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The p53 protein is a tumor suppressor crucial to maintaining genomic integrity. In the event of DNA damage, p53 is responsible for transcribing genes leading to cell death. A class of mutations which occur in the core domain (102-292) leads to thermodynamic destabilization and inability to bind its cognate DNA sequence. Small molecules which bind to and stabilize mutant p53 core domain have potential to be therapeutically useful. Two potential "hot spots" on the surface of the mouse p53 core domain have been discovered which can be targeted by small molecule compounds. One hot spot was discovered by soaking the crystal lattice with various organic solvents and locating the solvents in the electron density. Another potential hot spot was located in the high resolution structure of the mouse core domain where a molecule of tris(hydroxymethyl)aminomethane (Tris) was observed to bind on the surface of the protein, making numerous hydrogen bonding contacts. Urea denaturation experiments show an increase in stability (~.37kcal/mol) in the presence of 1mM Tris when compared to protein in the absence of Tris. Although the gain in stability is relatively modest, the structural data acquired in this study can guide the way to designing compounds with increased activity.

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Introduction

The p53 tumor suppressor protein is crucial to maintaining genomic integrity. In the event of DNA damage, p53 activates transcription of genes which lead to apoptosis or cell cycle arrest[1]. As many as 50% of all human cancers are associated with mutations to p53[2]. The p53 protein has four domains: an amino terminal transactivation domain (residues 1-44), a core DNA binding domain (102-292), a tetramerization domain (residues 320-356), and a carboxy-terminal regulatory domain (residues 320-356). An estimated 95% of all tumorogenic mutations to the p53 gene occur within the core domain[3]. Most of these mutations to the core domain occur as point mutations, which can be generally classified into two groups: 1) those which occur to amino acids making direct contact with DNA, thereby decreasing binding affinity, and 2) those which cause the core domain to be unstable, causing unfolding or misfolding, and therefore inability to bind DNA. To address mutations which cause the core domain to be unstable, attempts have been made to introduce small molecules which have the capability to bind to and stabilize the core domain, rescuing function. Among these attempts are those made by Freidler and co-workers[4], who have designed a peptide with micromolar affinity and the ability to rescue human p53 core domain function. Knowledge of areas on the p53 core domain surface which can potentially be targeted by designed small molecules can be helpful.

Body

During the period of April 1, 2002 to April 30, 2005 for which my work was funded a number of goals were accomplished.

1.) ***Synthesize a library of structurally diverse molecules that have been shown to pharmacologically rescue p53.*** Based on the published work of Foster et al.[5] , molecules which were reported to interact with the p53 core domain were synthesized with the purpose of assaying binding and determining the mode of binding via x-ray crystallography. In total four molecules were synthesized (Figure 1). All products were characterized *via* mass spectrometry and NMR spectroscopy.

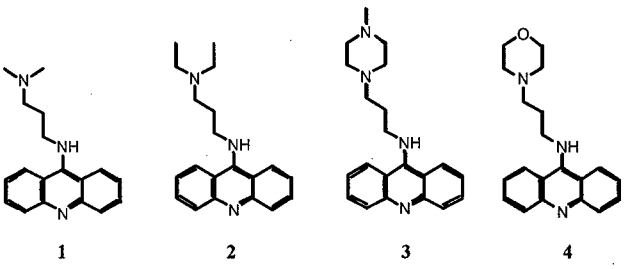

Figure 1: (**1.**)N'-Acridin-9-yl-N,N-dimethyl-propane-1,3-diamine, (**2.**) N'-Acridin-9-yl-N,N-diethyl-propane-1,3-diamine), (**3.**) Acridin-9-yl[3-(4-methyl-piperazin-1-yl)propyl]-amine, (**4.**) Acridin-9-yl-(3-morpholin-4-yl-propyl)-amine

2.) ***Crystallize the core domain of p53 in complex with a molecule shown to pharmacologically rescue p53.*** Using the molecules synthesized above, crystallization trials with the mouse p53 core domain were initiated. However, no co-crystals were ever grown. Further studies with the compounds revealed that they did not interact with the mouse p53 core domain at all. Our findings were independently confirmed by the findings of Fersht[6] et al. who also showed that the compounds published by Foster[5] et al. did not interact directly with the human p53 core domain.

3.) ***Structurally characterize the binding of peptides that have been shown to interact with and stabilize the p53 core domain using x-ray crystallography.*** We became

interested in a paper published by the Fersht[4] group detailing a number of peptides that were demonstrated to bind to and stabilize the p53 core domain. We then entered into a collaboration with the Fersht lab to solve the crystal structure of the peptides in complex with the mouse p53 core domain. With such a complex, we would then be able to use structure based drug design techniques to improve the desired properties of the peptides. Co-crystallization was attempted using both the mouse p53 core domain (92-292) and the human p53 core domain (94-32). No co-crystals were ever grown, although large well diffracting and well behaved crystals (1.55Å diffraction limit) of the mouse p53 core domain were obtained. At this point it was decided to use this crystal form as a tool to probe for "hot spots" which can potentially bind small molecules. Interestingly, other groups have also tried to grow co-crystals but were similarly unsuccessful.

4.) ***Soak high resolution crystals of the mouse p53 core domain with organic solvents in order to locate possible binding sites for small molecules.*** Crystals of the mouse p53 core domain were soaked with a number of organic solvents 1,6 hexanediol, acetonitrile, isopropanol, acetone, and phenol in an attempt to locate "hot spots" that would be amenable to binding small molecules[7; 8]. Typically, the crystals were soaked in ~35% organic solvent and the frozen. Data were collected at our home source and

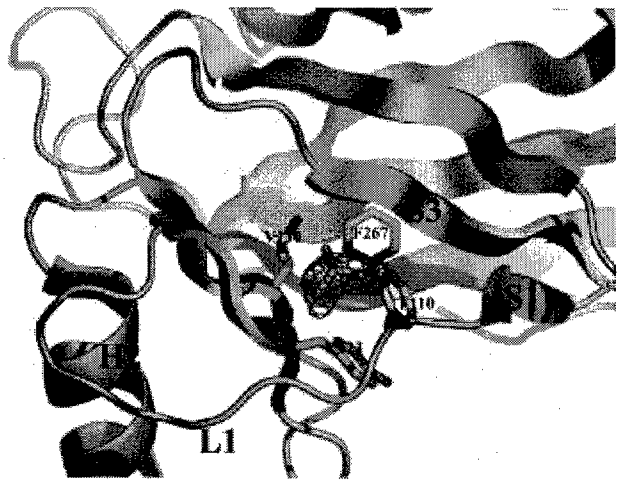

Figure 2: Fo-Fc omit map calculated at 2.5ó using the final structure with isopropanol omitted.

then solvent molecules were located by viewing f_o-f_c, f_o-f_o and $2f_o$-f_c electron density maps. Of the solvents tested, only one molecule of isopropanol could be confidently located and assigned (figure 2). The hydroxyl functional group of isopropanol was observed to be bridging two â-sheets, while its methylene groups point to a hydrophobic region.

5.) ***Characterization of the Tris binding site on the surface of the mouse p53 core domain.*** During refinement of the high resolution structure (1.55Å) of the mouse p53 core domain, continuous electron density was observed which could not be accounted for by the protein itself, or water. A molecule of tris(hydroxymethyl)aminomethane (Tris), which was present in the mother liquor during crystallization was built into the electron density and refined for several cycles, resulting in an improvement of the model statistics. Tris fits very well into the electron density and is observed to make numerous hydrogen bonding contacts to neighboring residues; Y126 and Q128 interact with hydroxyl groups while D268 makes contacts with the amino group of Tris (Figure 3). Additionally, the Tris molecule makes a water-mediated hydrogen bonding interactions to the backbone carbonyl of S266 and to the side chain nitrogen of Q101. It is interesting to note that D268 and Y126 are on opposing strands of anti-parallel beta sheets, in effect bridging S1 and S10. Further examination of the site of the Tris binding site reveals that Tris makes no inter-subunit

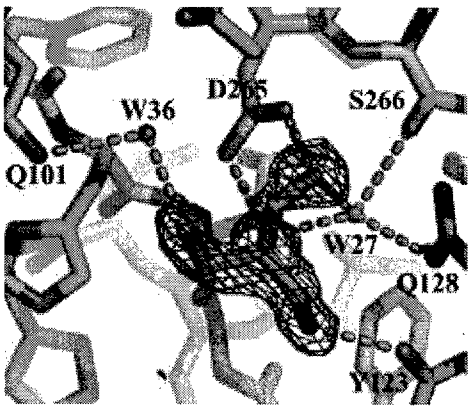

Figure 3: F_o-F_c omit density of Tris bound to the p53 core domain contoured at 2.06. Hydrogen bonding interactions are depicted in orange. Two molecules of water are depicted in red.

contacts in the crystal lattice and is therefore not likely to be an artifact of crystallization. To prove this, crystallization using Hepes as a buffer rather than Tris resulted in formation of crystals that were isomorphous in every property except for the absence of electron density in the proposed Tris binding site.

Although Tris clearly interacts with the mouse p53 core domain in the crystal structure, we were unsure of what effects, if any it has on the overall stability of the mouse p53 core domain. In order to characterize these possible effects, urea denaturation experiments were carried out in the presence and the absence of 1mM Tris. The data shows that addition of Tris provided a small, but noticeable increase in stability to the mouse p53 core domain (Figure 4). Without Tris, the mouse p53 core domain was observed have a midpoint of denaturation(U_m) of 2.39± .01M Urea. In the presence of 1mM Tris, the U_m was observed to be 2.61 ± .12M Urea. The increased stability was calculated to be ~.37kcal/mol. Differential Scanning Calorimetery(DSC) was also attempted, but artifacts due to the thermal instability of Tris could not be eliminated. Our search for molecules based on Tris which impart increased stability is ongoing, although at this point, no other molecules have been discovered.

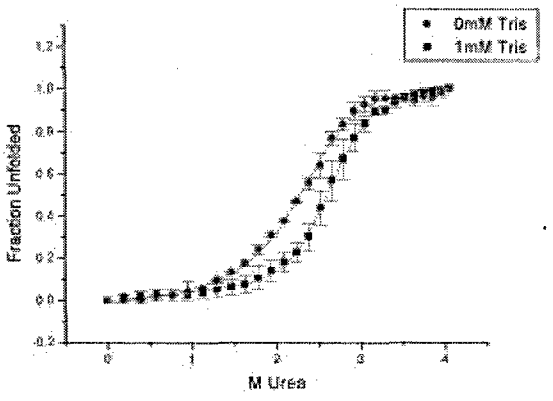

Figure 4: Urea denaturation curve of mouse p53 core domain in the absence of Tris (red circles), and in the presence of 1mM Tris (black squares). Fluorescence of a single buried tryptophan was measured at 356nm with excitation at 280nm. Measurements were done in duplicate for 0mM Tris and quadruplicate for 1mM Tris.

Nevertheless, our experiments with Tris demonstrate the feasibility of the concept that small molecules which bind to the core domain can impart stability.

Key Research Accomplishments:

- Synthesized a library of small molecules which were reported to bind to and stabilize the p53 core domain.

  Attempted to grow crystals of the small molecule-p53 core domain complex. It was discovered that this class of molecules does not interact with the mouse p53 core domain.

- Attempted to grow co-crystals of p53/peptides which Fersht et al. reported to bind to and stabilize the p53 core domain. Although both the mouse and human construct were used in crystallization trials, no co-crystals containing the protein/peptide complex were ever grown.
- Well behaved crystals of the mouse p53 core domain which diffracted extremely well(1.55Å) at a synchrotron light source were grown. Data was collected and the structure was solved and refined.

  Soaked this crystal form with several organic solvents in order to locate "hot spots" which might be able to accept small molecules. One molecule of isopropanol was observed to bind in a potential "hot spot".

- Refined a high resolution structure of the mouse p53 core domain and uncovered a binding site for tris(hydroxymethyl)aminomethane (Tris).
- Assayed the effects of Tris on mouse p53 core domain stability using urea denaturation experiments. Tris was found to stabilize mouse p53 core domain ~.37kcal/mol as compared to "native" protein.

Reportable outcomes

"High resolution crystal structure of the mouse p53 core domain: An analysis of protein flexibility and a framework for structure-based drug design" Ho, W.C., Zhao, K., Chai, X., and Marmorstein, R.M., *manuscript submitted.*

"Acridine derivatives activate p53 and induce tumor cell death through Bax" Wang, W., Ho, W.C., Dicker, D.T., MacKinnon, C., Winkler, J.D., Marmorstein, R., El-Deiry, W.S., *manuscript submitted.*

Conclusions

Several conclusions can be reached from this funded work. First, we have observed, along with others that the molecules reported by Foster et al. do not interact directly with the p53 core domain. It has been speculated that the effects of these molecules are due to their actions on other pathways. Second, we believe that we have located a "hot spot" on the surface of the mouse p53 core domain using isopropanol affinity as a probe. This region has shown affinity for functional groups contained in isopropanol—a hydroxyl group and two methylene groups. Third, our structure of the mouse p53 core domain is the highest resolution structure (1.55Å) of the p53 core domain reported to date. This work provides a more detailed picture of the p53 core domain structure. Last, we have successfully found a small molecule that binds to and stabilizes the core domain. The increase in stability contributed by Tris binding is small (~.37kcal/mol). However, we believe that the results of this study are valuable in that it demonstrates the feasibility of small molecules which can bind to a specific site on the core domain and stabilize it. Available structural data supports our biochemical data and elucidates the exact nature of the interaction and thus has the potential for rationally developing molecules with improved function.

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