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# The role of alpha-helical structure in p53 peptides as a determinant for their mechanism of cell death: necrosis versus apoptosis

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**Abstract**

Peptides derived from the N-terminal and C-terminal regions of the p53 tumor suppressor protein, linked to the membrane transduction domain of Antennapedia, have both been found to have significant cytotoxic effects selectively in human cancer cells. However, the N-terminal and C-terminal p53 peptides apparently display very different mechanisms for their anticancer effects. These differential effects can be attributed to dissimilar abilities to form distinctive 3-dimensional structures in extracellular-matrix-like aqueous solution that enable unique and selective cancer cell membrane penetration and effect. N-terminally based p53 peptides, with their ability to form distinctive S-shaped helix–loop–helix structures, are able to rapidly disrupt cancer cell membranes via toroidal-like pore formation causing necrosis; conversely, C-terminally based p53 peptides, due to their more random coil configuration, can be transduced across cancer cell membranes and bind to its intracellular target to cause a Fas pathway mechanism of apoptosis.


*Keywords:* p53; Necrosis; Apoptosis; Peptides; NMR solution structure; Alpha helical

**Contents**



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## 1. Introduction

The use of peptides in cancer therapeutics has recently become popular because of their potency, specificity, low toxicity and limitations of viral vector gene therapy approaches [1]. An important requirement in the use of peptide-therapy is the ability of these molecules to be efficiently transduced across the cancer cell membrane. In general, cellular plasma membranes are largely impermeable to proteins and peptides. Nonetheless, it was discovered that certain short peptide sequences, composed mostly of basic, positively charged amino acids (e.g. Arg, Lys and His), have the ability not only to transport themselves across cell membranes, but also to carry attached molecules (proteins, DNA, or even large metallic beads) into cells [2]. These basic sequences, mostly derived from DNA binding proteins are now commonly known as protein transduction domains (PTD) and have been successfully employed to transport cargo proteins across a variety of cell membranes [3]. It has been theorized that the ionic interaction between positively charged Arg residues of these PTDs and the negatively charged phosphate head group of the membrane lipid bilayer plays a key role in PTD membrane interaction [4]. However, the exact mechanism by which these PTDs operate is still largely unknown.

Due to its central role in tumor genesis, the p53 protein has long been a target for cancer therapeutics [5]. Our group has successfully used these PTDs linked to short peptide sequences derived from p53 end regions to selectively kill cancer cells. We have observed that a p53-based synthetic peptide (derived from the C-terminal residues 361–380 of p53) C-terminally linked to a 17 amino acid PTD from the *Drosophila* homeobox sequence of Antennapedia (Ant) (Cp53Ant-37) induced p53-dependent, Fas-mediated apoptosis in breast cancer cells containing either mutated p53 or overexpressed wild-type p53. However, Cp53Ant-37 was non-toxic to normal human breast cell lines and human marrow derived stem cells (CD34+) over a time course of 3–6 h [6]. Furthermore, our group has observed that another p53 based synthetic peptide (Np53Ant-32) derived from the N-terminal residues (12–26) of p53 C-terminally linked to the same 17 amino acid PTD sequence induced very rapid p53-independent necrosis in pancreatic cancer cells [7] and breast cancer cells [8], while being non-toxic to both normal cell types over a time course of 15 min.

It appears that the dramatic variation in the time course and type of cancer specific cell death effect between the Cp53Ant-37 and Np53Ant-32 sequences can be attributed, at least in part to differences in their mechanism of interaction with the cancer cell membrane. As an initial attempt to understand the mechanism by which these differential cancer cell membrane effects were mediated, we examined how changes in amino acid sequence of Cp53Ant-37 would affect peptide transduction, or as we referred to it as "efficiency as a transporter" [9]. Using sequence analysis, we observed a direct correlation between hydrophobic peptide characteristics (hydrophobic moment and helical wheel profile) and efficiency as a transporter. More specifically, we observed that when the PTD domain was changed from a 17-residue Ant to a 10-residue TAT, or when the Ant or TAT were placed on the N-terminal as opposed to the C-terminal end, the efficiency of transport and corresponding anticancer activity was decreased. On the basis of these results, we proposed that the use of biophysical parameters such as hydrophobic moment and helical wheel analyses may be useful predictive tools for choosing the best carrier arrangement for peptide therapies [9].

The next step to understanding how these peptides could display such dramatic differences in time course

and mechanism of cell death was to analyze actual peptide structures in physiologic environments initially using the Np53Ant-32 peptide [8]. Using bioinformatic and biophysical spectroscopic methods, we observed that the Np53Ant-32 peptide contained alpha-helical secondary structure consistent with membrane disruption capability. Moreover, using electron microscopy imaging, we found that this peptide caused rapid necrosis through the formation of uniform pores along the cancer cell membrane [8].

To more closely analyze structural effects, in relation to possible membrane interaction mechanisms, we used two-dimensional solution Nuclear Magnetic Resonance (NMR) spectroscopy, to determine actual peptide structure in two solution environments [10]. The first environmental type simulated an extra-cellular matrix in which the peptide would experience before it interacts with the cancer cell membrane, consisting of a buffered aqueous solution (AqS). The second type simulated the milieu the peptide would experience inside the membrane after penetration, consisting of an organic (mixed-solvent phase) membrane-mimetic solution (MmS). It was observed that in the AqS environment, the Np53Ant-32 peptide contained alpha-helical domains connected by loop structures, forming an S-shaped peptide backbone. In the membrane-mimetic MmS milieu, the helical domains found in AqS increased in length, all of which formed a U-shaped helix–coil–helix configuration. In both the AqS and MmS environments, the Np53Ant-32 peptide formed amphipathic structures with hydrophobic residues residing on one face while the polar residues aggregated on the opposite face [10]. We proposed that these alpha-helical structures could cause the formation of the pores we observed in cancer cell membranes by electron microscopy.

As the next step in understanding how peptide structure affects the cancer cell membrane interaction mechanism, we have now determined the AqS three-dimensional structure of the Cp53Ant-37 peptide, using the same two-dimensional NMR spectroscopic techniques as done above [10]. We can now compare the three-dimensional structures of the 2 p53 peptides in an aqueous extracellular-matrix like solution containing the same C-terminally linked PTD (Ant) sequence, allowing us to speculate on the role of these different sequences in contributing to dramatically different three-dimensional structures, and more importantly cause very different anticancer effects, namely either apoptotic or necrotic cancer cell death.

## 2. Methods

### 2.1. Peptides

Both the Cp53Ant-37 and Np53Ant-32 sequences were synthesized using solid phase methods and were purified by HPLC to >95% purity (Research Genetics, Huntsville, AL).

### 2.2. NMR structure determination

Both peptides were dissolved at a concentration of 4.0 mM in 10 mM phosphate buffer, pH 5.7 containing 5% DMSO-$d_6$. NMR experiments were carried out on a four-channel Varian $^{UNITY}$INOVA 600 MHz spectrometer (Varian, Palo Alto, CA) equipped with a triple resonance probe and *z*-axis pulsed field gradients (Dept. of Chemistry, College of Staten Island, Staten Island, NY). Experiments were collected between 10 °C and 37 °C. Only the 37 °C results are presented here. Proton assignments were accomplished with *To*tal *C*orrelated *S*pectroscop*y* (TOCSY) with a mixing time of 70 ms, *N*uclear *O*verhauser *E*ffect *S*pectroscop*y* (NOESY) (mixing times, 100 ms and 300 ms), and *D*ouble *Q*uantum *F*iltered *Co*rrelated *S*pectroscop*y* (DQFCOSY) experiments [11].

The assignments were assisted and confirmed by natural abundance using *H*eteronuclear *S*ingle *Q*uantum *C*oherence (HSQC) with $^{13}$C, $^{1}$H-HSQC and $^{13}$C-HSQC–TOCSY spectroscopies. Conventional phase-cycling was used instead of gradient-selection to avoid sensitivity loss. The WET scheme was used to suppress the water signal [12] in all experiments. The standard deuterium lock solvent of 5% DMSO-$d_6$ was used for all spectroscopic experiments.

NMR data were processed with the NMRPIPE [13] program and analyzed with the NMRVIEW [14] program. Unique NOE distance constraints were calibrated with the medium intensity at 2.7 Å for both peptide sequences. Two hundred structures were calculated for each of the 2 peptide sequences

using the DYANA [15] program, and the 4 best structures were selected for the display for both the Np53Ant-32 and Cp53Ant-37 sequences. Overall *r*oot *m*ean *s*quare *d*eviation (RMSD) between the calculated structures was 5–6 Å for the Np53Ant-32 and 10–15 Å for the Cp53Ant-37 sequence. The larger RMSDs for the Cp53Ant-37 peptide reflect its random and more fluctuating structure. Computer graphic rendering of NMR Structures was done using MolMol [16].

## 3. Results

### 3.1. Table of peptide sequences

Amino acid sequence of Cp53Ant-37 and Np53Ant-32 peptides (p53 regions underlined and numbered).

**Cp53Ant-37**

361 380

GSRAHSSHLKSKKGQSTSRHKKWKMRRNQFWVKVQRG

**Np53Ant-32**

12 26

PPLSQETFSDLWKLLKKWKMRRNQFWVKVQRG

## 4. Discussion

The Np53Ant-32 peptide exhibits definitive structures in an aqueous extracellular-like environment, as shown in Figs. 1 and 2 and shares much similarity in structure to antimicrobial peptides, and therefore may share similar mechanisms of membrane disruption. The resulting ionic surface area depiction (Fig. 3) shows a very compact positively charged shape, able to disrupt membrane surfaces.

The Np53Ant-32 peptide induced rapid p53-independent necrosis in a variety of cancer cells in our studies [7,8]. Over a time course of 15 min, we observed that all cancer cells in culture were killed, with uniform pore formation (observed with electron microscopy) occurring within 5 min. We speculate that this rapid phenomenon is largely initiated by the unique three dimensional structure depicted in Figs. 1-3 in the aqueous extracellular matrix. The S-shaped α-helical structure in Fig. 1 and its ionic surface area depiction

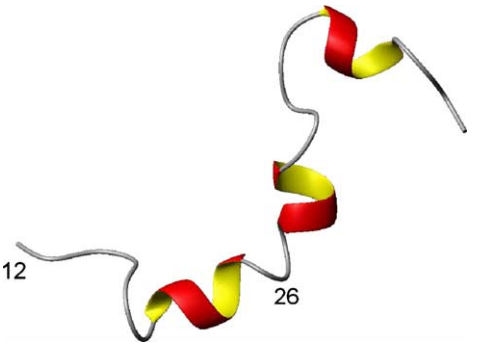


Fig. 2. Previously acquired Np53Ant-32 peptide NMR solution structure, depicting one of the best four, from Fig. 1, shown for clarity.

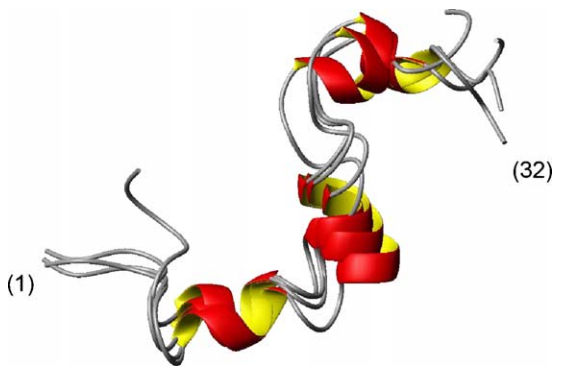


Fig. 1. Previously acquired Np53Ant-32 peptide NMR solution structures, depicting the best four calculated forms in AqS.

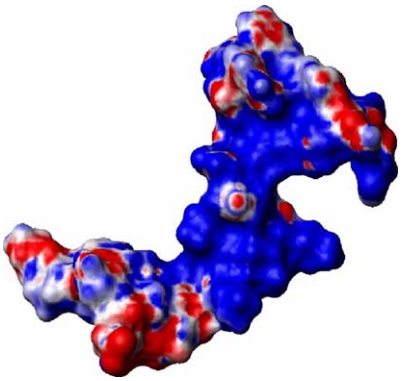

Fig. 3. Ionic Surface Area Depiction of the Np53Ant-32 peptide. Blue (Positive), Grey (Neutral), and Red (Negative).

(Fig. 3) closely resembles those categorized as α-helical antimicrobial peptide forms [17]. The presence of prolines at the $NH_2$ ends of Np53Ant-32 peptide may stabilize its α-helical structure thus promoting membrane interaction, by stabilizing inter-membrane helix packing, as seen recently in the studies of transmembrane proteins [18].

In general, most known α-helical antimicrobial peptides exhibit similar structural characteristics with the Np53Ant-32 peptide. An example of this group known as magainins is so efficient as antimicrobials that synthetic forms are being pharmaceutically developed [19]. These structures are known to interact with membranes by introducing so-called "positive curvature strain" on the lipid bilayer which induces the formation of toroidal pores [20], opening up the continuous bilayer. The peptide, once inserted into this toroidal pore complex, further stabilizes the porous structure by intercalating its basic, positively charged amino acids among the negatively charged phospholipid head groups along the inside pore wall. It is also known that these α-helical structures undergo a conformational phase transition, whereby the membrane binding process induces increased structure formation [21]. The Np53Ant-32 peptide undergoes such transition by increasing its α-helical content in a membrane-mimetic environment [10].

Np53Ant-32 peptide selectivity for cancer cells can be analogous to antimicrobial peptide selectivity for pathogen membranes. It is known that antimicrobial selectivity for the pathogen membrane is thought to occur because of differential phospholipid content type [17] and sterol content [22] between host and pathogen membranes. Moreover, it was observed that a peptide with the PTD (Ant) domain sequence alone exhibited differential abilities to insert into artificial lipid vesicles [23]. We suggest that given the similarity in residue content and three dimensional structure of the Np53Ant-32 peptide, similar mechanisms may cause our observed effect on cancer cells. It is well documented that cancer cells have major differences in phospholipid, cholesterol and protein content of their membranes as compared to normal cells. More importantly, the ability to have definitive α-helical structures in AqS enables this peptide to produce rapid and selective membranolytic properties.

When compared to the Np53Ant-32 sequence, the Cp53Ant-37 peptide exhibits little structure in an aqueous extracellular-like environment, as shown in Figs. 4 and 5. The resulting ionic surface area depiction (Fig. 6) shows a stretched out string-like form containing positively charged areas interspersed with neutral and negatively charged regions. The only indication of more distinctive structure is a discrete loop conformation with Lys-372 at the apex of the bend (Fig. 5). Preliminary results suggest that this structure changes negligibly in a membrane-mimetic environment by $^1$H-NMR studies.

The overall random coil structure (Fig. 5) of the Cp53Ant-37 peptide may allow it to function as a molecular crutch to overcome the inactive mutant p53 structure and cause apoptosis. Apoptosis is a complex-intertwined system of protein–protein interactive pathways that regulate cell death, of which p53 plays a major role. We have observed that the Cp53Ant-37 peptide induced a p53 dependent, Fas-FADD/APO-1 mediated apoptosis through interaction with the $NH_2$ terminus of FADD [6]. The peptide is known to bind to mutant p53 and reactivate its DNA binding ability and transcriptional activity, but new transcription and translation are not necessary for the induction of apoptosis [6]. This suggests that this peptide may also bind directly to the FAS-FADD/APO-1 complex, normally inactivated when p53 is mutated, to cause apoptosis. Recent NMR structural analysis of the bromodomain of the coactivator CREB Binding Protein (CBP) with the p53 C-terminal binding domain revealed a

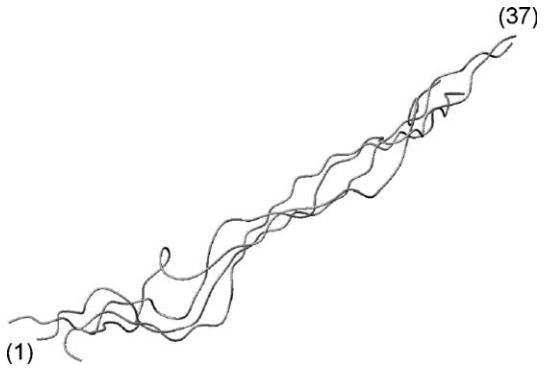


Fig. 4. Newly acquired Cp53Ant-37 peptide NMR solution structures, depicting the best four calculated forms in AqS.

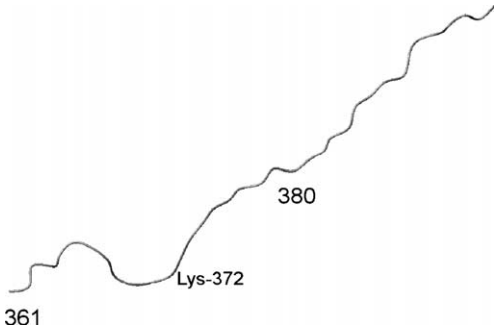


Fig. 5. Newly acquired Cp53Ant-37 peptide NMR solution structures, depicting one of the best four, from Fig. 4, shown for clarity. Note the curved loop region from residues 361–372, with the Lysine 372 labeled.

structure complex, where a peptide containing the same sequence as Cp53Ant-37 peptide, bound with an acetylated lysine conforming to the same curve-like structure as depicted in Fig. 5, with lysine 372 as the analogous acetylated bound form in the NMR structure [24]. This suggests that the flexible random coil configuration of Cp53Ant-37 peptide, with a loop around Lys-372 may allow for such complexes to form with other proteins such as FAS-FADD/APO-1, thus possibly contributing to the apoptosis we observed in our studies [6,9].

The structural differences between the Np53Ant-32 and Cp53Ant-37 peptides account for their differential effects. The less ordered structure of Cp53Ant-37 peptide gives it negligible membranolytic capability, thus allowing membrane penetration but not disruption. When compared to the Np53Ant-32 sequence, this peptide induced apoptotic cell death presumably through protein–protein interactions which triggered programmed cell death and not rapid membrane disruptive necrosis. From a sequence-structure effect comparison, it seems that when the Ant is C-terminally attached to the 15 residue p53 N-terminal region, it displays definitive regions of α-helical structure, but when attached to the 20 residue p53 C-terminal region, it induces a random coil configuration on the whole sequence. The possible inherent α-helical structure of the cargo sequence could modulate the Ant sequence ability to either form or not form α-helices. We are currently investigating these possible structural effects of the two different cargo sequences on Ant peptide structure using NMR by determining the individual solution structures of the Ant sequence and both the 15 residue p53 N-terminal sequence and 20 residue p53 C-terminal sequences alone.

The presence or lack of initial structure in aqueous solution between these two peptides invokes differences in membrane interactive capability. One insight into possible causes behind these differences comes from known anti-microbial peptide behavior. It seems that selective pathogen membranolytic activity among small cyclic peptides is directly correlated with their ability to have initial α-helical structure and to form more α-helical structure (conformational dynamics) in non-aqueous membrane-mimetic environments [25]. It has been observed that slight increases in peptide cyclic structure increase membranolytic selectivity against pathogens by 30 fold. Furthermore, it has recently been observed in small vasoactive intestinal peptides that decreasing α-helical content decreases membrane affinity for murine stomach membranes by 60 fold [26]. We suggest similar mechanisms for the vastly different behavior between the Np53Ant-32 and Cp53Ant-37 peptides. Although both peptides contain the same PTD (Ant) carrier sequence, the Np53Ant-32 peptide with its inherent ability to form α-helical structure in aqueous and organic solution enables it to have rapid and selective membranolytic effects and induce necrosis; alternatively, the Cp53Ant-37 peptide with its random coil configuration merely penetrates cancer cells and once inside the cell can adopt an induced fit interaction with binding sites of target effector proteins to cause selective apoptosis. This may allow a mutant p53 conformation

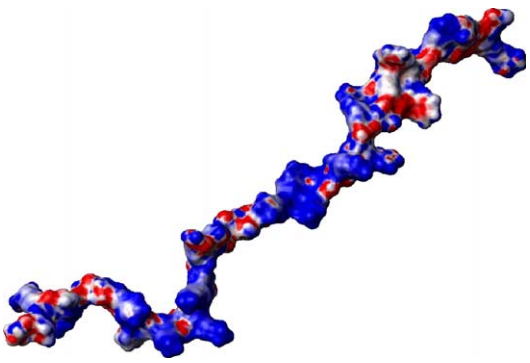

Fig. 6. Ionic Surface Area Depiction of the Cp53Ant-37 peptide. Blue (Positive), Grey (Neutral), and Red (Negative).

to undergo a gain of function conformation whereby it can now activate the Fas-FADD apoptotic pathway.

Interestingly, the use of both peptides together could represent a novel therapeutic regimen against cancer cells that could be developed into an innovative approach to drug resistance. If tumor cells treated with the apoptotic inducing Cp53Ant-37 peptide become resistant through loss of Fas-FADD components or its efficiency, then the subsequent use of the Np53Ant-32 peptide could be used for selective necrosis, allowing for completion of cell death. Scenarios such as this could be envisioned and developed where both peptides are used in "combination therapies", taking advantage of the very unique and selective cytotoxic capabilities of both peptide structures.

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