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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. A class of small molecules discovered via high throughput screening has been reported to bind to and stabilize the p53 core domain (Foster et al.). Our results show that these compounds do not interact directly with the p53 core domain at all. Another approach using peptides as lead compounds has resulted in a class of peptides that bind to the p53 core domain with low micromolar affinity. And, in addition, has shown the ability to stabilize mutant core domain. The structure of the p53 core domain in complex with these peptides in now being actively pursued.

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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. Most notable, a publication by Foster and co-workers(4) detailed a class of small molecules discovered by high thoroughput screening that seemed to interact directly with the core domain and rescue its function. The specific aims of this project are as follows: 1.) Synthesize a library of structurally diverse molecules that have been shown to pharmacologically rescue mutant p53. 2.) Crystallize the core domain of p53 in complex with a molecule shown to pharmacologically rescue p53. 3.) Determine the x-ray structure of p53 core domain in

complex with a molecule shown to pharmacologically rescue p53. 4.) Design and synthesize molecules which bind tighter to the core domain of p53.

Body:

The approved statement of work submitted with the grant proposal consisted of four goals:

1.) Synthesize a library of structurally diverse molecules that have been shown to pharmcologically rescue p53.

From Foster et al. (4), a subset of target molecules were synthesized for attempts at crystallography. The subset chosen were the acridines which have been shown in Foster et al. as well as a Pfizer international patent(5) to stabilize the p53 core domain. These molecules were successfully synthesized and shown below:

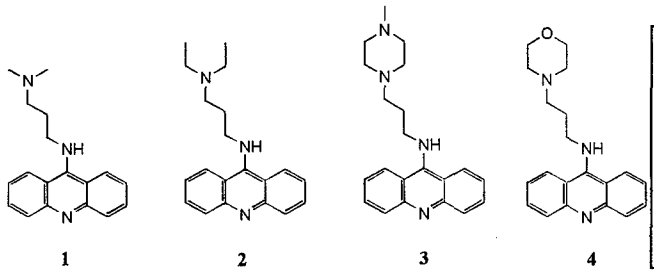

Figure 1: (**1.**)N'-Acridin-9-yl-N,N-dimethyl-propane-1,3-diamine, (**2.**) N'-Acridin-9-yl-N,N-diethyl-propane-1,3-diamine), (**3.**) Acridin-9-yl[3-(4-methyl-piperazin-1-yl)propyl]-amine, (**4.**) Acridin-9-yl-(3-morpholin-4-yl-propyl)-amine

2.) Crystallize the core domain of p53 in complex with a molecule shown to pharmacologically rescue p53.

Using the already purified mouse core domain (residues 92-292) multiple attempts were made to obtain co-crystals with the compounds. After a number of screening attempts, no co-crystals were ever observed. In light of this, we decided to assay the compounds for interaction with the p53 core domain. The acridyl ring system has unique and easily detected fluorescent properties. It has an excitation peak at ~400nm and an emission peak at ~454nm. Using a spectrofluorimeter with an excitation filter of 408nm and an emission filter of 465nm, we were unable to detect binding at

concentrations as high as 600μM of core domain using 4μM of the compound, a 150x excess. Similarly, larger constructs core and oligomerization domains (86-351) and -core, oligomerization and regulatory domains (89-390) did not display any binding activity. However, at identical concentrations of the compound using this assay, we were able to detect binding of the compounds to double stranded DNA, with a K_d of about 1.5uM. Considering this data, we were forced to conclude that the compounds do not bind directly to the p53 core domain as Pfizer had stated. At about the same time, the Fersht group in England published a paper in *Oncogene*(6) supporting our observations both *in vivo* and *in vitro*.

Given our findings concerning the compounds published by Foster et al., we became very interested in discovering small molecule leads that would directly bind to and stabilize the p53 core domain using structure guided methods. To this end, screening was initiated to find a crystal form of the mouse p53 core domain that diffracted to high resolution, with the goal of mapping potential binding surfaces of the core domain using small molecule probes such as isopropanol, acetonitrile, phenol, and acetones. This technique for mapping potential drug targets has been exploited for other systems(7, 8, 9). Briefly, various organic solvents are soaked into the crystal lattice, and then their interaction sites located in electron density. Using this approach, in which well diffracting crystals are crucial, it is possible to determine an 'active site' which would be targeted when designing small molecule therapeutics which interact with and stabilize the core domain.

Well diffracting crystals from this screening were obtained by the hanging drop method using a reservoir solution of 100mM Tris pH 7.0 and 16-18% PEG 2K MME at

20°C. Crystals appeared overnight and grew to typical size of 0.3mm x 0.1mm x 0.1mm in 2-3 days. Crystals were flash frozen in 100mM Tris pH 7.0, 20% PEG 2K MME, and 20% MPD.

A high resolution dataset was collected at the 19BM beamline at the Advanced Photon Source, Argonne National Laboratory using a Quantum CCD (charged coupled detector.) Crystals belonged to the spacegroup C2 with one molecule per asymmetric unit and diffracted to a maximum of 1.38Å. Data from 1.55Å - 1.38Å were eventually discarded due to weak intensity. This crystal form represents the most well diffracting of the p53 core domain to date. The structure was solved and refined to a final R_{free}/R_{factor} of 17.67 and 22.81 respectively. Anisotropic temperature factors were refined, providing a more inclusive picture of disorder of the p53 core domain.

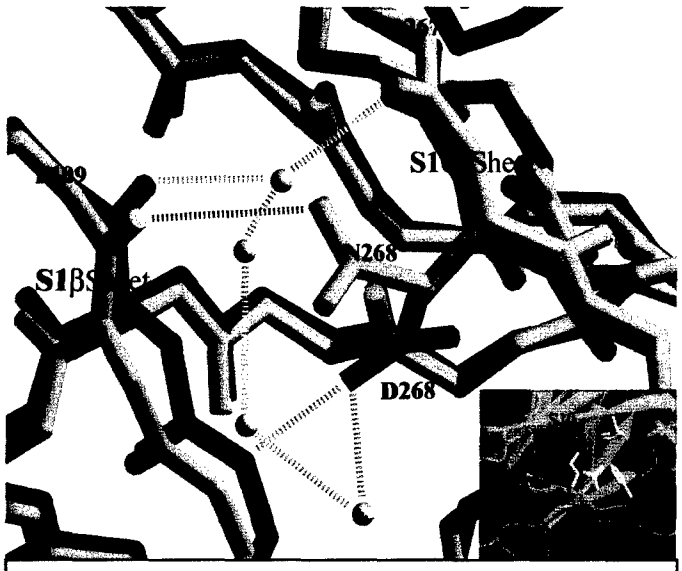

Figure 2: Overlay of the mouse p53 core domain (blue) with the human (green). Light blue spheres are water molecules, orange dashed lines represent hydrogen bonds within the mouse core domain, red dashed lines represent hydrogen bonds within the human core domain. Inset: Overview of the human core domain at the region of interest. (Adapted from(7))

Interestingly, in the human p53 core domain, the second site mutation N268D has been shown to provide thermodynamic stability to destabilized mutants, and in particular to rescue the V143A mutant. Introduction of the N268D mutation to the wild type imparts increased stability, suggesting that it provides global stability to the protein. In

the mouse core domain, this residue is a naturally occurring D268. D268 is a very close distance-wise from V143. In the human core domain, N268 is involved in a hydrogen bond to the backbone carbonyl of F109. From the high resolution structure of the mouse core domain, (Figure 2) we can see that the D268 is involved in hydrogen bonding interactions with the backbone nitrogen of L111. Furthermore, the side chain carbonyl of N268 is involved in a network of hydrogen bonds to water molecules, culminating with a water which interacts with backbone carbonyl carbons of both F109 and R267, forming a "bridge" in between the two. Based on this structural information, the increased hydrogen bonding interactions may help to stabilize the core domain by holding the S1 and S10 sheets together.

While carrying out the studies with the amino-acridines, we were also alerted to a report by Fersht and co-workers presenting a number of peptides that bound to human p53 core domain and restored its' function(10). Binding is relatively tight with a K_d of 0.5 μM. Based on these studies, I entered into a collaboration with Fersht's group to determine the structure of the p53 core domain in complex with these peptides. Human p53 core domain was cloned and large amounts were purified (Figure 3) in anticipation of crystal trials. I received peptides from Fersht's group and I am now attempting to grow co-crystals with the core domain. At the present, several promising crystal leads are being pursued.

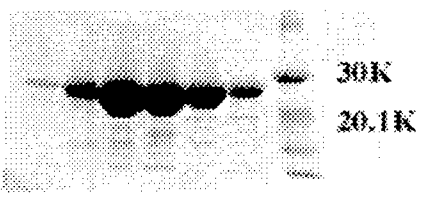

Figure 3: SDS-PAGE of purified human p53 core domain.

3.) ***Determine the x-ray structure of p53 core domain in complex with a molecule shown to pharmacologically rescue p53.***

This aim has yet to be completed.

4.) ***Design and synthesize molecules which bind tighter to the core domain of p53.***

This aim has yet to be completed.

Key research accomplishments

- Synthesized compounds that were reported by Foster et al. (4) to bind to and stabilize the p53 core domain.
- Determined that compounds from Foster et al. do not bind to the p53 core domain via a fluoresence based assay.
- Screened for a crystal of the mouse p53 core domain that diffracts to high resolution for the purposes of mapping binding surfaces using organic molecule soaking.
- Grew crystals of the mouse core domain which diffract to the highest resolution for the p53 core domain (1.55Å) known to date.
- Solved the structure of the high resolution data set and refined anisotropic thermal parameters to a final R_{free}/R_{factor} of 17.67 and 22.81 respectively. Soaking of this crystal form with various organic solvents is now underway.
- Cloned and purified the human p53 core domain (94-312) for the pu

Conclusions

Several conclusions can be reached as a result of the work directly funded by the Department of Defense. Firstly, it was established that the class of compounds published by Foster et al. (4) did not interact directly with the p53 core domain, even though it was hypothesized as such in their publication. This conclusion is supported by other published results. Second, the discovery of a crystal form of the p53 core domain which diffracts to high resolution (1.55Å) will permit a more detailed picture of the core domain to emerge based on the extra data available-for example, refinement of anisotropic thermal parameters. Furthermore, this highly stable crystal form will allow the mapping of the potential binding surfaces on the core domain based on co-crystals with probe molecules. This potentially valuable information can allow the *de novo* design of lead molecules that interact with the core domain and stabilize it. Third, the purification of the human core domain has allowed crystallography experiments to be performed using peptides that have been found to directly interact with the human p53 core domain and stabilize it. Structural information about this peptide/core domain complex can be extremely informative in the discovery of more effective molecules.

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