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COMPUTATIONAL PROTEIN CHEMISTRY OF P53 AND P53 PEPTIDES

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TABLE OF CONTENTS

1. ABSTRACT

Computational protein chemistry has potential to contribute to the development of new therapeutic approaches in medicine in several different ways, including indirectly by increasing understanding of the disease-associated changes in protein structure that are mechanistically important, which can have diagnostic implications, as well as directly in designing peptides to counteract the patho-physiologic effects of these changes. Studies of the role of the tumor suppressor protein p53 in the carcinogenic process provide examples of both types of contribution. Computational studies of the effects of mutations in p53 on its structure have provided insights into cancer mechanisms and have served to elucidate potential new diagnostic approaches based on the identification of changes in p53 structure. Computational studies of p53 peptides have contributed to identifying and optimizing the structural characteristics that contribute to their activity in selectively killing cancer cells.

2. INTRODUCTION

The p53 tumor suppressor protein functions in the transcription of growth inhibiting genes, apoptosis, cell cycle arrest and DNA repair (1, 2). Known as the "guardian of the genome", p53 regulates growth and division of cells that have sustained DNA damage in order to prevent uncontrolled cell proliferation, leading to cancer (1, 2). Thus, it is not surprising that over 50% of human cancers lack functional p53 because of mutation, deletion or inactivation (1, 2). Since p53 is vital to controlling cell growth and because it is so frequently altered in human cancers, understanding the relationship between alterations in its structure and its function are critical to understanding carcinogenesis. Furthermore, these features also make p53 an ideal target for designing novel interventions for cancer treatment and prevention, particularly those based on structurally specific peptides that can mimic or reactivate p53. Computational protein chemistry therefore can have an important role to play in understanding the contribution of p53 alterations in cancer and in the development of specific therapies to correct these defects.

The human p53 gene lies on chromosome 17p and encodes a nuclear, DNA-binding phosphoprotein of 393 amino acid residues (3, 4). P53 consists of several protein domains including an N-terminal transactivation domain (amino acid residues 1-42); a central sequence-specific DNA-binding domain (amino acid residues 90-295); and C-terminal domains for tetramerization (amino acid residues 323-355; p53 functions in cells as a tetramer), nuclear localization (amino acid residues 316-325) and negative regulatory control (amino acid residues 80-93 and 368-393) (3, 4).

The N-terminal transactivation domain contains many acidic residues and one of the five highly evolutionarily conserved sequences in p53 (amino acid residues 12-20) which is required for its transcriptional activation function (3, 4). The N-terminal domain also contains binding sites for several other cellular and viral proteins (TBP, hTAFII31, hTAFII70, p62, RP-A, CBP/p300, mdm-2, E1B55, E6 and Hbx) (3, 4). Some of these, such as the cellular protein mdm-2 and the adenoviral protein E1B55, can inhibit the transcriptional activation function of p53 (3, 4). Mdm-2 binding is also known to promote ubiquination and rapid degradation of p53, probably an important normal control mechanism for regulating the level and hence activity of p53 in cells (3, 4). The N-terminal domain also contains numerous phosphorylation sites which can interact with several protein kinases, and this may contribute to modulating the various functions of p53 (3, 4). The central domain interacts directly with DNA by binding to specific consensus sequences (one common one being 5'-

PuPuPuC(A/T(T/A)GPyPyPy-3 repeats separated by 0-13 bp) through several contact residues (K120, S241, R248, R273, A276, C277 and R280) (3, 4). The central domain also contains the other four highly evolutionarily conserved sequences in p53 (amino acid residues 117-142, 171-181, 234-258 and 270-286), binding sites for some cellular and viral proteins (53BP1, 53BP2 and SV40T), and a limited number of phosphorylation sites (3, 4). In addition, the central domain contains four metal-binding residues (C176, H179, C238 and C242) which interact with a zinc atom, and it is apparently necessary to maintain the normal structure and sequence-specific DNA-binding activity of the domain (3, 4). As noted, the C-terminus contains domains for tetramerization, nuclear localization and negative regulation, which contains many basic residues, as well as binding sites for cellular and viral proteins (TBP, XPB, XPD, CSB, S100b, Hbx, BZLF1, E4orf6 and 1E2) and several phosphorylation sites (3, 4).

For the purposes of this review, the focus will be on computational contributions to the study of the effects of mutations on the structure of the central DNA-binding domain and the development of p53 peptide-based therapeutic approaches from the N-terminal mdm-2 binding region and the extreme C-terminal negative regulatory region.

3. EFFECTS OF MUTATIONS ON P53 STRUCTURE

3.1. Computed conformational changes in mutant p53

More than a thousand mutations in p53 have been identified in human tumors (3, 4). The vast majority of these mutations (approximately 74%) are missense, which result in the expression of full-length p53 with single amino acid substitutions at selected sites (3, 4). These missense mutations are not randomly distributed throughout the p53 sequence, but rather tend to cluster at certain sites. The vast majority of these mutations (75-80%) are localized to the central DNA-binding domain with particular clustering within four “hot-spot” regions that coincide with the four regions in this domain that have been highly conserved throughout evolution (3, 4). The amino acid residues in these regions are thus presumed to be critical to maintaining the normal structure and function of p53. In some cases, these mutations not only abrogate the function of the mutant p53 molecule but also confer a dominant negative ability on the mutant molecule to interfere with the function of normal, wild-type p53 molecules (3, 4). It would therefore be of interest to be able to determine how mutations in p53 change its structure and whether all of these mutations that have been found in p53 in human tumors produce any common conformational effects. Current structure determination methods such as nuclear magnetic resonance (NMR) spectroscopy and X-ray crystallography currently have instrumental and sample methodology limitations that prevent the structure determination of the whole p53 molecule, so it is unlikely that structures for a large number of p53 mutants will be available in the near future. Computational protein chemistry approaches may be able to provide an answer.

In many ways, p53 represents a good model to examine the uses of computational approaches to answer protein chemistry questions because, as noted, many different mutations at many different sites have been identified, the mutations are largely confined to one discrete domain, and an x-ray crystal structure for this domain in the wild-type protein has been determined (5), which provides a well-defined starting point for computational approaches. Therefore, over the past several years, we have used computational approaches to examine the conformational effects of a number of mutations on the structure of the central DNA-binding domain of p53 (6-9).

Briefly, the basic computational approach employed in these studies was conformational energy analysis and molecular dynamics simulations. As noted, the starting structure was the x-ray crystal structure for the wild-type p53 protein from amino acid residues 94 to 312, including the tetrahedrally coordinated zinc atom, which was subjected to energy minimization and molecular dynamic simulations, as described below; the total conformational energy for the wild-type protein converged to a minimum value, and the last 50 isoenergetic structures on the trajectory were employed in computing the coordinate fluctuations (all less than 2.1 A) and the final average structure (6). For each mutation examined, the substituted amino acid residue was introduced into this wild-type average structure in place of the normally occurring amino acid residue at that position. The initial backbone dihedral angles for the substituted residue were the same as for the corresponding amino acid residue in the wild-type structure. Starting side chain conformations for each substituted residue were the lowest energy conformation ones for the given backbone conformation as determined by the Empirical Conformational Energy for Peptides Program (ECEPP) for the single residue minima for these amino acids (10). The structures were then subjected to nested energy minimizations and molecular dynamics simulations (6). In the first stage, only the substituted amino acid was allowed to move under energy minimization. In the second stage, to determine the lowest energy conformation for the side chain of the substituted amino acid, the side chain was subjected to a 50 psec cycle of molecular dynamics. In the third stage, the substituted amino acid and five neighboring amino acids on its amino and carboxyl terminal ends were allowed to move under energy minimization, since it is known that these residues most strongly influence the conformation of a given residue (11). In the fourth stage, energy minimization was performed in which the entire structure was allowed to move. For the molecular dynamics simulations, initial velocities were assigned randomly to each atom from a Maxwell-Boltzmann distribution characteristic of the target temperature of 300^{o}K, and the temperature of the system was raised from 0 to 300^{o}K in 25 degree increments every 5 psec. After equilibration, the simulations were performed until the total conformational energy converged to a minimum value for each structure using time steps of 1.0 fsec (200,000-600,000 steps or 200-600 psec). Continual monitoring of the RMS deviation from the starting structure and the energy of the lowest energy structure is performed so simulations can be stopped

when these parameters start to change rapidly in a reverse direction indicating that the structure is moving up and out of its minimum energy well. As above, the last 50 isoenergetic structures on the molecular dynamics trajectory were employed in computing the residue coordinate fluctuations and the average structure for each mutant, and the overall and individual residue RMS deviations between each mutant and the wild-type structure were determined in such a fashion that the RMS deviation of the coordinates of the backbone atoms of one average structure from the other is a minimum. These procedures rely on the Assisted Model Building with Energy Refinement (AMBER) force field, a united atom approach in which explicit hydrogen atoms are present only on polar groups, a distance-dependent energy cut-off for all long-range non-bonded interactions, and a distance-dependent dielectric constant of the form $D_0(1+r_{ij})$ to simulate the effects of solvation (6).

This approach has been applied to determining the conformation of the central DNA-binding domain of eleven p53 mutants (R175H, H179L, G245D, G245N, R248W, R249W, R249M, R249S, I255F, P278S and E286K) that occur in three of the conserved "hot-spot" regions. Coordinate fluctuations for all residues in each mutant were less than 2.1 A (and most were less than 1.5 A), indicating that the proteins converge on a specific structure that can be represented by the average structure in each case (6-9). The overall RMS deviations for the average structure of each mutant from the average structure of the wild-type protein were generally considerable, ranging from 3.4 to 6.8 A (mean=5.6 A) (6-9). On the other hand, the overall RMS deviations for the average structure of each mutant from every other mutant was generally less, ranging from 3.1 to 4.6 A (mean=4.0 A) (6-9). The individual residue RMS deviations for the average structure of each mutant compared to the wild-type protein demonstrate large deviations at discrete regions throughout the mutant proteins, some of which are remarkably similar from mutant to mutant. Relatively large (> 5A) RMS deviations from the wild-type structure can be seen in all mutants in the regions of amino acid residues 94-117, 175-185 and 282-294 (6-9). Regions of relatively large deviation that occur in most mutants, but not all, are amino acid residues 204-217 and 239-294 (6-9). In some cases, the sites of the mutations were near or within regions of large deviation, but in many cases, these regions were quite distant from the sites of mutation, indicating that common conformational changes can be produced by amino acid substitutions at diverse locations within the same protein structure. It is also of interest to note that removal of the zinc atom from the wild-type structure also results in major conformational changes that are most closely related to those observed in the H179L mutant (8); this is understandable since His 179, as noted, is one of the zinc-binding residues in the wild-type protein.

Finally, the effect of explicit solvation on two of these mutant structures has been examined, since it is known that in certain cases aqueous protein interactions can influence protein conformation. For the R249W and the R249S mutants, the structures were placed in an equilibrated three-dimensional shell of water molecules such that water molecules were evenly distributed in at least three layers around the protein, and the computations were repeated (7). The results were the same as those computed using the distance-dependent dielectric, suggesting that for the p53 central DNA-binding domain explicit consideration of solvation effects is unnecessary and can be simulated by the distance-dependent dielectric constant.

3.2. Correlations with experimental data

Several of the results obtained by this computational approach have been found to be consistent with experimental data, including comparison with immunologic analyses of epitope shifts, with analyses of phosphorylation site accessibility, and more recently with x-ray crystallographic data (6-9).

For example, the region of amino acid residues 94-117 which is found to undergo relatively large shifts in all mutants studied contains part of the epitope for the monoclonal antibody PAb1620 (12). Detection of the epitope has been considered to indicate a "wild-type" conformation for p53 because it is present in the native protein but absent in some (but not all) mutants (including the R175H mutant) (13). The computational results are consistent with this since the epitope region is found to be accessible in the wild-type protein but much less accessible in some of the mutants, such as R175H as well R249W and I255F (see Figure 1) (9). In other cases (e.g., R248W), although conformationally shifted in this region, the PAb1620 epitope remains somewhat accessible, consistent with persistent antibody detectability (9). Similarly, the region from amino acid residues 97-117 has been shown to be involved in p53 phosphorylation by several different kinases (7). The fact that two mutant forms of p53 (R249 and R249S) cannot be phosphorylated as easily or in the same pattern as wild-type p53 is consistent with the conformational shift seen in this region computationally; furthermore, a synthetic peptide for amino acids 97-117 was able to inhibit p53 phosphorylation by several kinases (7).

The region from amino acid residues 175-185, which contains one of the zinc coordination sites, also contains the epitope for another monoclonal antibody, DO-11, which has been detected in many p53 mutants (including the H179L mutant) but not in wild-type p53 (13). Once again, this is consistent with the computational results, since this region moves considerably in all the mutants, and in the H179L mutant in such a fashion that instead of being packed against the hydrophobic core of the domain (as it is in the wild-type), it is bent out and away from the rest of the structure and is exposed at the surface (9). Similarly, as noted, the region from amino acid residues 204-217 is also shifted in several p53 mutants, including R249W and I255F (see Figure 1) (9). In many of these, the region is exposed at the surface of the protein rather than concealed by packing against the hydrophobic core as occurs in the wild-type structure. This is consistent

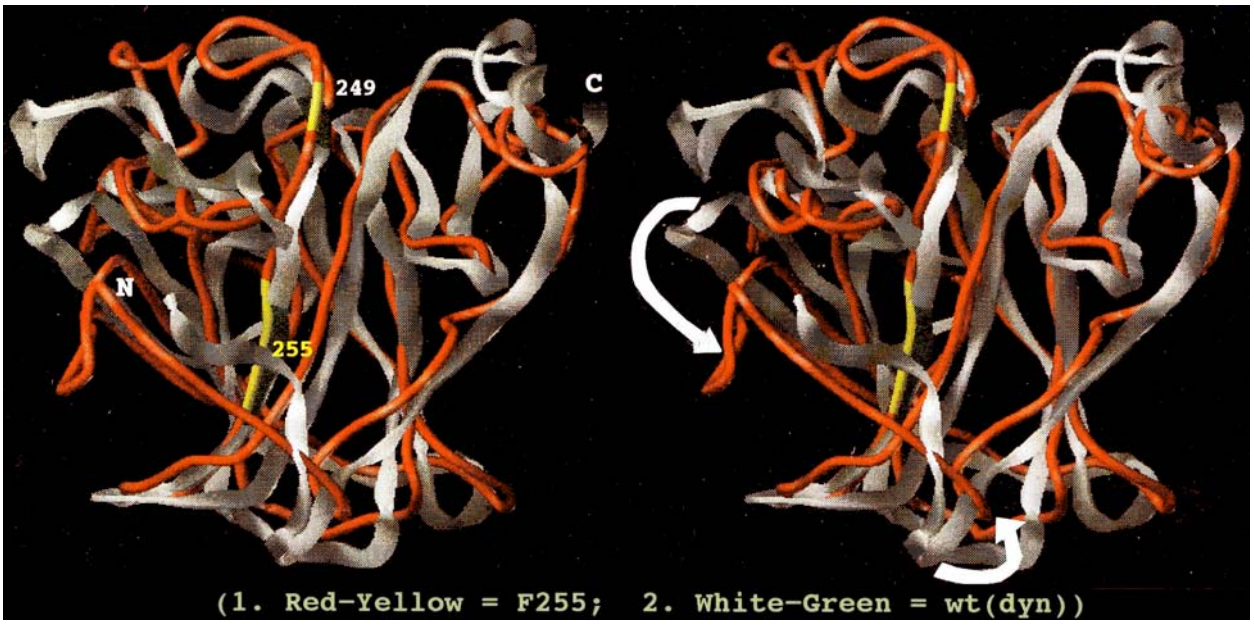

Figure 1. Stereo view of superposition of backbone tracings of DNA-binding core domain of wild-type and F255 mutant p53. Arrows indicate regions of predicted major conformational changes consistent with antibody epitope shifts.

with the results of detectability for the epitope of the monoclonal antibody PAb240, which has been considered an indicator of p53's "malignant" conformation (14). On the other hand, a few mutants studied show this region to be much less deviated leaving the PAb240 epitope still partially concealed in the core of the protein. For example, in the R248W mutant this region moves less than 5 A and the epitope is not fully exposed, consistent with the lack of antibody detectability in this case (14). Another region that was significantly shifted in most mutants (such as R175H) was amino acid residues 256- 264, which contains the epitope for monoclonal antibody DO-12 that is not detectable in the wild-type protein but is detectable in many mutants, including R175H (13).

Finally, recent preliminary results have been obtained comparing the computed structure of another p53 mutant (G245S) with its x-ray crystal structure. In this case, the overall RMS deviation between the computed and experimental structures was only 0.5 A with little apparent residue-by-residue variation (8, 9). However, it should be noted that NMR solution studies of the central DNA-binding core domain of wild-type and several p53 mutants (including G245S and R249S) have suggested that conformational changes in mutants are primarily localized to the site of the amino acid substitution, although in one case (V143A) long-range effects were noted (15). These results, however, are inconsistent with the epitope shifts and phosphorylation availability results from experimental studies noted above, so more research will be necessary to resolve these inconsistencies.

3.3. Potential applications

The ability to predict conformational shifts in mutant p53 proteins could have practical applications in clinical cancer studies. For example, the detection of epitope shifts that are associated with p53 mutations can be used as a biomarker to follow the development and progression of cancer, as well as the response to therapy.

For example, the H179L, R249W and I255F mutations in p53 have been associated with vinyl chloride exposure through the generation of reactive intermediates, chloroethylene oxide and chloroacetaldehyde, which produce etheno-adenine DNA adducts that are pro-mutagenic resulting in A->T tranversions at the second base of codon 179 and the first base of codons 249 and 255 in p53 (16, 17). All of these mutations have been associated computationally with a shift in the region of amino acid residues 204-217 in such a way as to result in exposure of the PAb240 epitope at the surface of the domain (6, 8). Thus, it has been possible to detect the PAb240 epitope via immunohistochemistry in the angiosarcomas of the liver from vinyl chloride-exposed workers that have these mutations but not in angiosarcomas of the liver without p53 mutations or in normal liver tissue (6, 8). In addition, the PAb240 epitope is also immunologically detectable using an enzyme-linked immunosorbent assay (ELISA) in the supernatant from cells in culture that also contain these p53 mutations, suggesting that it could also be detected in extracellular fluids *in vivo* in cases where the mutations are present (6). In fact, the serum of individuals with vinyl chloride-induced angiosarcomas of the liver that contain these mutations have been found to be positive for the presence of the PAb240 epitope via ELISA, whereas in the serum of individuals with angiosarcomas of the liver without p53 mutations and of normal healthy controls, the epitope was non-detectable (6). Furthermore, the level of the epitope in serum has been found to parallel the clinical course of the angiosarcoma, decreasing with successful therapy and increasing with disease recurrence (18). In fact, detection of this PAb240 epitope biomarker was found to occur before the clinical detection of recurrent disease, suggesting that detection may be possible before the

clinical detection of *de novo* neoplastic disease (18). Thus, serum positivity for this biomarker has been noted in vinyl chloride-exposed individuals without any clinically detectable neoplastic disease. The presence of the biomarker was found to occur with a highly statistically significant dose-response relationship with regard to estimated, cumulative vinyl chloride exposure, suggesting that the generation of the biomarker was indeed the result of the exposure's mutational effect (χ^2 for trend = 43.5, $p<0.00001$) (19). A statistically significant increased occurrence of the biomarker can even be identified in individuals who have been exposed to vinyl chloride only at levels below the current permissible exposure limits, suggesting that even at these low levels of exposure a significant residual risk of genotoxicity exists (19). The detection of this PAb240 epitope biomarker may therefore be useful for refining the current risk assessment for permissible vinyl chloride exposure to prevent the occurrence of these cancer-related mutations (20).

Since p53 mutations that produce detectability of the PAb240 epitope are presumed to occur during the carcinogenic process for many different tumors, this biomarker may be generally useful to identify individuals in at-risk cohorts who have the highest probability for the development of neoplastic disease, to allow for early intervention and prevention. For example, in a study of banked serum samples from workers with asbestosis and silicosis at high-risk for the development of lung cancer and mesothelioma, serum positivity for this biomarker had a positive predictive value of 0.67 for the subsequent development of malignancy with an average lead time to diagnosis of 5.3 years, and serum positivity was found to be statistically significantly correlated with mutations in the resultant tumors ($p=0.017$) (21). In another study of banked serum samples from individuals at risk of lymphoid malignancies, positivity was associated with a highly statistically significant increased occurrence of subsequent cancer with an average lead time to diagnosis of 7 years (22).

As suggested above, detection of this PAb240 epitope biomarker may also be useful for monitoring the progress of therapy. For example, serum biomarker levels have been found to decrease following surgical resection of breast cancers and colon cancers (23, 24); in the latter case post-operative levels after one week were statistically significantly lower than preoperative levels (24).

In addition, even greater utility for these markers is possible if they can be coupled with new therapeutic or prophylactic interventions that target the specific molecular defect identified, namely, mutations in p53. Once again, computational protein chemistry can play a role in the development of such interventions.

4. P53 PEPTIDE-BASED THERAPEUTIC APPROACHES

4.1. C-terminal p53 peptides

The latent activity of the core domain of p53 normally appears to be negatively regulated by other regions of the protein, including the C-terminal positively charged amino acid segment from residues 368-393 and the N-terminal proline-rich segment from residues 80-93 (25, 26). For example, the specific DNA-binding of p53 is apparently allosterically regulated by the C-terminal domain and can be activated in vitro by C-terminal truncation, phosphorylation or acetylation of specific amino acid residues in the C-terminus or by binding of the anti-p53 monoclonal antibody PAb421 which recognizes an epitope in this C-terminal region (25). Furthermore, the addition of chemically modified, synthetic C-terminal p53 peptides have been found to restore the in vitro sequence-specific DNA-binding function to a mutant p53 (R273H) (27). In addition, intranuclear microinjection of this peptide into SW480 colon carcinoma cells carrying an endogenous R273H mutation restored transcriptional activation of a p53-responsive reporter construct (28). Another p53 C-terminal 22-amino acid peptide (corresponding to residues 361-381), fused to a 17-amino acid segment of the Antennapedia (Ant) homeobox domain to facilitate cellular uptake, was found to suppress growth and induce apoptosis of SW480 cells (29). Using this p53-Ant peptide (residues 361-382 of p53 with the 17 residue segment of Ant on the C-terminus; GSRAHSSHLKSKKGQSTSRHKKWKMRR NQFWVKVQRG), we have found that the peptide can induce rapid apoptosis in various cancer cell lines (breast, prostate, colon, lung, mesothelioma) and premalignant cell lines (colonic adenoma) carrying endogenous p53 mutations or over-expressed wild-type p53 but is essentially non-toxic to nonmalignant human cell lines containing normal levels of wild-type p53 (30). The peptide also had no effect on three null-p53 tumor cell lines (30). This peptide was found to bind to mutant, and to a lesser extent wild-type, p53 in cell extracts, but it did not bind to mutant p53 with a C-terminal truncation, suggesting that binding was occurring outside the region of residues 360-393 or at least that other regions of p53 were more critical for binding (30). The observed apoptotic effect of the peptide was found to occur in the absence of new protein synthesis and to involve the Fas/APO-1 signaling pathway with activation of the Fas-APO-1-specific protease FLICE, activation of caspases and eventual PARP cleavage (30).

We have used computational methods, similar to those described above, to model the structure of the C-terminal p53 peptide as well as potential complexes of the peptide with other regions of p53 (30). We have found that the C-terminal peptide sequence from residues 369-382 forms an energetically favorable complex with the N-terminal regulatory sequence from residues 84-89 which is an $(Ala\text{-}Pro)_3$ repeat. In this complex, the C-terminal peptide adopts an energetically stable α-helical conformation, and the N-terminal sequence adopts an energetically stable DADAC helical conformation. In the complex, the two helices align in an anti-parallel fashion and intercalate in such a way that the proline residues of the N-terminal sequence form favorable contacts within the grooves of the □-helix of the C-terminal peptide. The two helices are aligned such that most of the side chains of the lysine residues of the C-terminal peptide (Lys^{370}, Lys^{373}, Lys^{381} and Lys^{382}) point away from the face of the peptide that makes contact with the N-terminal sequence. Several of the CH_2 groups of the side chains of Lys^{372} and Arg^{379} contact the proline residues, and the side chain CH_3 group

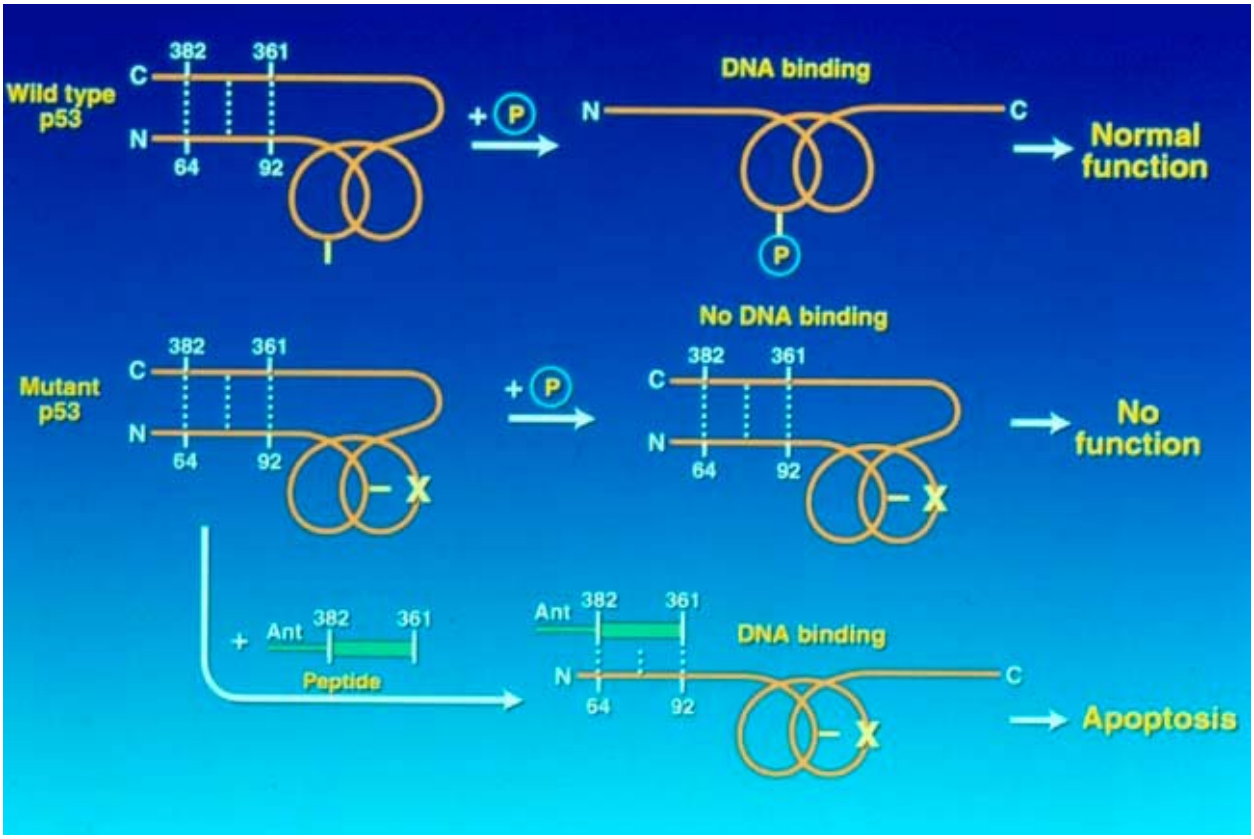

Figure 2. Proposed model for mechanism of action of C-terminal p53 peptide in restoring mutant p53 function.

of Thr[377] also contacts the second proline residue. The complex therefore has amphipathic properties in which the interface between the two sequences tends to be non-polar, whereas the hydrophilic lysine residues point away on the opposite face of the C-terminal peptide helix (30).

Furthermore, since a helical conformation of the peptide may be important for this complex formation, we have used other computational approaches to address the issue of the most advantageous choice and positioning of a leader sequence that not only would allow membrane penetration but also would serve to stabilize a helical structure. Alpha-helical probability profiles for various configurations of peptide and leader sequence were computed using probabilities from the protein database or using the Ising model based on helix nucleation and growth equilibrium constants determined experimentally from block copolymers for each of the twenty naturally occurring L-amino acids modified by the inclusion of the effects of charges on these parameters; similarly, hydrophobic moment and helical wheel analyses were also performed on the various peptide-leader sequence configuration (31). These approaches examined the C-terminal sequence linked to the Ant leader sequence, an HIV-1 TAT leader sequence (GRKKRRQRRR), and a modified HIV-1 TAT-a leader sequence (GRKKRRARRR) on its N-terminus or C-terminus. All approaches suggested that the C-terminal peptide with the Ant leader sequence on its C-terminus would be the most hydrophobic and the most helical, generating an amphipathic α-helix. Experimental results confirmed that this configuration was much better than the others in terms of the peptide's cellular penetration and ability to induce apoptosis (31). All three of these leader sequences are positively charged , and placement of positive charges on the C-terminus of the p53 peptide would be expected to stabilize an α-helical conformation augmenting the activity of the peptide, whereas placement of positive charges on the N-terminus would be expected to destabilize α-helical structure with loss of activity of the peptide (32), consistent with the experimental results.

These results suggest that a direct interaction between the N-terminal regulatory domain of p53 and the C-terminal peptide or the C-terminal regulatory region of intact p53 in an amphipathic α-helical conformation is possible and potentially important for function. The N-terminal regulatory region is thought to cooperate with the C-terminal regulatory region to negatively regulate normal p53 activity (26). This suggests at least one simplistic model to explain the peptide's mechanism of action (see Figure 2). In this model, normal p53 is kept in its latent form by direct interactions between the N-terminal regulatory region and the C-terminal regulatory region which prevent the central DNA-binding domain from being functional. Appropriate cellular signals, e.g., phosphorylation of certain residues in p53, cause a conformational change in p53 such that the interaction between the N-terminal regulatory region and the C-

terminal regulatory region is removed allowing the activity of the central DNA-binding domain to proceed. With certain p53 mutations, the conformation of p53 is changed so that it is unresponsive to these signals that activate its latent activity, and therefore the mutant p53 is non-functional because the interaction between the N-terminal regulatory domain and the C-terminal regulatory domain remains intact. The C-terminal peptide, however, can act as a direct competitive inhibitor of this interaction by binding to the N-terminal regulatory region preventing the C-terminal regulatory region of the intact protein from binding, thus allowing the latent activity to be again expressed. In a damaged cell such as cancer cells with mutant p53, this reactivated mutant p53 would trigger the apoptotic mechanism in order to remove the damaged cell. In other damaged cells such as cancer cells that over-express wild-type p53, the large amounts of activated wild-type p53 could also trigger the apoptotic cascade. However, in normal cells with low levels of wild-type p53, in the absence of damage, the activated p53 would be expected to have no effect.

This model does not preclude the possibility that the C-terminal peptide could bind to other regions of p53 or indeed other proteins to exert its effect. For example, we have identified other potential binding complexes with sites in the central DNA-binding domain from residues 97-117, a region known to undergo considerable conformational change in mutant p53, and in the C-terminal domain from residues 320-350 (30). These possibilities would be consistent with the results of others that suggest the C-terminal peptide can interact with the central DNA-binding domain (residues 99-307) and the C-terminal domain (residues 320-393); they also reported weak binding of the C-terminal peptide to the N-terminal region (residues 1-100), consistent with our model (33). As noted, we did not find any evidence of direct binding of the C-terminal peptide to the same C-terminal region in intact p53, but that would not preclude binding of the peptide to residues 320-360. Still others have failed to find evidence of interaction of the peptide to the central DNA-binding domain ((26), but this discrepancy may be dependent on the different salt concentrations employed leading to differential states of binding. Indeed, it is possible that under the right conditions the peptide binds to all three domains of p53, and these may all be required for its reactivating function. We are currently involved in studies of p53 molecules with selective deletions of potential binding sites for the peptide to measure direct binding of the peptide by surface plasmon resonance *in vitro* as well as activity of the peptide in inducing apoptosis in cells with these deletions in p53 *in vivo*. Preliminary results with p53(del320-363) suggest that this region may indeed contribute to the peptide's activity. Additional computational studies may indicate other sites in p53 that could contribute to the peptide's activity and/or could be useful as therapeutic peptides themselves.

4.2. N-terminal p53 peptides

Another way in which p53's activity is regulated in cells is via its binding to other proteins. For example, as noted, control of the lifetime of the p53 molecule in cells, which is a critical factor in its ability to control cell cycle events, is dependent upon binding of p53 to the oncogene-encoded mdm-2 protein which targets it for ubiquination and degradation (3, 4). When appropriately phosphorylated, p53 no longer interacts with mdm-2 resulting in the prolongation of its half-life and its ability to reverse cell cycle events that lead to cellular transformation (3, 4). Thus, it is possible that blockade of p53-mdm-2 interactions could counter cell-transforming events. Others have demonstrated that a peptide sequence designed to interact with mdm-2 (MPRFMDYWEGLN) can induce apoptosis in osteosarcoma cells that over-express mdm-2; this peptide had no effect on cells with homozygous deletions of p53 suggesting that it was functioning by disrupting the p53-mdm-2 interaction (34).

The region of p53 involved in mdm-2 interactions includes residues 12-26 (PPLSQETFSDLWKLL) which can be divided into two partially overlapping sub-domains, an evolutionarily conserved segment from residues 12-20 and a segment containing the residues that actually contact the binding domain of mdm-2 from residues 17-26 (3, 4). The x-ray crystal structure for the complex of the mdm-2 binding domain of p53 with the mdm-2 protein has been elucidated demonstrating that the mdm-2 binding sequence of p53 adopts an α-helical conformation when bound in the complex (35). Therefore, we used computational methods for determining helix probability profiles as described above to predict the configuration of the N-terminal p53 sequence and leader sequence that would be most likely to elicit an α-helix. As above, the results indicated that addition of the Ant leader sequence to the C-terminus of the N-terminal p53 peptide resulted in stabilization of an □-helical conformation due to the positive charge distribution (36). Indeed, when synthesized, this peptide was found to be very effective in killing cancer cells of various types but to have no effect on normal cells (36, 37). However, unexpectedly the peptide was found to be equally cytotoxic for cancer cells that were p53-null as for cancer cells that had mutant or wild-type p53, suggesting that a p53-independent mechanism of cell death was involved (37). Extensive analysis revealed that this was non-apoptotic cell death most closely resembling necrosis with very rapid destruction of the plasma cell membrane probably through membrane pore formation, as seen by electron microscopy (37). Furthermore, solution structure determination, using circular dichroism (CD) spectroscopy, as well as predictive bioinformatic sequence analysis, revealed that in an aqueous solution environment, this peptide contains alpha helical secondary structure elements possessing a very high hydrophobic moment, thus possibly allowing it to rapidly cause selective cancer cell membrane disruption (37).

In order to further investigate how the predicted structure for this peptide might be contributing to its biological activity in promoting selective and rapid tumor membrane disruption, we performed two-dimensional NMR on the peptide in both an aqueous cytosolic-like and mixed organic membrane-like solution environment. In the aqueous environment, the N-terminal p53-Ant peptide primarily contained three α-helical domains connected by loop structures assuming an overall S-shaped form. In the

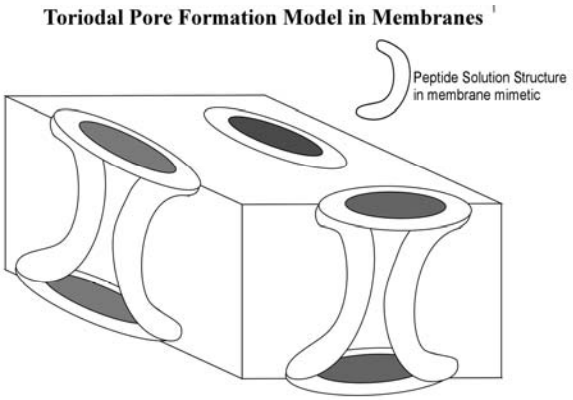

Figure 3. Proposed model for mechanism of action of N-terminal p53 peptide in producing necrosis through membrane pore formation.

ganic membrane mimetic environment, the helical domains found in water increase in length forming three classes of structures, all of which form a U-shaped helix-coil-helix ensemble. In both solvent systems, this peptide forms amphipathic structures such that its hydrophobic residues coalesce on one face while the polar residues aggregate on the opposite face. The organic-phase U-shaped structural motif has been observed in a number of peptides containing 20-30 amino acid residues that adopt helix-loop-helix structures and that are known to be membrane-active, including melittin, the active component of bee venom that induces disruption of red cell membranes (38), and the antimicrobial peptide magainin, that causes lysis of bacterial membranes (39). A cardinal feature of this structural motive for all these peptides is its amphipathic nature which would allow them to react with the hydrophobic and hydrophilic components of the membrane simultaneously. Such amphipathic, U-shaped helix-loop-helix structures have been observed to form quaternary structure toroidal pores within the membrane thereby promoting membrane lysis (40, 41), which is possible also for the N-terminal p53-Ant peptide (see Figure 3) and would be consistent with our observations for the membrane effect of this peptide. In addition, recent analysis of the Ant sequence alone has shown that its ability to interact and penetrate membrane components varies with membrane cholesterol content, which may contribute to the selectivity of effect of this peptide for cancer cells (42). Additional computational studies of this structural motif can contribute to identifying and optimizing the peptide's structural characteristics that contribute to its membrane activity and cancer cell selectivity.

5. CONCLUSION AND PERSPECTIVES

Computational protein chemistry methods are likely to play an increasing role in biomedical sciences. With the completion of the human genome project, an enormous number of genetic sequences are available with little detailed experimental data available concerning the likely structures of their protein products. In addition, for inherited polymorphisms in proteins or acquired mutations in proteins that may contribute to disease susceptibility there is limited experimental data on the effects of these changes on protein structure. As suggested by the example of p53 above, computational protein chemistry approaches can help to shed light on these issues. In addition, peptide-based interventions for various diseases are increasing because of their potency, specificity and low toxicity with as many as 30 peptide-based drugs currently on the market and several others recently approved for clinical use (43). As suggested by the example above for p53, computational protein chemistry approaches can be of value in elucidating the mechanisms of action of such peptides, as well as in optimizing the design of their structures to achieve the best biologic effect.

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7. REFERENCES

1. Soussi, T.: The p53 tumor suppressor gene: from molecular biology to clinical investigation. *Ann NY Acad Sci* 910:121-139 (2000)

2. Sigal, A. and V. Rotter: Oncogenic mutations of the p53 tumor suppressor: the demons of the guardian of the genome. *Cancer Res* 60, 6788-6793 (2000)

3. Choisy-Rossi, C., P. Reisdorf and E. Yonish-Rouach: The p53 tumor suppressor gene: structure, function and mechanism of action. *Results Probl Cell Differ* 23, 145-172 (1999)

4. Hupp, T.R., D.P. Lane and K.L. Ball: Strategies for manipulating the p53 pathway in the treatment of human cancer. *Biochem J* 352, 1-17 (2000)

5. Cho Y., S. Gorina, P.D. Jeffrey and N.P. Pavletich: Crystal structure of a p53 tumor suppressor-DNA complex: understanding tumorigenic mutations. *Science* 265, 346-355 (1994)

6. Brandt-Rauf, P.W., J.M. Chen, M.-J. Marion, S.J. Smith, J.-C. Luo, W. Carney and M.R. Pincus: Conformational effects in the p53 protein of mutations induced during chemical carcinogenesis: molecular dynamic and immunologic analyses. *J. Protein Chem* 15, 367-375 (1996)

7. Adler, V., M.R. Pincus, T. Minamoto, S.Y. Fuchs, M.J. Bluth, P.W. Brandt-Rauf, F.K. Friedman, R.C. Robinson, J.M. Chen, X.W. Wang, C.C. Harris and Z. Ronai: Conformation-dependent phosphorylation of p53. *Proc Natl Acad Sci USA* 94, 1686-1691 (1997)

8. Chen, J.M., S.J. Smith, M.-J. Marion, M.R. Pincus and P.W. Brandt-Rauf: Common conformational effects in the p53 protein of vinyl chloride-induced mutations. *J Protein Chem* 18, 467-472 (1999)

9. Chen, J.M., R. Rosal, M.R. Pincus and P.W. Brandt-

Rauf: Common conformational effects of p53 mutations. *J Protein Chem* 20, 101-105 (2001)

10. Vasquez, M., G. Nemethy and H.A. Scheraga: Computed conformational states of the 20 amino acid residues and the prototype residue γ-aminobutyric acid. *Macromolecules* 16, 1043-1049, 1983

11. Ponnuswamy, P.K., P.K. Warme and H.A. Scheraga: Role of medium-range interactions in proteins. *Proc Natl Acad Sci USA* 70, 830-833 (1973)

12. Ravera, M.W., J. Carcamo, R. Brissette, A. Alam-Moghe, O. Dedova, W. Cheng, K.C. Hsaio, D. Klebenov, H. Shen, A. Bluwe and W. Mandecki: Identification of an allosteric binding site on the transcription factor p53 using a phage-displayed peptide library. *Oncogene* 16, 1993-1999 (1998)

13. Vojtesek, B., H. Dolezalova, L. Lauerova, M. Svitkova, P. Havlis, J. Kovarik, C.A. Midgley and D.P. Lane: Conformational changes in p53 analyzed using new antibodies to the core DNA binding domain of the protein. *Oncogene* 10, 389-393 (1995)

14. Legros, Y., A. Meyer, K. Ory and T. Soussi: Mutations in p53 produce a common conformational effect that can be detected with a panel of monoclonal antibodies directed toward the central part of the p53 protein. *Oncogene* 9, 3689-3694 (1994)

15. Wong, K.-B., B.S. DeDecker, S.M.V. Freund, M.R. Proctor, M. Bycroft and A.R. Fersht: Hot-spot mutants of p53 core domain evince characteristic local structural changes. *Proc Natl Acad Sci USA* 96, 8438-8442 (1999)

16. Hollstein, M., M.-J. Marion, T. Lehman, J. Welsh, C.C. Harris, G. Martel-Planche, I. Kusters and R. Montesano: P53 mutations at A:T base pairs in angiosarcomas of vinyl chloride-exposed factory workers. *Carcinogenesis* 15, 1-3 (1994)

17. Trivers, G.E., H.L. Cawley, V.M.G. DeBenedetti, M. Hollstein, M.-J. Marion, W.P. Bennett, M.L. Hoover, C.C. Prives C.C. Tamburro and C.C. Harris: Anti-p53 antibodies in sera of workers occupationally exposed to vinyl chloride. *J. Natl Cancer Inst* 87, 1400-1407 (1995)

18. Li, Y., M. Asherova, M.-J. Marion and P.W. Brandt-Rauf: Mutant oncoprotein biomarkers in chemical carcinogenesis. In: Biomarkers: medical and workplace applications. Eds: Mendelsohn M., Mohr L., Peeters P., Joseph Henry Press, Washington, DC 345-353 (1998)

19. Smith, S.J., Y. Li, R. Whitley, M.-J. Marion, S. Partilo, W.P. Carney and P.W. Brandt-Rauf: Molecular epidemiology of p53 mutations in workers exposed to vinyl chloride. *Am J Epidemiol* 147, 302-308 (1998)

20. Brandt-Rauf, P.W., J.-C. Luo, T.-J. Cheng, C.-L. Du, J.-D. Wang, R. Rosal, T. Do and M.-J. Marion: Molecular biomarkers and epidemiologic risk assessment. *Hum Ecol Risk Assess* 8, 1295-1301 (2002)

21. Husgafvel-Pursiainen, K., K. Annio, P. Oksa, T. Suitiala, H. Koskinen, K. Hemminki, S. Smith, R. Rosenstock-Leibu and P.W. Brandt-Rauf: Mutations, tissue accumulations, and serum levels of p53 in patients with occupational cancers from asbestos and silica exposure. *Environ Mol Mutagen* 30, 224-230 (1997)

22. Lehtinen, T., T. Luostarinen, J. Dillner, A. Aromaa, M. Hakama, T. Hakulinen, P. Knecht, P. Leinikki, J. Lumio, M.L. Lahdeaho, J. Maatela, L. Teppo and M. Lehtinen: Serum p53 accumulation and altered antibody response to Epstein-Barr virus proteins precede diagnosis of haemopoietic malignancies or lymphoid origin. *Br J Haematol* 93, 104-110 (1996)

23. Rosanelli, G.P., G.H. Wirnsberger, P. Purstner and P. Steindorfer: DNA flow cytometry and immunohistochemical demonstration of mutant p53 protein versus TPS and mutant p53 protein serum levels in human breast cancer. *Proc Am Assoc Cancer Res* 34, A1353 (1993)

24. Shim, K.S., K.H. Kim, B.W. Park, S.Y. Lee, J.H. Choi, W.S. Han and E.B. Park: Increased serum levels of mutant p53 protein in patients with colorectal cancer. *J Korean Med Sci* 13, 44-48 (1998)

25. Hupp, T.R. and D.P. Lane: Allosteric activation of latent p53 tetramers. *Curr Biol* 4, 865-875 (1994)

26. Muller-Tiemann, B.F., T.D. Halazonetis and J.J. Elting: Identification of an additional negative regulatory region for p53 sequence-specific DNA binding. *Proc Natl Acad Sci USA* 95, 6079-6084 (1998)

27. Hupp, T.R., A. Sparks and D.P. Lane: Small peptides activate the latent sequence-specific DNA binding function of p53. *Cell* 83, 237-245 (1995)

28. Abarzua, P., J.E. LoSardo, M.L. Gubler, R. Spathis, Y.A. Lu, A. Felix and A. Neri: Restoration of the transciption activation function to mutant p53 in human cancer cells. *Oncogene* 13, 2477-2482 (1996)

29. Selivanova, G., V. Lotsova, M. Okan, M. Fritsche, M. Strom, B. Groner, R.C. Grafstrom and K.G. Wiman: Restoration of the growth suppression function of mutant p53 by a synthetic peptide derived from the p53 C-terminal domain. *Nat Med* 3, 632-638 (1997)

30. Kim, A.L., A.J. Raffo, P.W. Brandt-Rauf, M.R. Pincus, R. Monaco, P. Abarzua and R.L.Fine: Conformational and molecular basis for induction of apoptosis by a p53 C-terminal peptide in human cancer cells. *J Biol Chem* 274, 34924-34931 (1999)

31. Yin, L., R.V. Rosal, P.W. Brandt-Rauf and R.L. Fine: Correlation between hydrophobic properties and efficiency of carrier-mediated membrane transduction and apoptosis of a p53 C-terminal peptide. *Biochem Biophys Res Commun* 298, 439-449 (2002)

32. Vasquez, M. M.R. Pincus and H.A. Scheraga: Helix-

coil transition theory including long-range electrostatic interactions. *Biopolymers* 26, 351-372 (1987)

33. Selivanova, G., L. Ryabchenko, E. Jansson, V. Iotsova and K.G. Wiman: Reactivation of mutant p53 through interaction of a C-terminal peptide with the core domain. *Mol Cell Biol* 19, 3395-3402 (1999)

34. Wasylyk, C., R. Salvi, M. Argentini, C. Dureuil, I Delumeau, J. Abecassis, L. Debussche and B. Wasylyk: P53 mediated death of cells overexpressing MDM2 by an inhibitor of the MDM2 interaction with p53. *Oncogene* 18, 1921-1934 (1999)

35. Kussie, P.H., S. Gorina, V. Marechal, B. Elenbaas, J. Moreau, A.J. Levine and N.P. Pavletich: Structure of the MDM2 oncoprotein bound to the p53 tumor suppressor transactivation domain. *Science* 274, 948-953 (1996)

36. Kanovsky, M., A. Raffo, L. Drew, R. Rosal, T. Do, F.K. Friedman, P. Rubinstein, J. Visser, R. Robinson, P.W. Brandt-Rauf, J. Michl, R.L. Fine and M.R. Pincus: Peptides from the amino terminal mdm-2-binding domain of p53, designed from conformational analysis, are selectively cytotoxic to transformed cells. *Proc Natl Acad Sci USA* 98, 12438-12443 (2001)

37. Do, T.N., R.V. Rosal, L. Drew, A.J. Raffo, J. Michl, M.R. Pincus, F.K. Friedman, D.P. Petrylak, N. Cassai, J. Szmulewicz, G. Sidhu, R.L. Fine and P.W. Brandt-Rauf: Preferential induction of necrosis in human breast cancer cells by a p53 peptide derived from the MDM2 binding site. *Oncogene* 22, 1431-1444 (2003)

38. Pincus, M.R.: The physiological structure and function of proteins. Academic Press, NY (2001)

39. Dathe, M. and T. Wieprecht: Structural features of helical antimicrobial peptides: their potential to modulate activity on model membranes and biological cells. *Biochim Biophys Acta* 1462, 71-87 (1999)

40. Hallock, K.J., D.K. Lee and A. Ramamoorthy: MSI-78, and analogue of the magainin antimicrobial peptides, disrupts lipid bilayer structure via positive curvature strain. *Biophys J* 84, 3052-3060 (2003)

41. Naito, A, T. Nagao, K. Norisada, T. Mizuno, S. Tuzi and H. Saito: Conformation and dynamics of melittin bound to magnetically oriented lipid bilayers by solid-state (31)P and (13)C NMR spectroscopy. *Biophys J* 78, 2405-2417 (2000)

42. Christiaens, B., S, Symoens, S. Verheyden, Y. Engelborghs, A. Joliot, A. Prochiantz, J. Vanderkerckhove, M. Rosseneu, B. Vanloo and S. Vanderheyden: Tryptophan fluorescence study of the interaction of penetratin peptides with model membranes. *Eur J Biochem* 269, 2918-2926 (2002)

43. Mullin, R.: Biopharmaceuticals - Rohm and Haas takes on peptides. *C&E News* 81, 12 (2003)

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