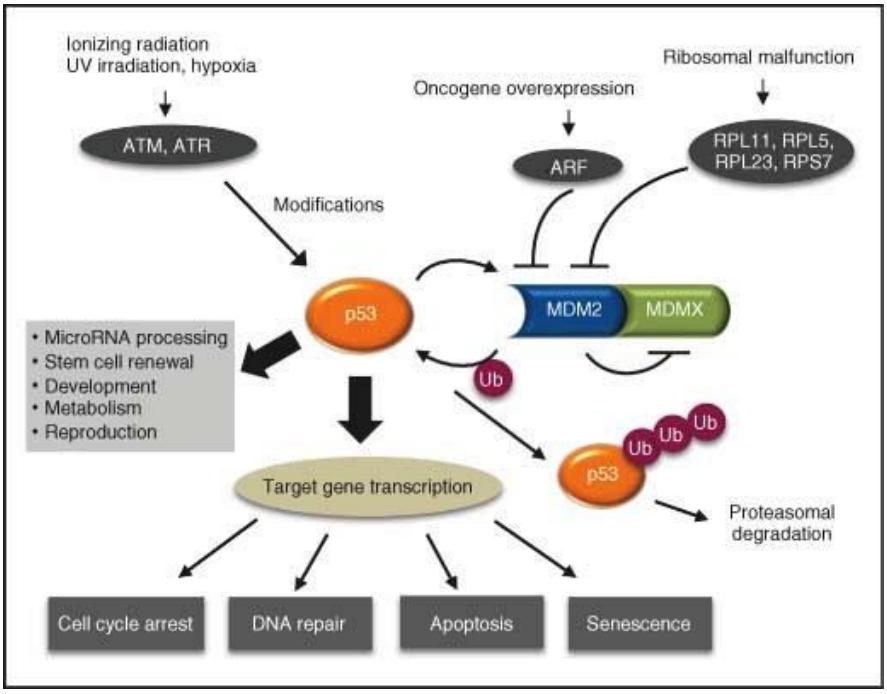
Table 1. Amino acid equivalents arranged in peptide sequence order from the carboxyl group to the amino group (C-N terminus)

Reagent	4 Equivalents (mg)
Amino Acids:	
Leucine	282.4
Lysine	375
Lysine	375
Histidine	382
Arginine	477
Serine	306
Threonine	318
Serine	306
Glutamine	488.5
Glycine	237.8
Lysine	375
Lysine	375
Serine	306
Lysine	375
Leucine	282.4

*Equivalence is a ratio of the moles of any component to the number of moles of the limiting reactant.

FIGURES

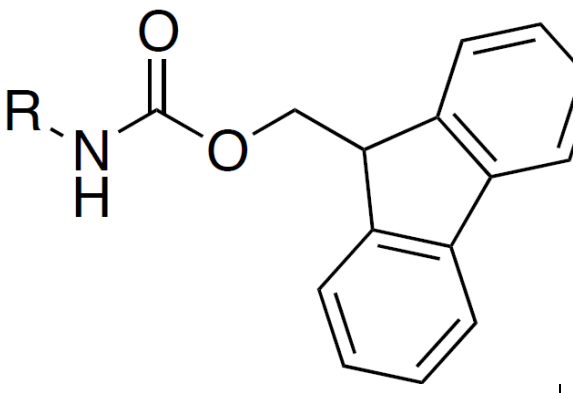
A


Ionizing radiation
UV irradiation, hypoxia
Ribosomal malfunction
Oncogene overexpression
ATM, ATR
ARF
RPL11, RPL5,
RPL23, RPS7
Modifications
p53
MDM2
MDMX
• MicroRNA processing
• Stem cell renewal
• Development
• Metabolism
• Reproduction
Ub
Ub
Ub
Ub
p53
Target gene transcription
Proteasomal
degradation
Cell cycle arrest
DNA repair
Apoptosis
Senescence

B

Figure 1. Normal p53 function in human cells. A) The diagram shows the the interaction between p53 and mdm2 and a number of the pathways involved in p53 function including the transcriptional genes that are regulated by p53. Image quoted from "Chakraborty et al. 2011, p.3." B) DNA binding domain of p53 bound to DNA. The purple represents the DNA strand, in pink are alpha helices, in white are loops and in yellow are beta strands. The scaffold for the p53 DNA binding site is provided by an immunoglobulin like β-sandwich. The DNA (in purple) major groove (pink helices) binds to a loop-sheet-helix motif on p53. Image developed from PDB 1tup text file from "Cho & Gorina 1994, p.348" using Jmol.

A)



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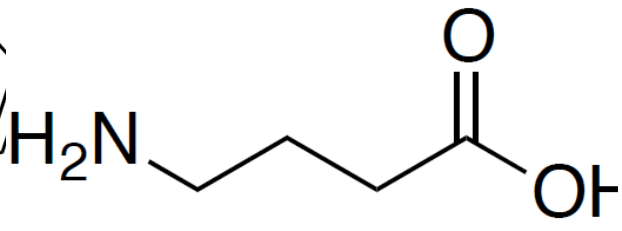
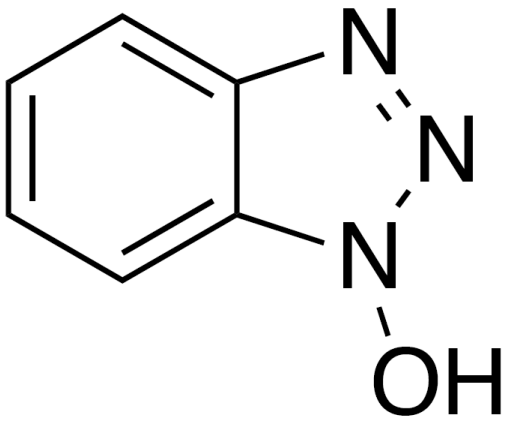


C)



D)

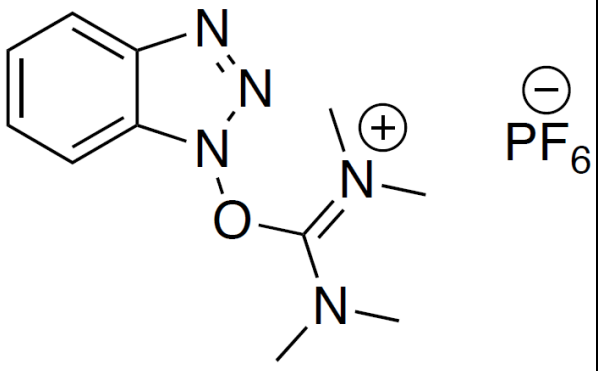


Figure 2. Protecting group, linker and coupling reagents used to facilitate synthesis of the C1 peptide. A) The chemical structure of the Fmoc protection on the Rink Amide AM and some of the amino acid residues used to synthesize the C1 peptide. B) GABA, the linker used in the synthesis of the C1 peptide. It enables conjugation of a capping residue. C) The chemical structure of the HoBt coupling reagent that was used to couple amino acids onto the C1 peptide. D) The chemical structure of the HBTU coupling reagent that was used to couple amino acids onto the C1 peptide. All images developed using Chemdraw software.

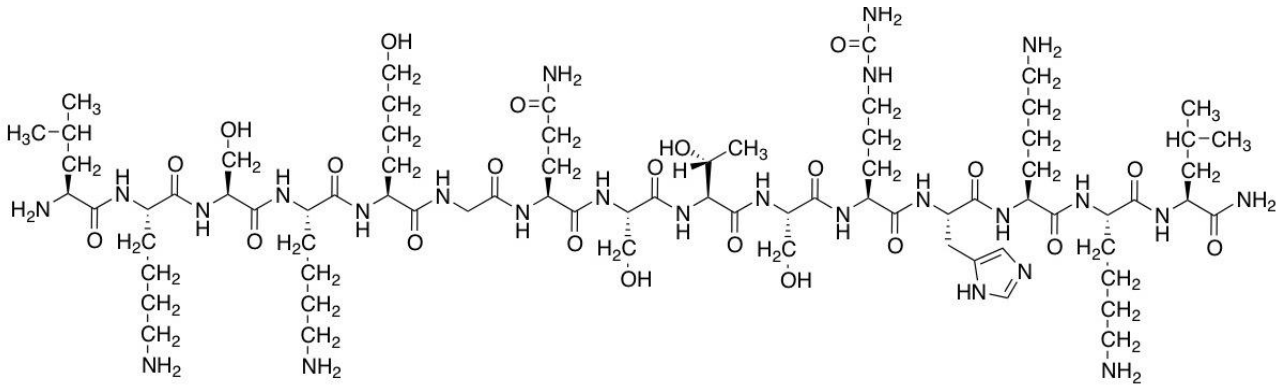


Figure 3. The desired peptide, termed C1 peptide. The C1 peptide is diagrammed in figure 3 above. The peptide was selected because it was likely to bind to p53 (Hupp et al, 1995). The peptide was synthesized using a peptide synthesizer (see Materials and Methods).

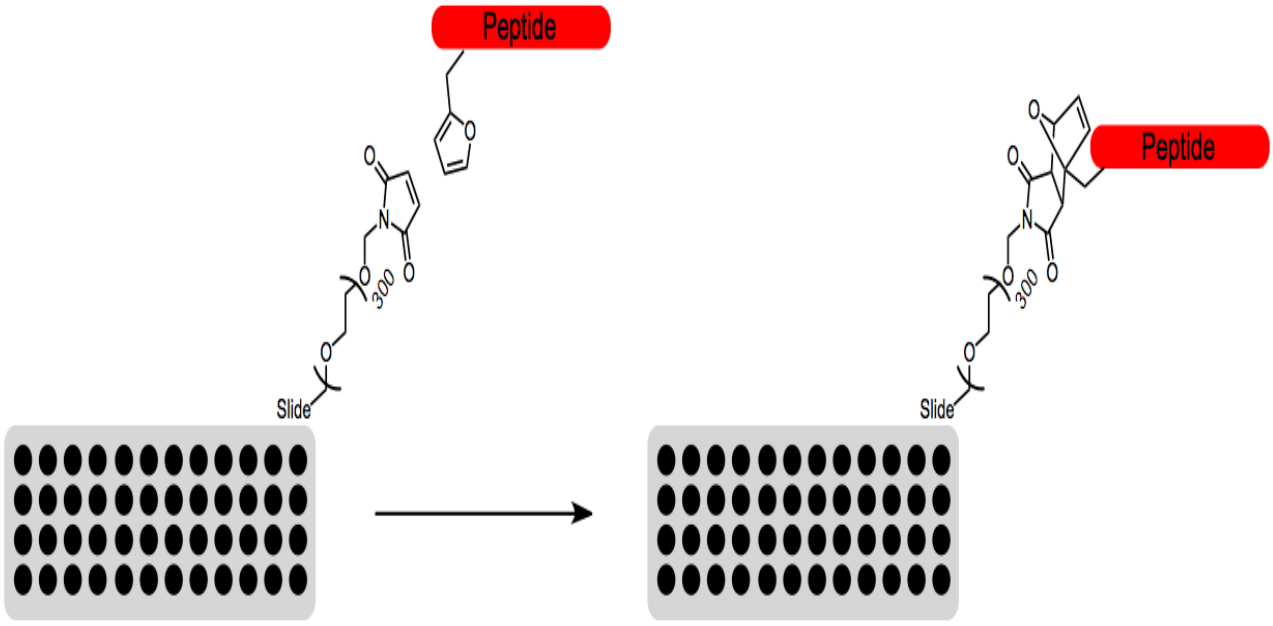

Peptide
Slide
300
Peptide
Slide
300

Figure 4. Microarray printing with the Nffa C1 peptide. The Nffa cap on the C-terminal of the C1 peptide enables binding of the C1 peptide to the maleimide-coated glass slides with a NanoPrint LM360 Micro-spotting prints. The Nffa and the maleimide react through a Diels-Alder cycloaddition reaction.

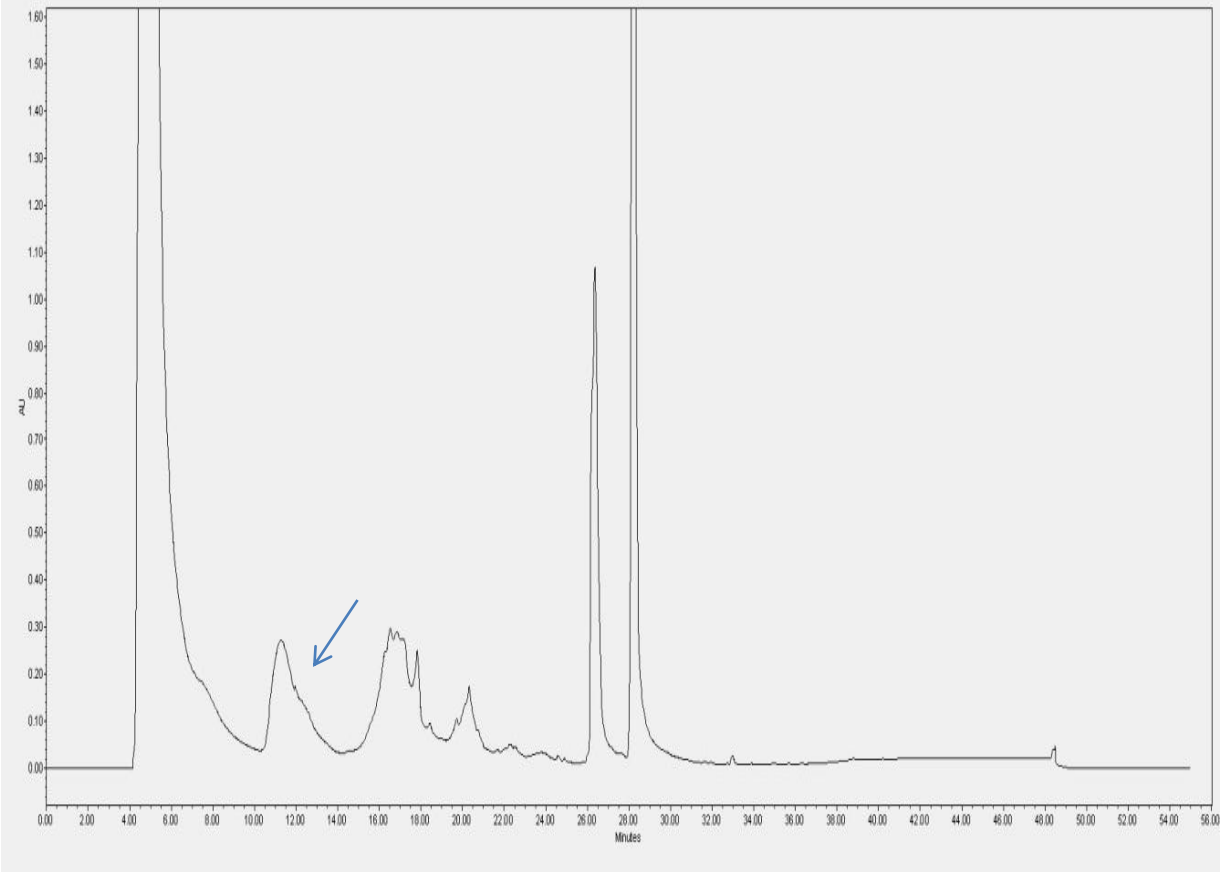
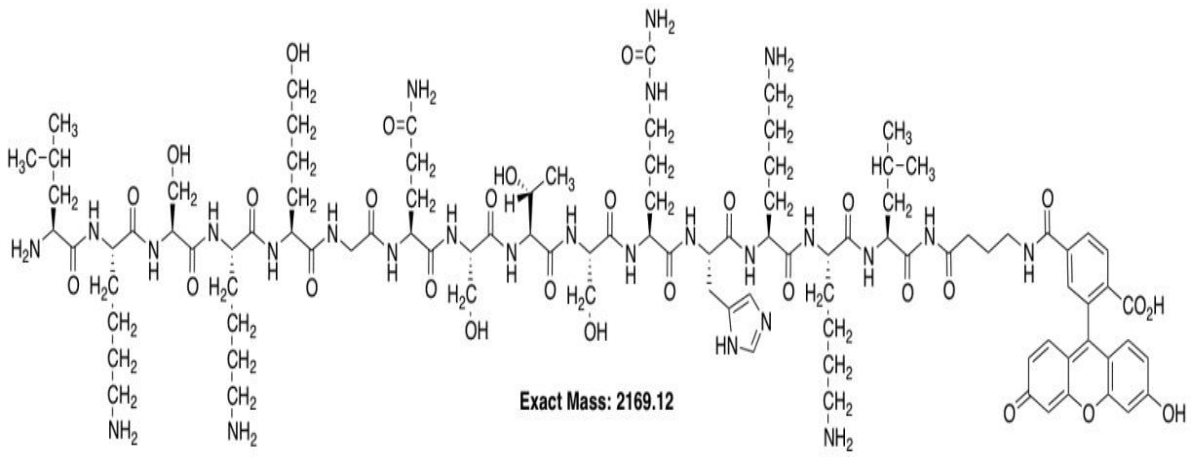
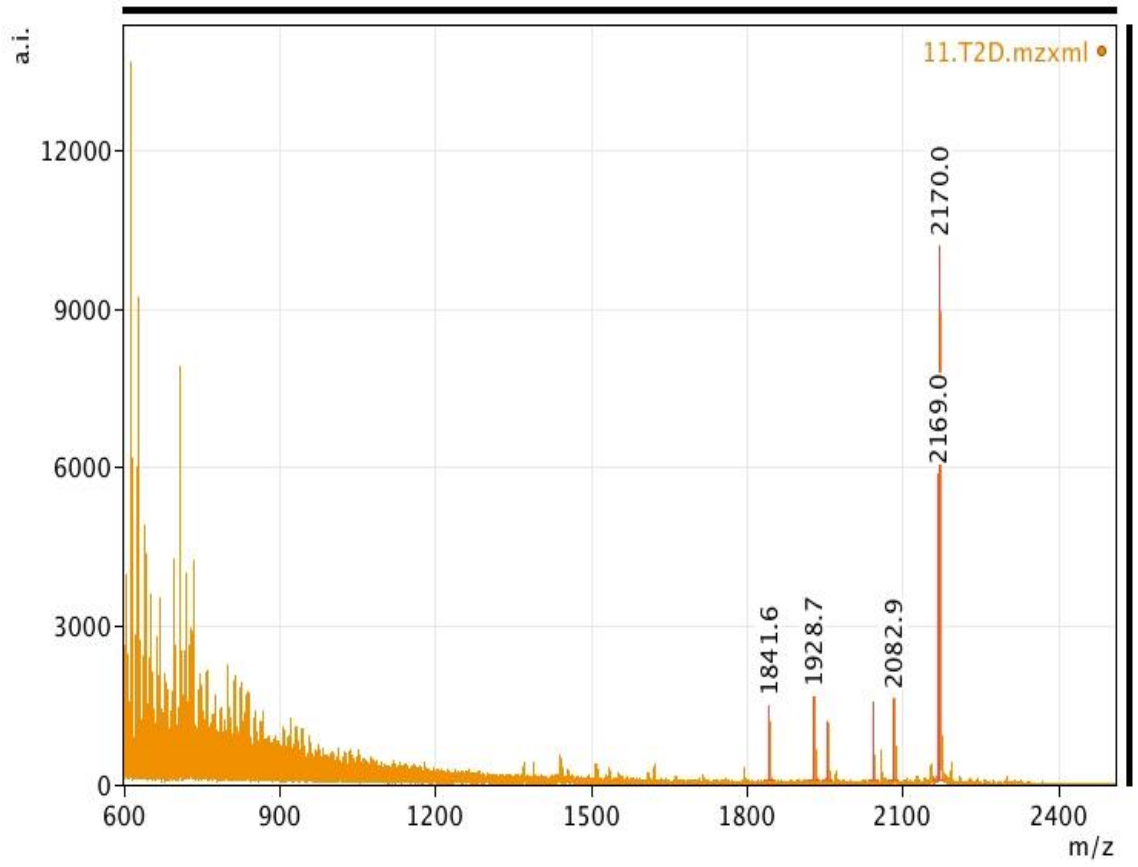

AU
Minutes

Figure 5: Analytical HPLC of the Fluorescein capped C-1 peptide. The fluorescein cap was added to C1 by reacting it with the GABA residue, deprotecting the fmoc from GABA, and conjugating NHS-fluorescein, and then removing the peptide from the solid matrix with 95% TFA. HPLC analysis was used to examine the purity of this preparation. The peak containing fluorescein-capped C1 is indicated with an arrow.


Exact Mass: 2169.12

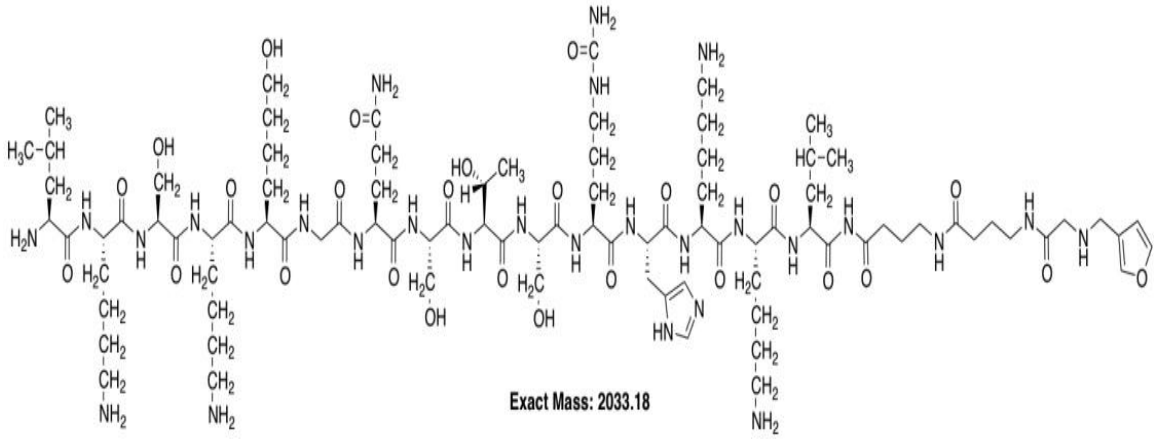
A)


a.i.
11.T2D.mzxml
12000
9000
6000
3000
0
2170.0
2169.0
1841.6
1928.7
2082.9
600
900
1200
1500
1800
2100
2400
m/z

B)

Figure 6. The chemical structure and mass spectrometry graph of the C1 peptide capped with fluorescein. A) The chemical structure of the C1 peptide capped with fluorescein. This was the structure of the C1 peptide that was theoretically deduced. This structure was verified by the mass spectrometry that we performed. The calculated mass of the peptide was matched with the 2169.0 peak. This verified the presence of the peptide in the sample that was at hand. B) The graph from mass spectrometry of the C1 peptide capped with fluorescein. After analytical HPLC, we did MALDI mass spectrometry on the compound to verify the presence of the desired peptide.

A)


Exact Mass: 2033.18

B)

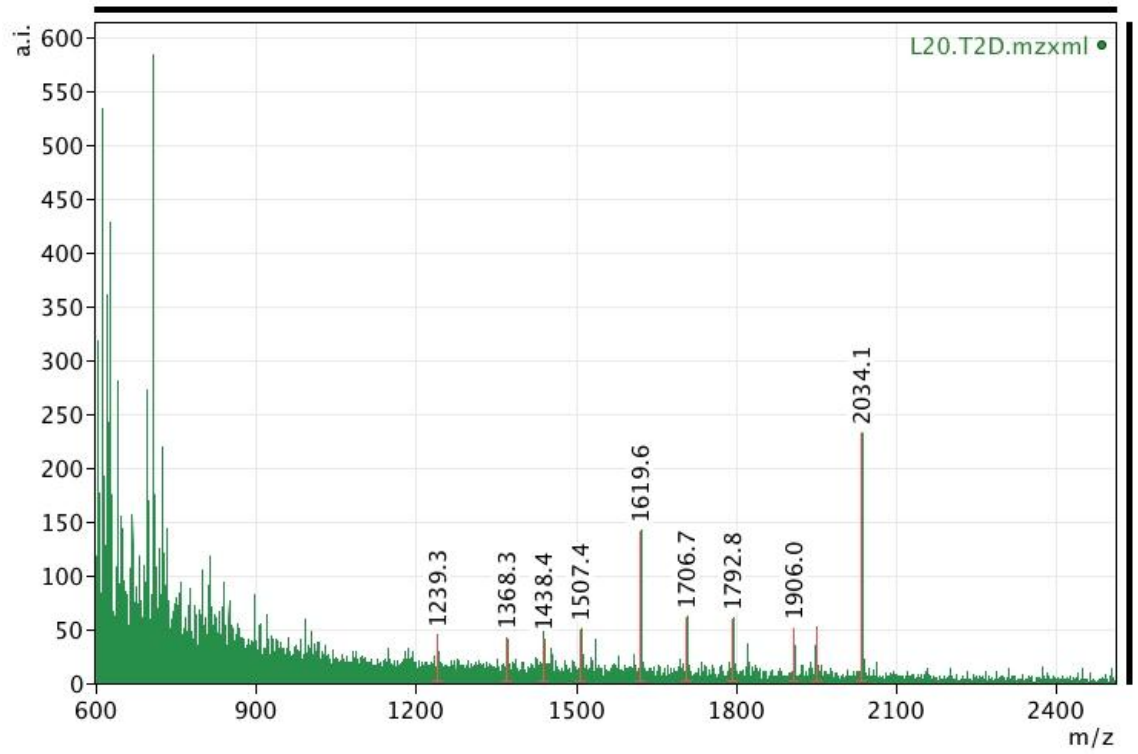

a.i.
L20.T2D.mzxml
600
550
500
450
400
350
300
250
200
150
100
50
0
1239.3
1368.3
1438.4
1507.4
1619.6
1706.7
1792.8
1906.0
2034.1
600
900
1200
1500
1800
2100
2400
m/z

Figure 7. The Mass Spectrum and the chemical structure of the Nffa capped C-1 peptide. A) The chemical structure of the Nffa capped C1 peptide. This structure was developed according to what would be the known sequence of the peptide. The presence of a peptide of this structure was verified by mass spectrometry as shown in Figure 7. B) The mass spectrum of the C1 peptide capped with Nffa. The spectrum was used to verify the presence of the correct structure of the peptide capped with Nffa. The 2034.1 peak indicated the mass of the peptide.

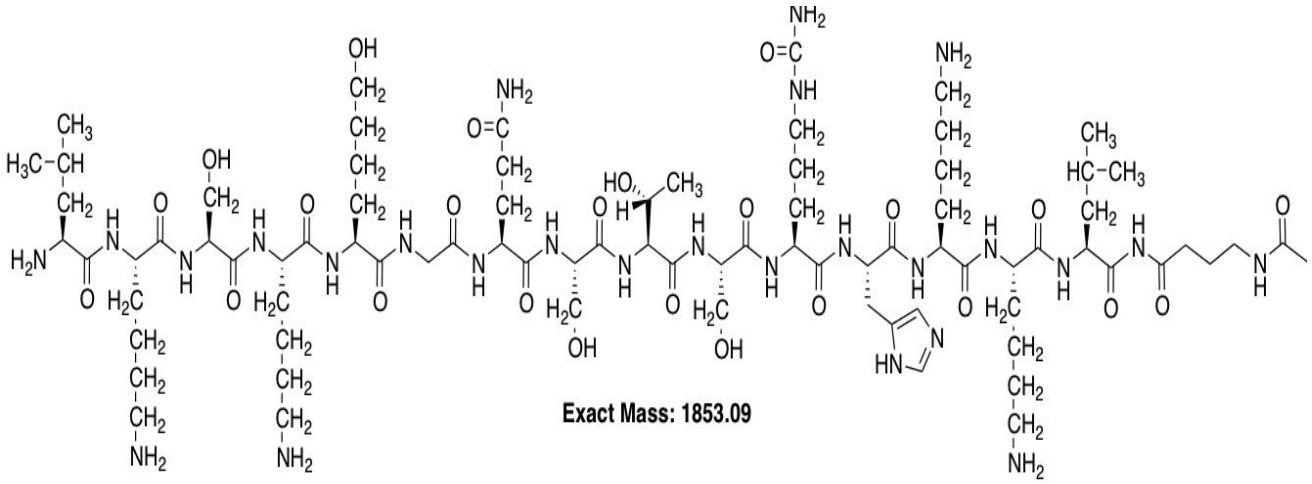

Exact Mass: 1853.09

Figure 8. The sequence of the Acetyl capped C-1 peptide. The acetyl cap ($COCH_3$) can be seen at the N terminus.

Mamba 49

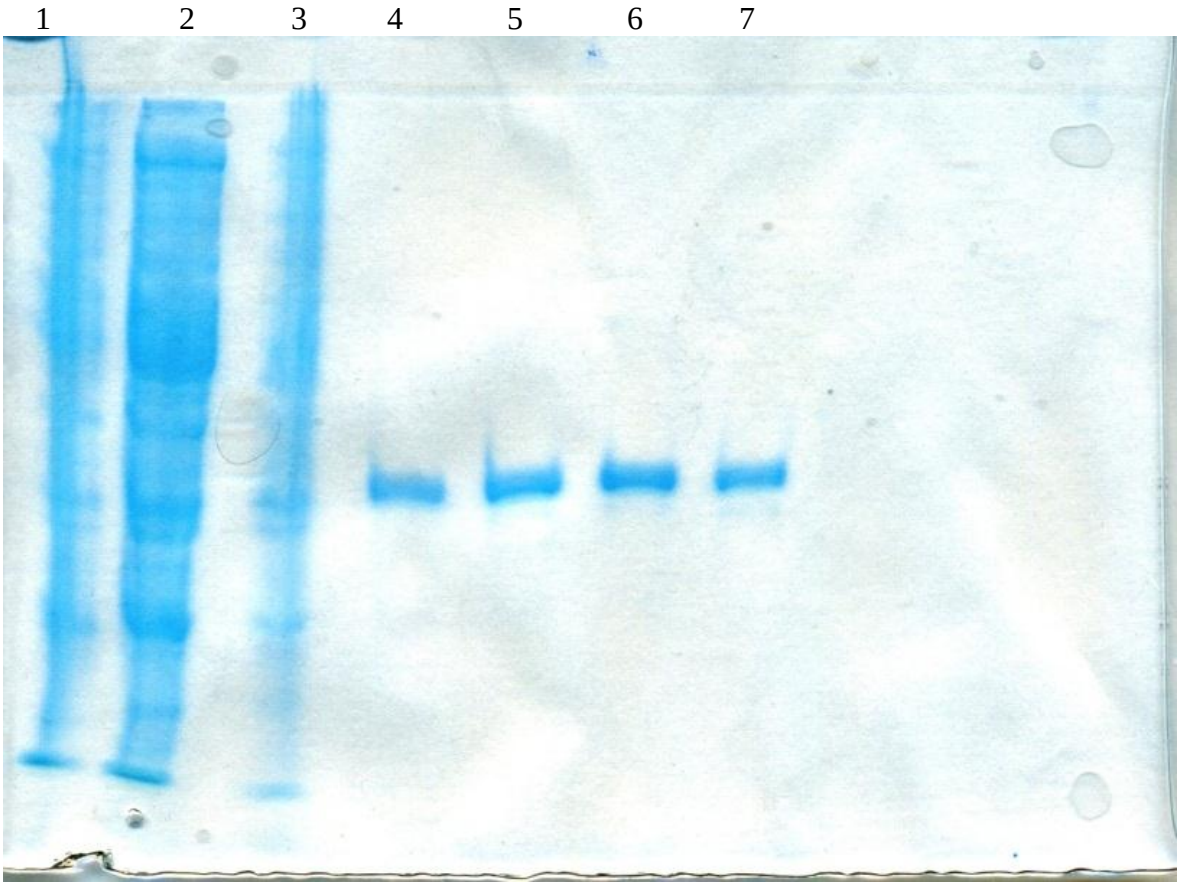


Figure 9. A SDS protein gel testing the purity of p53 at different steps of the purification process. This figure shows the protein gel testing the purity of p53 during the nickel column purification process. The four distinct bands were samples from the four fractions that were collected and the smeared bands were a sample of the total cell lysate before purification. Lane 1 is 20 µL of total cell lysate. Lane 2 is the clarified total soluble protein fraction. Lane 3 is 20 µL of the nickel affinity column flow through (unbound proteins). Lanes 4-7 are 20 µL of fractions 1-4 of purified protein that were collected.

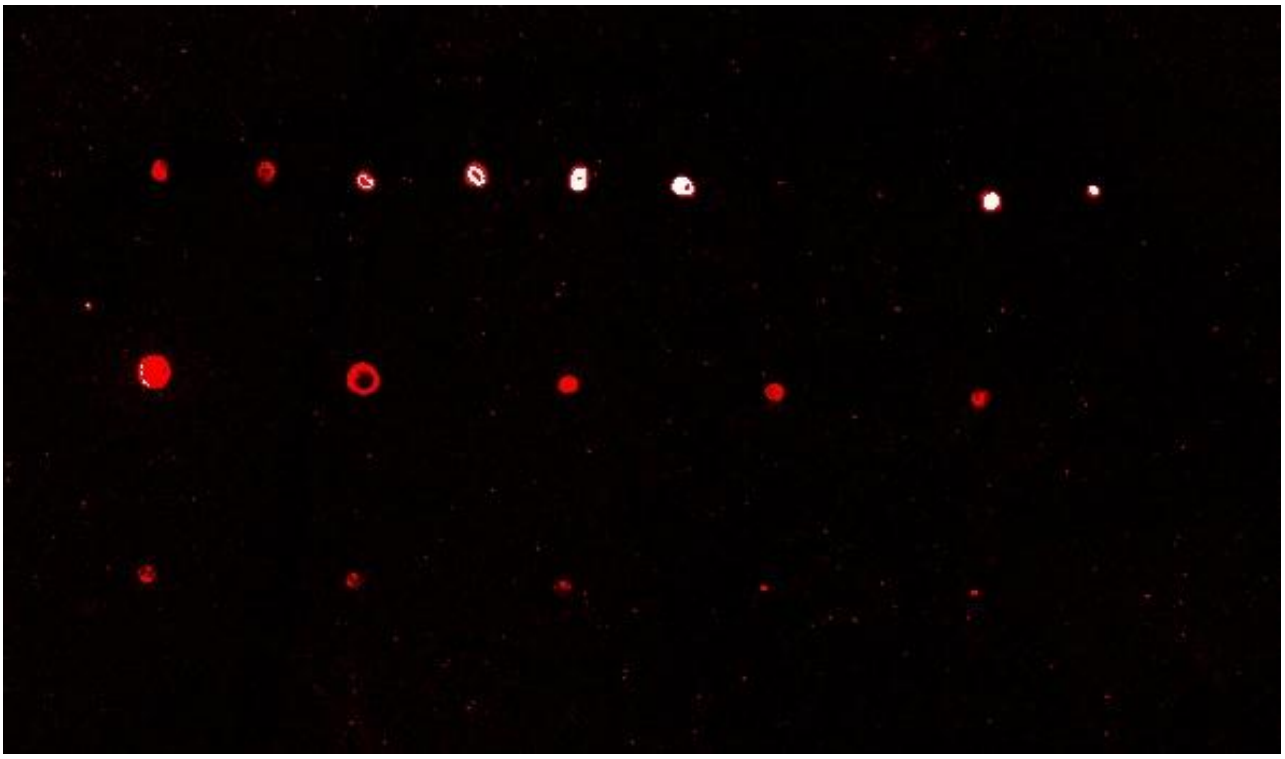


Figure 10. Microarray slide showing emitted light from binding of p53 to the C-1 peptide. This picture shows the microarray after probing with a single concentration of p53 protein. The different size of the C1 spots corresponds to different concentrations of the peptide. We made a 20mM stock of the C1 peptide, and then diluted it 5-fold. The p53 protein concentration was varied on different blocks of the slide. Since the spots on the second and third rows on the microarray are fluorescent, the C-1 peptide bound to p53.

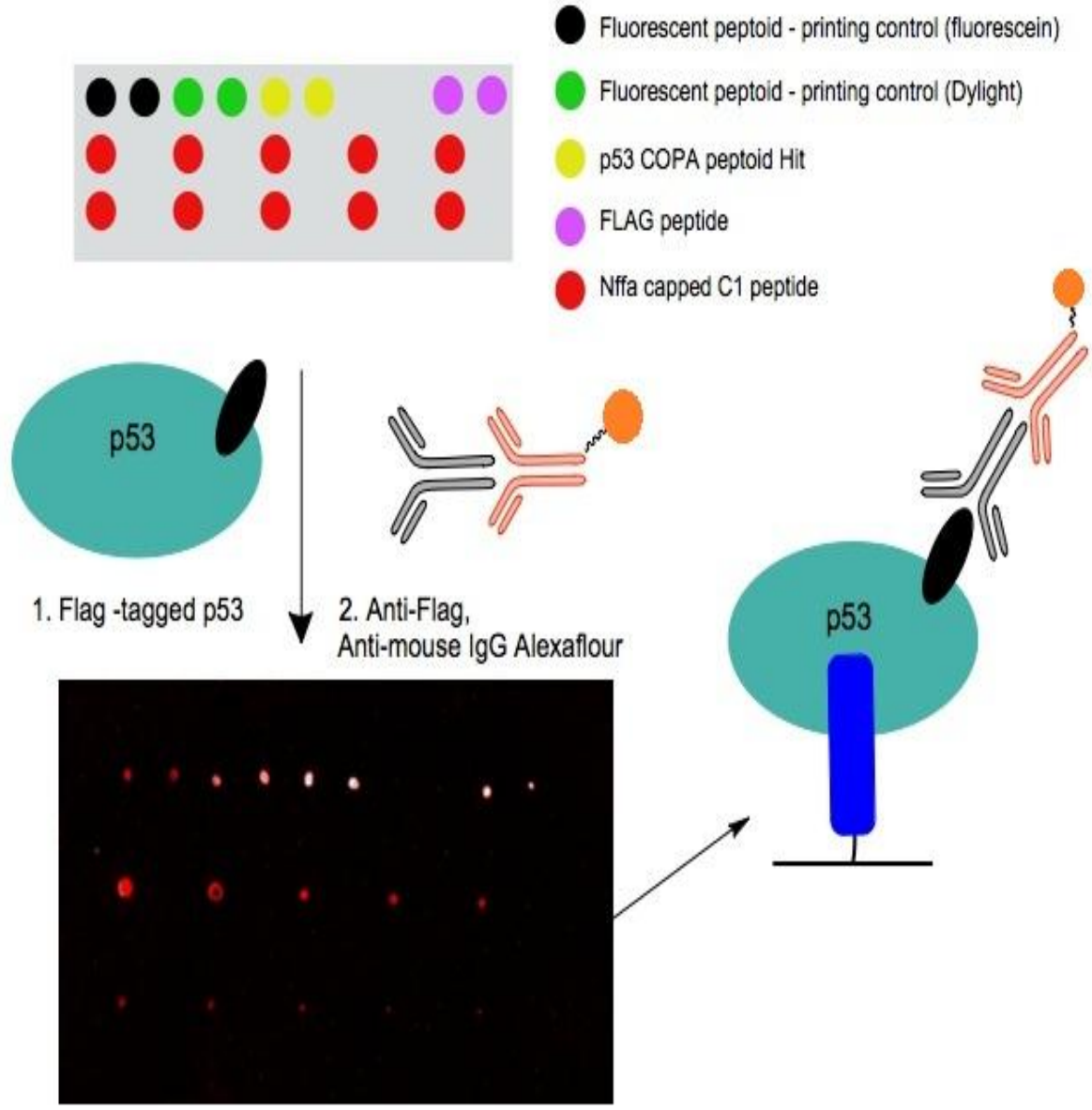

Fluorescent peptoid - printing control (fluorescein)
Fluorescent peptoid - printing control (Dylight)
p53 COPA peptoid Hit
FLAG peptide
Nffa capped C1 peptide
p53
1. Flag -tagged p53
2. Anti-Flag,
Anti-mouse IgG Alexaflour
p53

Figure 11.Labeled diagram of the controls used during this procedure and the data procured. The black spot controls show a positive signal indicating that the array scanner can detect the fluorescent tag on the secondary antibody. The green spots are also fluorescent printing controls. The green and black spots were both used to control for error in fluorescence of the peptoid. The yellow spots were a control for p53 effectivity; the p53 COPA peptoid hit was already shown to bind p53. The purple spots are a positive control because a flag peptide would bind to the primary anti-flag mouse antibody. The red spots represent a dilution series of the Nffa capped C1 peptide and they emitted decreasing amounts of fluorescent light as was expected. The blue rectangle is the Nffa capped C1 peptide that is bonded to the slide through the Nffa cap. The binding of p53 causes fluorescence because ultimately the secondary antibody is fluorescent (peach ball).

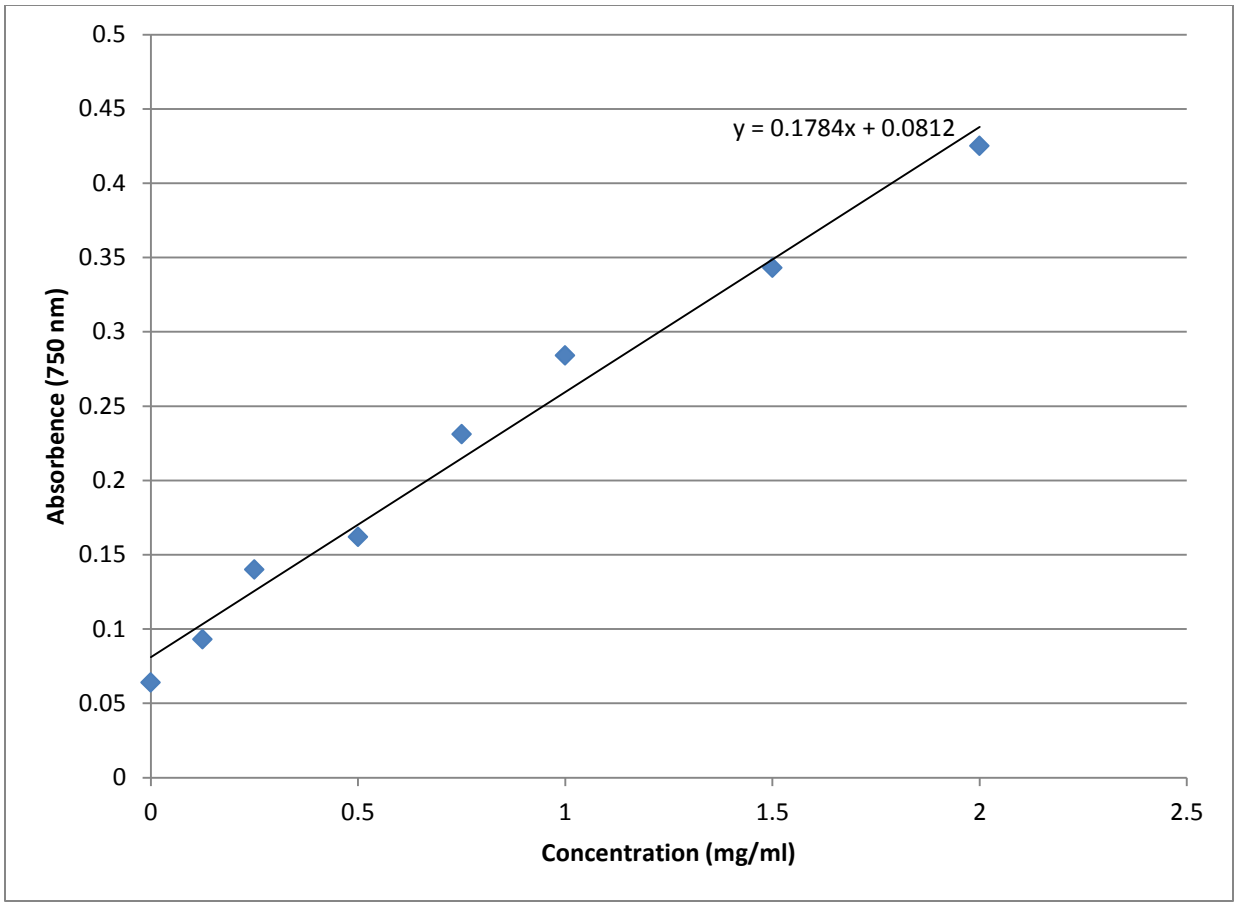

y = 0.1784x + 0.0812
Absorbence (750 nm)
Concentration (mg/ml)
0.5
0.45
0.4
0.35
0.3
0.25
0.2
0.15
0.1
0.05
0
0
0.5
1
1.5
2
2.5

Figure 12. The BSA Protein Assay that was used determined the concentration of purified p53. At 2.296 absorbance, the concentration of p53, according to the graph was 1.6 mg/ml. This concentration was too low so the p53 was concentrated again.

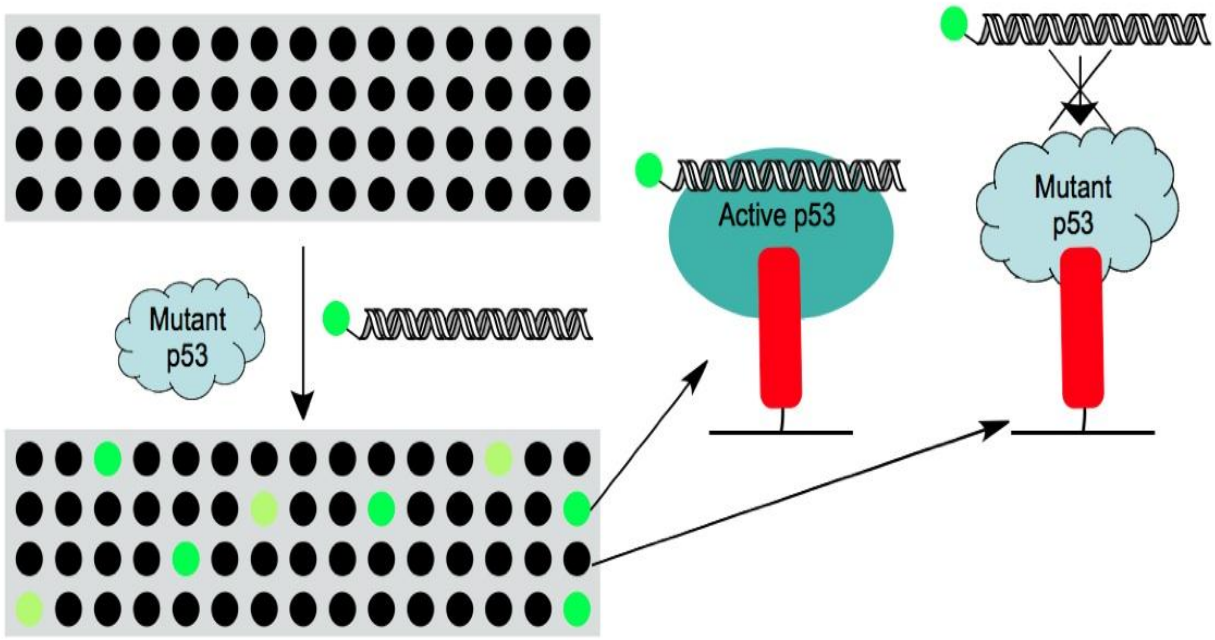

Mutant
p53
Active p53
Mutant
p53

Figure 13. Microarray DNA binding assay. A slide spotted with methylated versions of the C1 peptide (black) is treated with dysfunctional mutant p53 and fluorescently labeled DNA. If the peptoid (red bars) is capable of reactivating the mutant p53 (center), the fluorescent DNA will bind and light up the spot on the microarray (bottom). Peptoids incapable of binding DNA (right) will not fluoresce.

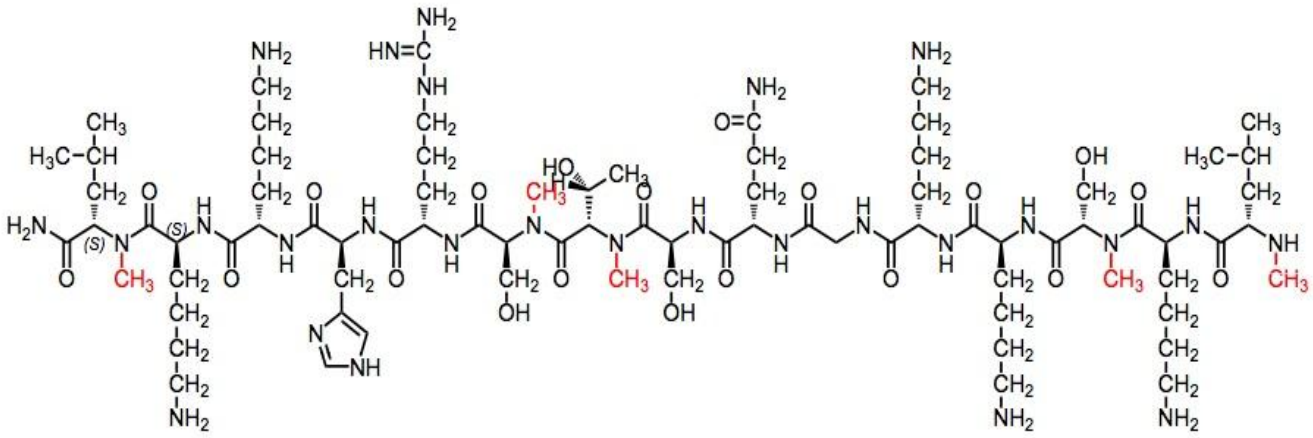


Figure 14. The most methyl substituted PTA of the C1 peptide (C-N terminus). This would be the structure of the C1 peptide that would be the most stable and most rigid of the PTA library. Methyl groups would be substituted for hydrogen bonds to decrease the polarity of the $-NH_2$ bond. The methyl groups would also decrease the flexibility of the structure of the peptide. If this structure still binds to p53, this could be an effective drug in the rescue of p53.