Investigate the Potential of Small Molecule Drug Candidates for the Inhibition of p53 Mutant Aggregation and Cancer Cell Proliferation

by

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LIST OF ABBREVIATIONS

ThT	Thioflavin T
DLS	Dynamic Light Scattering
AFM	Atomic Force Microscopy
TEM	Transmission Electron Microscopy
AC	Acetylcholine chloride
PepR248W	Peptide Sequence WRPILTIITL
PepR248Q	Peptide Sequence QRPILTIITL
PepR248	Peptide Sequence RRPILTIITL
PLA	Polyarginine
ORT	Polyornithine
CNV	Canavanine
CIT	Citrulline
hBMVEC	Human brain microvascular endothelial cells
Cga	N-α-carbamoyl-L-glutamine 1-amide
BH	Betaine hydrochloride
SEM	Standard error of the mean
CIS	Cisplatin
K	Aggregation rate

ABSTRACT

The master's thesis study focuses on the identification of novel small molecule drug candidates for inhibiting cancer causing p53 mutant peptide aggregation and tumor growth. p53 protein is a tumor suppressor protein, and controls cellular function and unwanted cell proliferation. When p53 is mutated it loses its function. Mutations of p53 are present in almost about 50-70% of all cancers. In a recent study, it has been reported that the p53 mutations cause aggregation and subsequent loss of p53 function, negative dominance and cell toxicity leading to advanced cancers. Further, p53 mutant aggregation has been observed in several cancers. Hence, there is growing interest in finding therapies for p53 mutant aggregation associated cancer.

The objective of the thesis study include studying the inhibitory effect of small molecule drugs on p53 aggregation in vitro, their inhibitory potential on p53 mutant cancer cells proliferation in vitro, and finally a nanoformulation of p53-anti aggregation drug candidates to treat p53 aggregation associated cancer with increased therapeutic efficacy. Characterization tools used for this study include biochemical assays, transmission electron microscopy, confocal microscopy, atomic force microscopy, dynamic light scattering, and cellular assays. The results of the thesis study show potential of small molecule drugs for treating cancer due to p53 aggregation.

Keywords: Aggregation, p53 peptide, small molecules, cancer.

Chapter 1

Introduction

p53 is a tumor suppressor protein. Under normal conditions, it arrests cell proliferation and promotes DNA repair. So p53 is hailed as the "guardian of genome". However, p53 is also one of the most frequently mutated proteins in human cancers, which affect over fifty percent of all cancers. The mutations inactivate p53 by changing the DNA-binding ability of mutant p53 and the interaction with downstream proteins or DNA, which will lead to the failure of p53 function [1]. In recent studies, it has been reported that the p53 mutations cause aggregation and subsequent loss of p53 function, negative dominance and cell toxicity leading to advanced cancers [2]. p53 aggregation has been observed in ovarian, breast, lung, bladder, colon, pharynx, and brain cancers [3, 4]. Hence it is important to find effective therapies to overcome this protein mutation-aggregation related cancer. Diversity of therapies has been emerged to rescue p53 mutant functional loss [5]. Theories of restoring p53 downstream functions and promoting mutant p53 degradation catch intensive attention [5]. But, therapeutic drugs for p53 aggregation and cancer is relatively new and need further research. As evidence shows mutant p53 aggregation plays a critical role in cancer, studying the aggregation tendency and the development of successful new drugs is important for cancer therapy.

Hence, the goal of the thesis study is to identify new natural small molecule drugs for p53 mutant aggregation associated cancer. Usage of natural small molecules for p53 aggregation

associated cancer, hasn't been explored before. Recently published studies suggest that the aggregation of specific segments of p53 can reveal the aggregation of whole sequence of p53 [6]. For the thesis study, we use mutant p53 segments to study the aggregation and to find natural small molecule drugs to inhibit aggregation of and also cancer cells proliferation inhibition.

Small osmolyte molecules have been widely used to stabilize protein aggregation and correcting protein misfolding [7]. In many studies, mutant p53 is aggregating in cancer cell line, and more than half of the cancer is induced by the loss of function of p53, caused by the mutation and misfolding in protein [3]. Hence, small osmolytes could have potential in p53 aggregation inhibition. In this thesis, we study the inhibitory effect of charged, aminoacid, and non-charged osmolyte molecules for p53 aggregation associated cancer.

Further, recent report suggests that p53 aggregation can increase chemoresistance [8]. Chemoresistance impacts the curing effect and limits the usage of chemodrug in the clinic. Cisplatin is a kind of classic and effective chemotherapeutic drug for cancers, however cisplatin-induce chemoresistance, and minimizes the therapeutic effect [9]. So in our study, we test whether there will be any improvement of chemotherapy if we use small molecule drugs that show inhibitory effect on the inhibition of aggregating of mutant p53 segments leading to chemosensitization.

Finally, in order to increase the therapeutic effect of drugs, nanotechnology approaches is utilized. Nanoformulations have unique advantages to overcome some harsh conditions of traditional drug delivery, due to poor drug availability [10]. Liposomes are introduced to help deliver drug, because of the ability to encapsulate various drug as well surface conjugate with tissue specific

target moieties [11]. In this thesis study, we develop liposomal anti-aggregating drugs, to improve the therapeutic efficacy of p53 anti-aggregation drugs.

Research Objectives

The research objectives of this thesis will be achieved by following specific aims:

Specific Aim1: Identify natural small molecule drugs for p53 mutant aggregation and cancer cells proliferation inhibition

Specific Aim2: Investigate the effect of small molecule drugs on p53 mutant associated chemoresistance.

Specific Aim3: Develop liposomal nanoformulations for delivery of anti-p53 aggregation drugs with increased therapeutic efficacy

Thesis Organization

This thesis is organized into seven chapters. Chapter 2 is a general background of p53 protein and aggregation, as well as approaches used to characterize the aggregation. Potential drug therapeutics approaches for rescuing p53 function are also briefly reviewed. The intention of the background is to provide the context for the motivation and goals of this work and to include an outline of the specific objectives of this work. Chapter 3 is a paper published in the protein and peptide letters that addresses the effect of cationic osmolytes on p53 aggregation. Chapter 4 is another paper published in biochemical and biophysical research communications, on polyarginine and its analogues on p53 mutant associated cancer cell proliferation inhibition. Investigation into the role of p53 aggregation on chemoresistance is presented in Chapter 5. Chapter 6 describes the development of liposomal drug depot for delivery of p53 anti-aggregation

compounds. Finally chapter 7 includes general conclusions and a summary of the contributions of this work.

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Chapter 2

Background

2.1 p53 protein and aggregation

2.1.1 p53 protein

p53 protein, was dubbed the 'guardian of genome' due to its ability to inhibit cancer by maintaining various cellular functions. p53 exists as a tetrameric nuclear protein that can interact with many diverse proteins or DNA， which will start cell cycle arrest DNA repair, apoptosis, transformation angiogenesis or differentiation and metabolism [1]. The p53 protein consists of 393 amino acids. The four major functional domains are transcriptional activation domain (amino acids 1-42), DNA binding domain (amino acids 102-292), oligomarization domain (amino acids 323-356), and regulatory domain (amino acids 360-393) (Figure 1) [2]. When stresses, like DNA damage, nutrient starvation, heat shock, virus infection, pH change hypoxia and oncogene activation, p53 gets activated and regulate normal cellular response [3]. When uncontrolled cell growth results, p53 induces p21 expression leading to cell cycle arrest. When damage is beyond repair, p53 triggers programmed cell death, and it is thought that p53-mediated apoptosis is the primary cause of tumor suppression. Many apoptosis-involved genes are triggered by p53, especially some in the intrinsic pathway of apoptosis. Some proapoptotic member of Bcl-2 family, like BAX, can be upregulated by p53. Other p53-target genes also involved in cell-cycle control include miR-34a and 14-3-3 proteins [4]. Altogether, the presence of a functional p53 is important for the different checkpoints of the cell cycle, thereby giving cells time to fix DNA

damage.

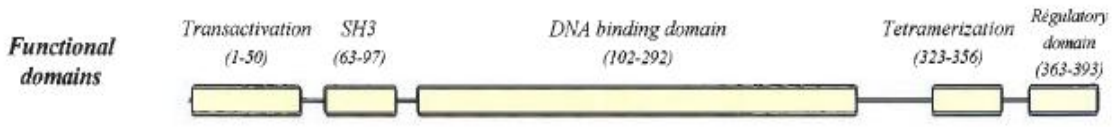

Figure 2.1 Schematic of the p53 protein structure [2]

2.1.2 p53 protein aggregation and cancer

When the p53 gene is mutated, the protein misfolds and aggregates, resulting in an inactive form of the protein [5-7]. This leads to abnormal cells to grow in an exponential manner. The p53 protein mutation is thought to be responsible for about 50 percent of all the cancers and as high as 70% for all lung cancers [8, 9]. More frequently p53 loses its function by single point mutation, resulting in DNA binding capacity (contact mutant), or destabilize the protein (conformational mutants). Several hot spot mutants (both contact and conformational) are frequently observed in most cancers with mutations at aminoacid locations 175, 245, 248, 249, 273, and 282 [10]. Recent publications show that, contact and conformational mutant p53 aggregation is observed in tumor tissue samples from patients, biopsies, as well as in several cancer cell lines indicating the correlation between aggregation and tumor growth [5, 11]. p53 aggregation has been shown to observed in ovarian, breast, lung, and skin cancers [11]. Studies reveal several hot spot mutants with aggregation forms in tumor tissues. The studies further report that the aggregated mutant p53 proteins loses their functionality. Hence, development of novel drug depots that target the p53 mutant aggregations could aid in new directions in treating cancer.

Recent work on potential reactivation of the p53 mutant protein *in vivo* [12, 13] gives encouragement in developing novel anti-cancer drugs for the treatment of p53 mutations aggregation associated cancer. Reactivating some wild-type p53 functions becomes an attractive therapeutic strategy [12, 14]. Some compounds can bind to a site in p53 formed in the Y200C

mutant function and stabilize the structure of this mutant [14]. As a result, it increases the p53 level with a wild-type conformation and activity. Other compounds bind to multiple mutant p53 proteins, like PRIMA-1, interacting with DNA binding domain, therefore it promotes the proper fold of mutant protein and restoration of p53 functions [15]. On the other hand, aggregation of mutant p53 is observed to be responsible for half of the cancer cells, and the therapeutic strategies for aggregation inhibition is recently gained interests. Recent studies are investigating the aggregation tendencies of p53 conserved segment in vitro [16]. Study by Sorangi et al. [17] reported in *Cancer Cell*, show that the inhibition of p53 mutant aggregation by a designer peptide, rescues p53 functionality, and inhibits tumor growth in ovarian cancer models, indicating the importance of p53 aggregation in cancer. Hence it is important to further study and develop novel therapeutic strategies for p53 aggregation associated cancer.

2.1.3 Small molecule drug inhibitors

Osmolytes or natural small stress molecules are believed to stabilize these organisms under harsh conditions. They accumulate to high intracellular concentrations preventing the misfolding/ denaturation of proteins and other labile macromolecular structures, and thereby preserving their functions from environmental stresses [18]. Further, the presence of these molecules had proved highly effective in preserving enzymatic activities against heating, freezing and drying [18, 19]. The ability of these molecules to stabilize biological functions under extreme conditions make them ideal drug candidates for stress related pathologies such as cancer, neurodegenerative diseases and AIDS [18]. Several studies have shown the effectiveness of these molecules in the inhibition of neurodegeneration causing peptide aggregation such as abeta 42 and prion peptides [18, 20, 21].

Interestingly, p53 mutant cancers may share a common aggregation mechanism with neurodegenerative diseases. Recently Silva et. al. [5] has shown that the mutant form of p53

exhibits prion aggregate-like behavior, suggesting the possibilities for similarities between the neurodegenerative peptides and p53 mutant aggregation mechanism in cancer. Malignant tumors featuring p53 mutations have exhibited aggregation-like behavior and have been detected in breast cancer, ovarian cancer, and skin cancer tissues [22, 23]. Hence, osmolytes, which have been shown to be extremely effective in inhibiting abeta 42, and prion peptide aggregation inhibition, could be ideal drugs for treating p53 aggregation related cancer pathology. Although several small molecule drugs have been studied for inhibiting the p53 mutant related aggregation, the effect of osmolyte molecules on p53 mutant aggregation and folding is yet to be explored [12-13].

2.1.4 Effect of p53 and chemoresistance

Cisplatin is widely used as an effective chemotherapeutic drug for curing many types of cancers. The mechanisms of cisplatin-mediated cytotoxicity include DNA damage, and apoptosis signal activation [24]. However, this efficacy of cisplatin is limited by cheomoresistance of cancer cell. For example, it has been reported that over 70% of lung cancer patients exhibit cisplatin chemoresistance [25].

The loss of p53 activity in tumors bearing wild type p53 is believed to correlate with its location in the cytoplasm. When p53 is mutated and aggregated it has been shown to localize in the cytoplasm [11]. It has been reported when p53 is located to the cytoplasm the tumors are less responsive to chemo and radiotherapy [26]. Hence, the understanding of p53 aggregation, and its effect on chemoresistance could give additional information into chemoresistance. In our study, we are study whether the cisplatin therapeutic efficacy can be improved when involving with drugs that can inhibit mutant p53 aggregation.

2.1.5 Nanotechnology based drug delivery approach

Current cancer therapies usually have trouble to minimize the mortality and morbidity because of the adverse effects on normal tissues. Nanotechnology approaches have the advantage to remedy some of the problems faced by conventional systemic chemotherapeutic tumor drug delivery [27-29]. Nanoparticles start drawing more attention, because the property of nanoparticles shows potential to deliver drug efficiently but keep away from normal tissues. By designing the nanoparticles structure, other advantages include higher bioavailability and plasma solubility, lower systemic toxicity, patient compliance improvement and increasing therapeutic efficacy of short half-live molecules. Hence a nanocarrier-based delivery approach of the p53 anti-aggregation formulation is needed for effective delivery. Several nanoformulations are used in drug delivery. Polymeric nanoparticles, and liposomes have advantages among them due to their ability to incorporate or surface coat variety of drugs. In this thesis liposome based delivery approach is developed.

2.2 Aggregation and Polymer formulation Methods

2.2.1 Peptide Aggregation

p53 has capacity to form amyloid both in vitro and in cancer cells. And the segments that they contain can be predicted to form amyloid. Studies have shown that p53 250-257 (PILTIITL) region is conserved across different species and also has highly amyloidogenic tendency in vitro [16]. According to this property of segments of p53, we can predict the aggregation tendency of mutant p53 by aggregating segment of them. For the thesis study, we utilize this peptide region, to use model wildtype and mutant peptides for the aggregation study. This reduces the cost of studying the full protein aggregation, which is extremely expensive, and hence wouldn't be possible for the thesis study.

2.2.2 Liposome Formulation

Liposomes are defined as microscopic/nanosize spherical-shaped vesicle, with an internal liquid sac wrapped with one or multiple lipid bilayers [30, 31]. Liposome membrane consists of the same material as a cell membrane. With such property of bilayer, liposomes are able to carry both hydrophilic and lipophilic molecules. Hydrophilic drug are encapsulated inside the liposomes, while the lipophilic drug are encapsulated in lipid bilayer. Figure 2 depicts the various liposome morphology and size [31]. Liposome provides selective passive targeting to cancer cells. Its capacity of encapsulation increases the efficacy by enhancing the solubility and stability of the encapsulated drugs. Further, the liposome improves the drug bioavailability in vivo. Other advantages of liposome include low toxicity of phospholipids content, drug protection against degradation effect induced by pH change and light, and minimization of side effects.

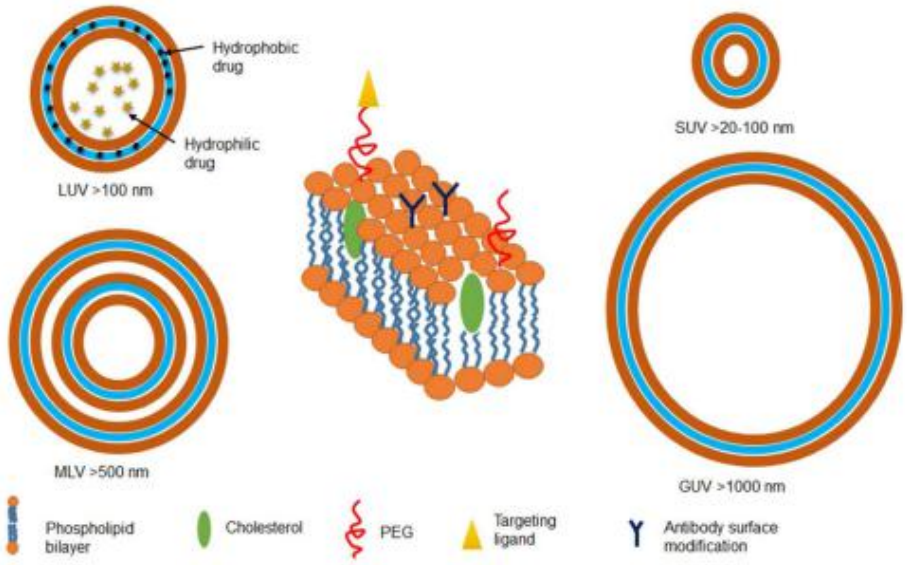

Figure 2.2. Types of liposomes, morphology, and sizes [31]

2.3 Characterization Methods

2.3.1 Spectroscopic Techniques

2.3.1.1 Thioflavin T fluorescence

ThT is a benzothiazole dye, that exhibits enhanced fluorescence 440/482 upon binding to aggregated proteins/ peptides/ amyloid fibrils, and is frequently used to diagnose pathological protein aggregation [32, 33]. In our experiments, we perform ThT binding experiments to evaluate the aggregation tendency of p53 peptide samples.

2.3.1.2 Congo red Binding

Congo red, similar to ThT, is widely used technique to evaluate amyloid aggregation level in vitro and in vivo. Congo red can bind with amyloid form of protein or peptide but not with the monomeric form, which makes it a proper method to determine amyloid fibrils in the samples.
Congo red absorbance and congo red fluorescence are two major techniques for peptide studies. By comparing congo red absorbance, congo red fluorescence and Thioflavin T fluorescence data, the conclusion will be more reliable.

2.3.1.3 Dynamic Light Scattering

DLS is used to predict particle size. In our studies DLS will be performed to predict the size of the peptide aggregates with and without the small molecule drugs.

2.3.2 Microscopy Techniques

2.3.2.1. Confocal Microscopy

Confocal microscopy is widely used to optical imaging method where higher resolution is necessary. In our study we use the confocal microscope to image cells, after immunofluorescence staining.

2.3.2.2 Atomic Force Microscopy

Atomic force microscopy, scanning probe microscopes with dimensional resolution approaching 0.1nm, makes it possible for visualizing material up to nanoscale. AFM analysis is a fast and not invasive method to observe the size and shape of the sample. In our cases, AFM provides us the convenience to compare the size of liposome with different content and measure the aggregation status of the peptide. As for AFM, samples will be spotted on mica grids and AFM measurements will be performed under taping mode conditions.

2.3.2.3 Transmission electron microscopy

TEM is used to visualize the aggregation/ disaggregation process of p53 mutant peptides with and without the small molecules. For TEM imaging, the samples are spotted on holy carbon copper grids, and then stained with 1% uranyl formate. The samples are imaged using the EMAL and MIL facilities at UM Ann Arbor.

2.3.3 Cell Assays

For the cell assays proliferation and immunofluorescence methods are used. For the proliferation XTT assay is used in the study. It is a colorimetric method that correlates with the cell metabolism and viability. Immunofluorescene is used to visualize expression of cellular markers due to various treatments. For the immunofluorescence, cells will be stained with primary and secondary antibodies, and imaged using confocal microscope.

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Chapter 3

Inhibition of p53 Mutant Peptide Aggregation *In Vitro* by Cationic Osmolyte Acetylcholine Chloride

(Modified from the paper published in Protein and Peptide Letters, 24(4): 353-357) Zhaolin Chen, and Mathumai Kanapathipillai

3.1. Abstract

Mutations of tumor suppressor protein p53 are present in almost about 50% of all cancers. It has been reported that the p53 mutations cause aggregation and subsequent loss of p53 function, leading to cancer progression. Here in this study we focus on the inhibitory effects of cationic osmolyte molecules acetylcholine chloride, and choline on an aggregation prone 10 amino acid p53 mutant peptide WRPILTIITL, and the corresponding wildtype peptide RRPILTIITL in vitro. The characterization tools used for this study include Thioflavin- T (ThT) induced fluorescence, transmission electron microscopy (TEM), congo red binding, turbidity, dynamic light scattering (DLS), and cell viability assays. The results show that acetylcholine chloride in micromolar concentrations significantly inhibit p53 mutant peptide aggregation in vitro, and could be promising candidate for p53 mutant/ misfolded protein aggregation inhibition.

3.2. Introduction

Many human disorders are caused by defects in protein folding due to genetic mutations or adverse environmental conditions and are commonly referred to as protein misfolding diseases

[1]. The disorders result in dysfunction in cellular process either due to protein aggregation, instability, lack of folding or defective trafficking[1, 2]. While too much of an incorrectly folded protein causes amyloidosis, lack of a correctly folded protein is thought to be involved in cancer [3, 4]. It mainly affects a protein called p53, which play a major role in the body's cancer resistance network [4-7]. When the p53 gene is mutated, the protein misfolds and aggregates, resulting in an inactive form of the protein. This leads to abnormal cells to grow in an exponential manner. p53 protein mutation/misfolding is thought to be responsible for about 50 percent of all the cancers [6]. Hence, it is imperative to find drugs that target p53 misfolding/aggregation.

Osmolytes, found in organisms that live in extreme environments accumulate to high intracellular concentrations preventing the misfolding/ denaturation of proteins and other labile macromolecular structures, and thereby preserving their functions from environmental stresses [8]. They accumulate to high intracellular concentrations preventing the misfolding/ denaturation of proteins and other labile macromolecular structures, and thereby preserving their functions from environmental stresses. The ability of these molecules to stabilize biological functions under extreme conditions make them ideal drug candidates for stress related pathologies such as cancer, neurodegenerative diseases. Further, several studies have shown the effectiveness of natural osmolytes in the inhibition of neurodegeneration causing peptide aggregation such as abeta 42 and prion peptides [9-12].

Among the different osmolytes, cationic osmolytes acetylcholine chloride and choline has shown to accumulate in large quantities to overcome osmotic stress in *Lactobacillus plantarum* [13]. Further, acetylcholine chloride has shown to have anticancer effects in lung cancer studies [14]. Recently Silva et al. [15] has shown that the mutant form of p53 exhibits prion aggregate-like behavior, suggesting the possibilities for similarities between the neurodegenerative peptides and

misfolded p53 aggregation mechanism in cancer. Hence, osmolytes, which have been shown to be extremely effective in stabilizing protein folding and aggregation, could be ideal candidates for treating p53 aggregation related cancer pathology. Although several small molecules, and designer peptides have been studied for inhibiting the p53 mutant functionality, and aggregation, the effect of cationic osmolyte molecules on p53 aggregation and folding is yet to be explored [16-18]. Hence the study on their inhibitory effect on p53 mutant peptide aggregation, could reveal, novel information of p53 mutant peptide aggregation inhibition.

Human p53 protein has been shown to possess many single mutated regions with highly amyloidogenic propensity. Among them p53 R248W is one of the p53 hot spot mutants that has shown to occur in high frequency [19, 20]. Hence in order to understand its amyloidogenic potential, and to screen for potential drug candidates to stabilize this mutant, we have chosen a 10 amino acids peptide sequence WRPILTIITL (248-257) to mimic the R248W amyloidogenesis. The mutant peptide sequence is a good model for the proof on principle study reported in this paper, and to minimize the costly study from using the full-length p53 mutant protein. In addition, the peptide sequence RRPILTIITL (p53 248-257), was used for the corresponding wildtype peptide sequence. In this paper, the inhibitory effects of acetylcholine chloride and choline on the R28W mutant mimetic peptide aggregation under *in vitro* conditions are studied in detail.

3.3. Materials and Methods

3.3.1. Materials

p53 mutant and wild peptides were custom synthesized from Genscript. All other materials were

purchased from Sigma Aldrich (St.Louis, MO).

3.3.2. *In vitro* aggregation formation

p53 peptide aggregation was induced with small modification from a previously reported method [18]. Briefly, peptides of 0.7 mM concentration were dissolved in 1 mM LiOH, 100 μM of tris buffer pH7, 50 μM ThT, and with or without the cationic osmolytes of concentrations ranging from 70 μM - 0.7 mM. The mixture was maintained at cold temperatures due to the rapid aggregation kinetics at room temperature. The peptide aggregation was characterized by Thioflavin-T (ThT), turbidity, Transmission electron microscopy (TEM), congo red, and dynamic light scattering (DLS) measurements.

3.3.3. ThT fluorescence

Briefly, peptides of 0.7 mM concentration, were dissolved in 1 mM LiOH, 100 μM tri buffer, pH7 and 50 μM ThT with or without the cationic osmolytes of concentrations ranging from 70 μM- 0.7 mM. The samples were prepared at cold temperature, and the aggregation kinetics was measured immediately. ThT fluorescence was measured at 440/482 nm excitation and emission using a spectrophotometer in the lab. Measurements were performed every 2 minutes for an hour at room temperature. The experiments were repeated for three independent experiments.

3.3.4. TEM

Peptides were aggregated using the same procedure used for the ThT measurement. For TEM imaging, the samples were then spotted on holy carbon copper grids, and then stained with 1% uranyl formate. The samples were imaged using JEOL TEM microscope at 80 kV, at the microscope facility in the UM Ann Arbor medical school.

3.3.5. Congo Red

Congo red (10 mg) was dissolved in 50 ml of DI water/ 10% ethanol mixture. The solution was then filtered through 0.22 μm filter, to remove undissolved particulates. 15 μl of the filtered

congo red was mixed with 85 µl of 50 µM peptide aggregates samples with and without acetylcholine chloride. The samples were then characterized using the spectrophotometer and the absorbance measurements were recorded from 600 -700 nm. The final sample absorbance measurements were subtracted from congo red absorbance alone. The experiments were repeated for three independent experiments.

3.3.6. Turbidity

Turbidity of aggregated peptide samples with and without acetylcholine chloride were performed using spectramax spectrophotometer in the lab. Turbidity measurements were performed at 600 nm wavelength. The experiments were repeated for three independent experiments.

3.3.7. DLS

DLS measurements were performed using a Malvern zetasizer instrument in the lab. Similar aggregation procedures used for the ThT, congo red and turbidity assays were used for the aggregation size measurements. Briefly, peptides of 0.7 mM concentration, were dissolved in 1 mM LiOH, 100 µM tri buffer, pH7 with or without the cationic osmolytes of concentrations ranging from 70 µM- 0.7 mM. Size based on scattering intensity was measured of 3-independent experiments.

3.3.8. XTT assay

To assess the cellular toxicity of cationic osmolytes, XTT assay was performed. SH- SY5Y cells were obtained from ATCC, and cultured according to the protocol. For the XTT assay, SH-SY5Y cells were cultured in 96 well plates at a density of 10,000 cells/well. After 24 hours, acetylcholine chloride with concentrations ranging from, 10 µm -1 mM were added, and the cells were incubated for an additional 48 hours. XTT assay was performed according the manufacturers protocol.

3.3.9. Statistical Analysis

Data were collected from three or more replicates for each experiment, and they are presented as mean ± standard error of the mean (SEM). P-values were determined from the results of at least 3-independent experiments. For statistical significance, unpaired t-test was used. The unpaired t-test is a good method to compare two population means, and would be appropriate to compare the treatment versus the no-treatment group in the study. The significance of the experiments was assessed by, comparing the acetylcholine chloride treatment with the no-treatment group.

3.4. Results and Discussion

Schematic of the concept used for the study is shown in Figure 1. In order to test the effect of the cationic osmolytes on the p53 peptides aggregation, first we tested the WRPILTIITL p53 mutant peptide (PepR248W) and the wild type peptide In order To test the effect of the cationic osmolytes on the p53 peptides aggregation, first we tested the WRPILTIITL p53 mutant peptide (PepR248W) and the wild type peptide RRPILTIITL (PepR248) using ThT measurement. ThT is a benzothiazole dye, that exhibits enhanced fluorescence upon binding to aggregated proteins/ peptides/ amyloid fibrils and is frequently used to diagnose pathological protein aggregation [21, 22]. The measurements show that the p53 mutant peptide PepR248W showed significant aggregation, compared to the corresponding wild type peptide PepR248 (Figure 1). Next, we tested the inhibitory effect of acetylcholine chloride, and choline on the PepR248W peptide aggregation. ThT fluorescence at 440/482 nm excitation and emission wavelength was measured every 2 minutes for 60 minutes to monitor the aggregation kinetics. The data show that acetylcholine chloride significantly inhibit PepR248W aggregation, while choline showed no effect on the inhibition (Figure 2 (i)). To assess the morphology of the samples at the end of the 48 h aggregation study, electron microscopy (TEM) images were obtained at UM-Ann Arbor core facility. The TEM images (Figure 2 (ii)) were in agreement with that of the corresponding

ThT sample measurements. We then did a dose dependent study of acetylcholine chloride to study the concentration effect of acetylcholine chloride on the mutant peptide PepR248W aggregation inhibition. The thioflavin T measurements show that the acetylcholine chloride concentrations as low as 200 μM were effective in inhibiting peptide aggregation, revealed by the ThT and congo red experiments (Figure 3A, B).

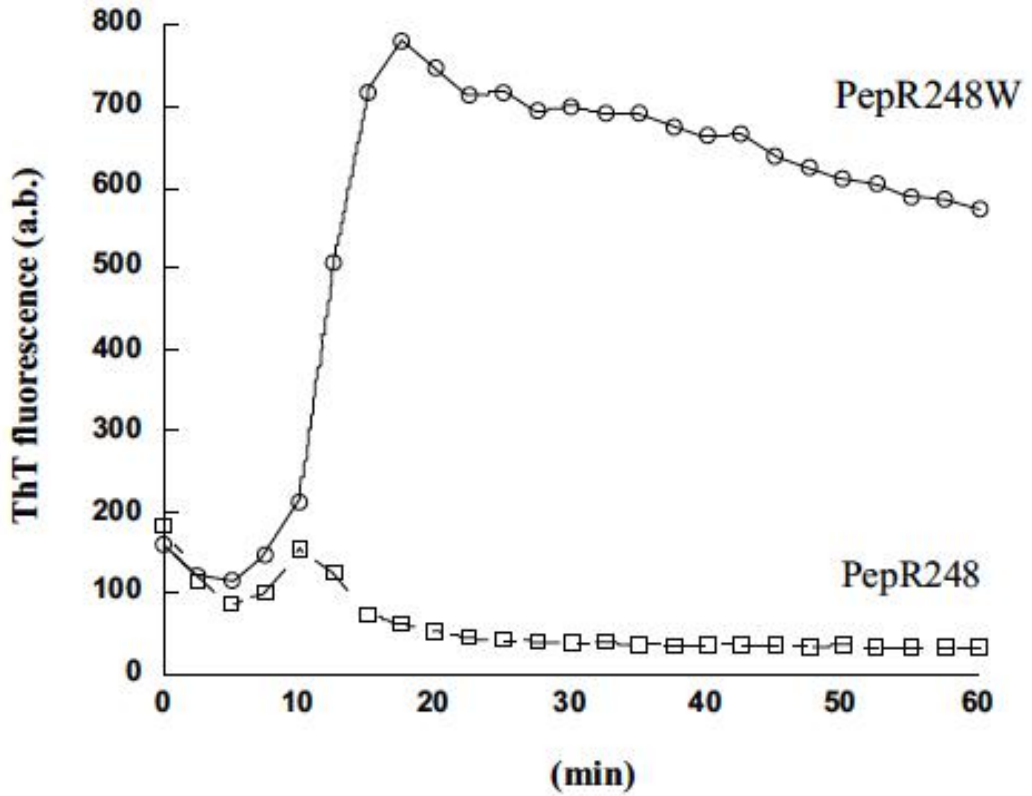

Figure 3.1. i). ThT fluorescence of 0.7 mM p53 peptides depicting the aggregation kinetics of the peptides with time. Mutant peptide PepR248W show increase thioflavin T fluorescence compared to the corresponding wildtype peptide PepR248.

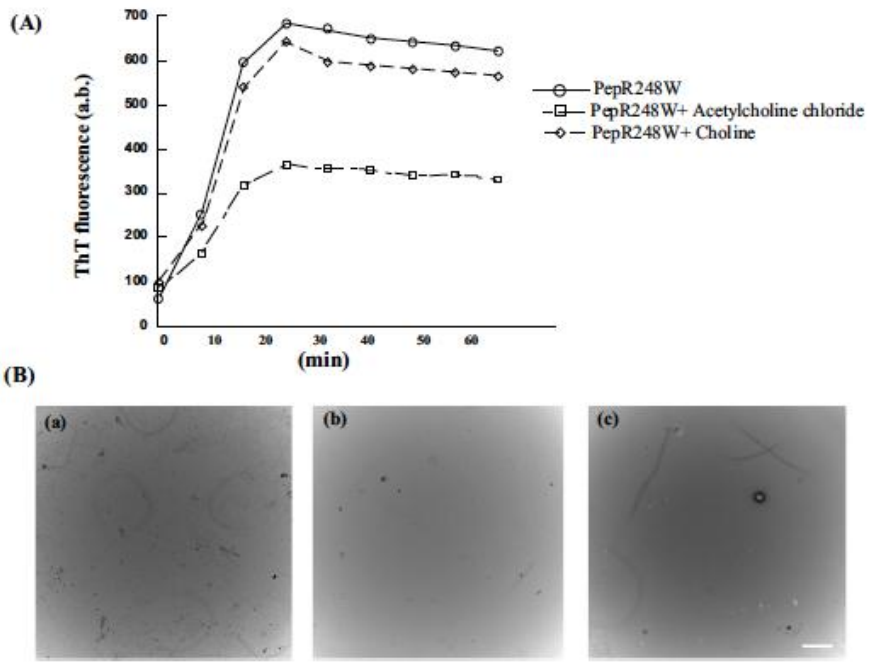

Figure 3.2. Mutant peptide PepR248W aggregation kinetics was studied with and without cationic osmolytes of same molar concentrations. **A).** ThT fluorescence depicting the inhibitory effect of acetylcholine chloride. **B).** Corresponding TEM images of (a) PepR248W only, (b) and (c), PepR248W aggregated in the presence of acetylcholine chloride, and choline. TEM images show less fibril and aggregation formation in the presence of acetylcholine chloride. Scale bar 500 nm.

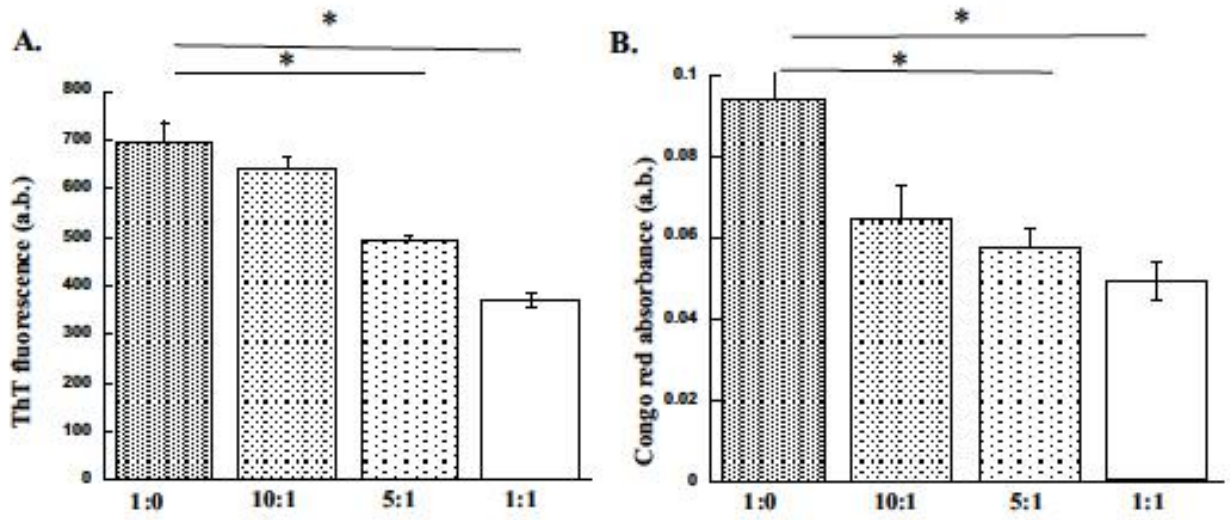

Figure 3.3. Dose response of acetylcholine chloride on the PepR248W aggregation **A).** ThT fluorescence **B).** Congo red, show the concentration dependent inhibitory effect of acetylcholine chloride. The ratios indicate peptide to acetylcholine chloride molar concentrations. * p-value < 0.01. Data are expressed as mean ± SEM.

To further confirm the aggregation potential of acetylcholine chloride, as a complementary technique, turbidity measurements were performed. Samples were measured at 600 nm wavelength, and results show significant turbidity for samples with peptide without acetylcholine chloride (Figure 4A), which corroborates with ThT fluorescence, and congo red measurement data. To assess the significance of the aggregation inhibition by acetylcholine chloride, we performed t-test analysis. The ThT, congo red, and turbidity measurements indicate that when the peptide to acetylcholine chloride molar ratio is below 10, significant aggregation inhibition could be achieved, reflected by p-values < 0.01 (Figure 3A, 3B and 4A). We also performed DLS measurements (Figure 4B) of the corresponding samples. Mutant peptide samples without any treatment showed average size around 1.2 μm while the acetylcholine chloride treated samples were around 800 nm and 900 nm in size. The size measurement corroborates with the ThT, TEM, turbidity, and congo red measurements. Finally, we used cell viability test to assess whether acetylcholine chloride has any toxicity effects on normal cells. Human neuroblastoma SH-SY5Y cells were incubated with acetylcholine chloride of concentrations ranging from 100 μM – 1 mM, and XTT assay was performed after 48 hours. Assay show, no significant toxicity effect of acetylcholine chloride up to 1 mM concentrations (Figure 5). This is significantly higher than the effective treatment of 200 μM acetylcholine chloride needed for peptide aggregation inhibition (Figure 3A, B). Hence, acetylcholine chloride could have therapeutic potential in treating p53 aggregation inhibition with minimal toxicity.

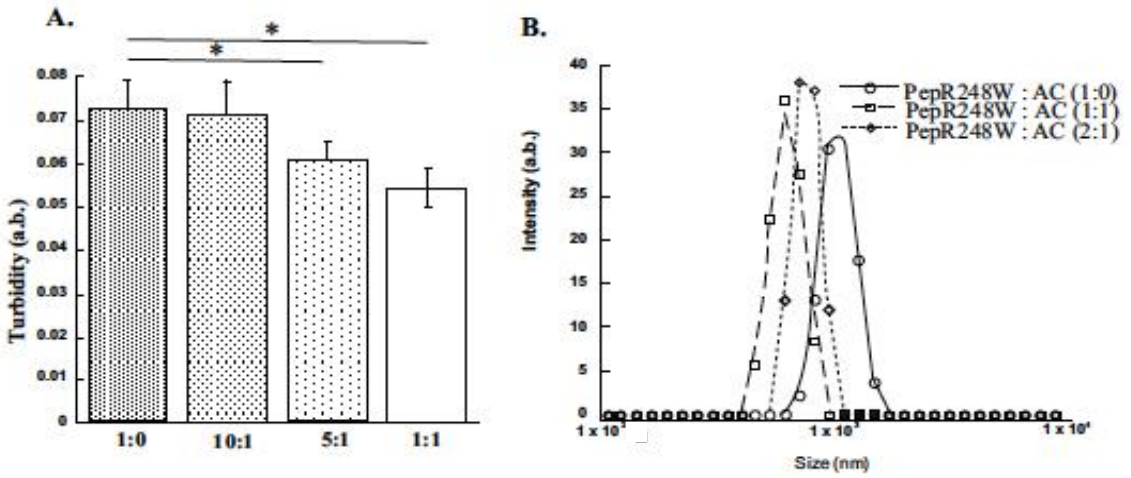

Figure 3.4. **A.**Turbidity measurement performed on the PepR248W aggregation samples at 600 nm. The ratios indicate peptide to acetylcholine chloride molar concentrations. * p-value < 0.01. Data are expressed as mean ± SEM. **4B.** DLS measurements of the peptide PepR248W aggregation with and without acetylcholine chloride. The size measurements show a decrease in peptide size aggregations in the presence of acetylcholine chloride.

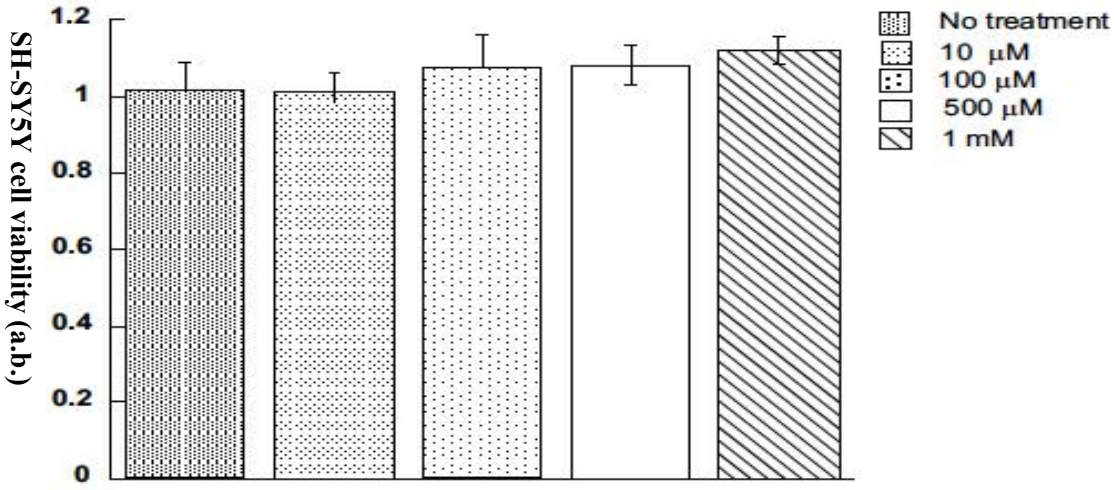

Figure 3.5. XTT assay, showing no significant effect of acetylcholine chloride concentrations ranging from 10 µM – 1 mM on the cell viability of SH-SY5Y cells.

In this study we show that cationic osmolyte acetylcholine chloride has the potential to inhibit mutant p53 peptide aggregation and hence could have therapeutic potential in treating the p53 mutant protein aggregation in cancer. The mechanism by which osmolyte molecules protect macromolecules has not been completely understood so far. It is widely believed that the

stabilization effect is achieved by the preferential hydration in the intermediate surface ofthe protein, resulting in preferential separation of small stress molecules from peptide/protein monomers and hydration of them by solvent molecules [23-25]. Hence we believe that the preferential hydration of p53 peptide by acetylcholine chloride may have reduced the possibility of formation of aggregation and subsequent fibrillar structures of the peptide. In addition, the acetyl group in the molecule seems to be essential for the preferential hydration, as the stabilization effect is not observed in the presence of choline. The stabilization could have further strengthened by specific hydrophobic interaction between the molecules and peptide that may have aid in the disruption of hydrogen bond formation between partially aggregated peptide monomers. The results also strengthen by our previous findings where, ectoine and hydroxyectoine shown to have significant inhibitory effect on Alzheimer's Aβ 42 peptide [9], and insulin amyloid fiber formation *in vitro* [10], further indicating the potential of osmolytes in stabilizing protein aggregation.

Different osmolyte stress molecules differ in their ability to protect against specific stresses. It is therefore necessary to identify the osmolyte or mixture of those under different stress conditions. Hence, the study would pave way to design high-throughput screening methods and assays to develop novel synthetic multifunctional osmolyte molecules or chimeric molecules to elucidate their structural influences on protein aggregation inhibition to treat cancer due to p53 mutant aggregation. Lastly, the finding reported here will open the potential of new cationic small molecule candidates to treat cancer associated p53 aggregation.

3.5. Acknowledgements

We would like to thank the University of Michigan Dearborn department of Mechanical engineering, and the College of Engineering and Computer Science for funding. We would like to thank the University of Michigan Microscope and Imaging (MIL) facility for the use of TEM and confocal microscopes.

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Chapter 4

Polyarginine and Its Analogues Inhibit p53 Mutant Aggregation and Cancer Cell Proliferation *In Vitro*

(A paper published in Biochemical Biophysical Research Communications)Zhaolin Chen, Jun Chen, Venkateshwar Keshamouni, and Mathumai Kanapathipillai
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4.1. Abstract

Arginine, a cationic amino acid is known to stabilize proteins under harsh conditions. It is widely used to stabilize protein aggregation, and to correct protein folding during protein production. Hence it would be a good therapeutic candidate for treating protein aggregation related diseases. Recent reports suggest, that the aggregation of tumor suppressor protein p53 is one of the leading causes of tumor progression. When mutated, p53 protein aggregates, loses its function leading to unwanted cell growth and ultimately results in tumor. Here in this study we focus on the inhibitory effects of polyarginine and its analogues polyornithine, canavanine, and citrulline on the inhibition of p53 mutant peptide aggregation, and p53 mutant cancer cell proliferation inhibition *in vitro*. Biochemical assays and cell toxicity studies were used to characterize the study. The results show that polyarginine, and polyornithine, in micromolar concentrations, significantly inhibits p53 mutant peptide aggregation, and the cell proliferation of p53 mutant

cancer cells. Hence they could be promising candidates for treating p53 mutant/misfolded protein aggregation associated cancer.

Keywords : Cancer, p53 aggregation, arginine, inhibition, cell proliferation

4.2. Introduction

Protein aggregation and misfolding are major hallmarks of diseases such as cancer and aging. In cancer, the tumor suppressor protein p53 due to mutations, misfolds and aggregates resulting in the loss of function of the protein [1, 2]. p53 protein mutation/misfolding is thought to be responsible for about half of all the cancers [3, 4]. Hence, it is important to find therapeutic candidates that could target p53 misfolding/aggregation. Several studies show that the mutant p53 aggregation is observed in tumor tissue samples, biopsies, as well as in several cancer cell lines indicating the correlation between aggregation and tumor growth [5, 6]. The studies further report that the aggregated mutant p53 proteins, loses their functionality [1, 5]. Recent work on potential reactivation of the p53 protein *in vivo* gives encouragement in developing novel anti-cancer drugs for the treatment of p53 mutations associated cancer [7, 8]. In a recent study by Sorangi et al. [9] show that the inhibition of p53 mutant aggregation by a designer peptide, rescues p53 functionality, and inhibits tumor growth in ovarian cancer models, indicating the importance of p53 aggregation in cancer. Hence, development of novel drug candidates that target the p53 mutant aggregations could aid in new directions in treating cancer.

Arginine has been widely used to suppress protein aggregation, and inter-protein interactions during protein purification. Several studies report the disaggregation and stabilization effect of arginine [10-12]. It has been reported that its mechanism of stabilization is different from osmolytes induced protein stabilization. Studies show that arginine prevents the association of

denatured or partially folded proteins, but not change the equilibrium of the folding process [12]. Arginine also has been shown to have inhibitory potential against amyloid aggregation due to hydrophobic interactions [13]. Arginine analogues such as ornithine, canavanine, and citrulline also could have protein stabilization effects. The ability of these molecules on modulating p53 aggregation has not been explored yet, and hence the study on their inhibitory effects on p53 aggregation and cancer cell growth, could reveal novel therapeutic strategies in rescuing p53 mutant aggregation in cancer.

Here in this paper, we study the inhibitory potential of polyarginine (PLA) and its analogues polyornithine (ORT), canavanine (CNV), and citrulline (CIT) on the p53 mutant peptide QRPILTIITL aggregation, and their ability to inhibit p53 mutant aggregation associated cancer cell growth. Human p53 protein has been shown to possess several mutation and conserved regions with highly amyloidogenic propensity [9, 14, 15]. Mutations of the amino acid at the 248 location, is shown to present in several tumor and biopsy samples [9, 16]. Previously we have shown that peptide with amino acid sequence WRPILTIITL (p53 248-257), carrying the R248W mutant, exhibited enhanced aggregation under *in vitro* conditions [17]. Here, in this study we are utilizing the amino acid sequence QRPILTIITL (p53 248-257), bearing the R248Q mutant, to test the inhibitory potential of arginine and its analogues. We then test the effect of these molecules on p53 mutant, p53 wild type, and p53 null cancer cells proliferation. Results show, polyarginine and polyornithine have p53 mutant aggregation dependent tumor cell proliferation inhibition effect, and hence could be potential therapeutic candidates for treating p53 aggregation prone cancer.

4.3. Materials and Methods

4.3.1. Materials

p53 mutant peptide was custom synthesized by Genscript. All other materials were purchased from Sigma Aldrich (St.Louis, MO).

4.3.2. *In vitro* aggregation formation

QRPILTIITL peptide aggregation was performed as previously reported, with small modifications [9]. Briefly, peptides of 0.7 mM concentration were dissolved in 1 mM LiOH, 100 µM of tris buffer pH7, 50 µM ThT, and with or without the polyarginine and its analogues of concentrations ranging from 7 µM - 0.7 mM. The mixture was maintained at cold temperatures, until the aggregation kinetic measurements, to avoid the rapid aggregation at room temperature. The peptide aggregation was characterized by Thioflavin-T (ThT), Transmission electron microscopy (TEM), and dynamic light scattering (DLS) measurements.

4.3.3. ThT fluorescence

For the ThT measurement, peptides of 0.7 mM concentration, were dissolved in 1 mM LiOH, 100 µM tri buffer, pH7 and 50 µM ThT with or without the arginine compounds of concentrations ranging from 7 µM- 0.7 mM. Spectramax M3 spectrophotometer was used to measure the ThT fluorescence at 440/482 nm excitation/emission. The ThT binding kinetics was performed every 2 minutes for an hour at room temperature. Three independent experiments were performed to confirm the data.

4.3.4. DLS

Malvern zetasizer instrument was used for the DLS measurements. Similar conditions used for the ThT and turbidity assays were used for the size measurements. Briefly, samples were diluted in double distilled water, and the size based on scattering intensity was measured.

4.3.5. XTT assay

Effect of cell growth and toxicity was studied by XTT assay. Lung cancer cell lines, A549, and H1299, are regularly cultured in Dr.Keshamouni's lab. The cells were cultured in RPMI medium, 10% fetal bovine serum, and 5% antibiotic-antimycotic. P53 mutant cell lines H719 (R248Q), and SK-BR-3 (R175H) were purchased from ATCC, and cultured according to the manufacturer's protocol. Cells at a density of $2x10^4$ cells/well, were cultured in 96 well plates for 24 hours, and then treated with polyarginine and analogues for 48 hr. Cell viability was measured by XTT assay.

The toxicity of polyarginine and its analogues on normal human brain microvascular endothelial cells (hBMVEC) was studied by the XTT assay. Cells were maintained in M199 medium, 10% fetal bovine serum, and 5% antibiotic-antimycotic. For the study, 2 x 10^4 cells/well, were cultured in 96 well plates over night. Cells were then subsequently treated with 1-5 μM concentrations of polyarginine, and polyornithine, and 1-5 mM concentrations of canavanine, and citrulline for 48 hours. XTT assay was performed at 470 nm using plate reader, and cell viability was determined according to the manufacturer's protocol.

4.3.6. Confocal microscopy

For Immunofluorescence, SK-BR-3 cells were cultured in 8-well chamber slides, and treated with polyarginine and analogues for 48 hours. The cells were then fixed, and stained for p21 expression. Images were taken using a Nikon A-1 spectral confocal at the UM-Ann Arbor microscopy image analysis laboratory (MIL).

4.3.7. Statistical Analysis

Data were collected from four or more replicates for each experiment. Three independent experiments were carried out, and they are presented as mean ± standard error of the mean (SEM), and P-values were determined. For statistical significance analysis unpaired T-test was used to determine the significance of polyarginine and its analogues treatment with that of the no-treatment (control) group.

4.4. Results

The aggregation of the QRPILTIITL p53 mutant peptide was studied by thioflavin-T, and dls. We tested the inhibitory effect of polyarginine, polyornithine, canavanine, and citrulline on the QRPILTIITL peptide aggregation. ThT fluorescence at 440/482 nm excitation and emission wavelength was measured every two minutes to monitor the aggregation kinetics. The data show that polyarginine and polyornithine significantly inhibit p53 mutant peptide aggregation (Figure 1 (i)). Canavanine and citrulline on the other hand did not exhibit inhibitory effects. To assess the size of the aggregated samples, at the end of the 30 min aggregation study, DLS measurements were performed. The DLS measurement (Figure 1 (ii)) were in agreement with that of the corresponding ThT sample measurements indicating more aggregation and fibers in the untreated samples compared to the polyarginine and polyornithine treated samples. We then did a dose dependent study of polyarginine to assess the concentration effect on the peptide aggregation inhibition. The thioflavin T measurements show that the polyarginine concentrations as low as 7 μM were effective in inhibiting 700 μM peptide aggregation (Figure 1 (iii)). Similar finding was observed with DLS measurements (Figure 1 (iv)).

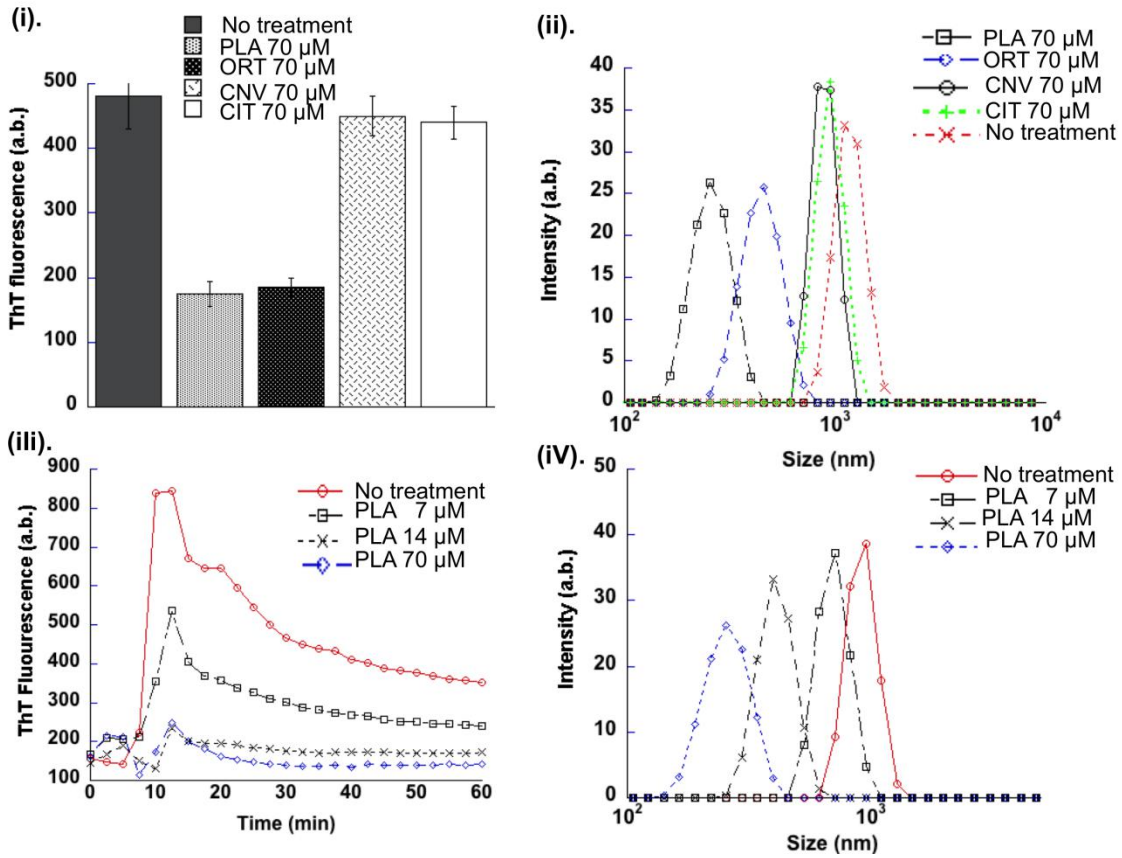

Figure 4.1. i). ThT fluorescence of 0.7 mM p53 mutant peptide after 1 hour incubation with and without polyarginine, polyornithine, canavanine, and citrulline. Polyarginine and polyornithine exhibit p53 peptide aggregation inhibition. * $p < 0.01$. Data are expressed as mean ± SEM ii). Corresponding dynamic light scattering (DLS) measurements show reduction in size of the aggregates in the presence of polyarginine and polyornithine. (iii).ThT fluorescence of p53 mutant peptide QRPILTIITL aggregation inhibition as a function of polyarginine dose. (iv). DLS size measurements of peptide aggregation in the presence of polyarginine (PLA) with varied concentration, similar to that of thioflavin T samples.

Next we tested the ability of these inhibitory molecules on p53 mutant cancer cell proliferation. For this we used four different cancer cells lines, p53 mutant cells (H719 (R248Q), SK-BR-3 (R175H)), p53 wild type cells (A549), and p53 null cells (H1299). As can be seen from Figure 2, polyarginine (PLA), and polyornithine (ORT) significantly inhibit the p53 mutant cell lines H719, and SK-BR-3. On the other hand, both PLA and ORT up to 4 uM of concentrations did not show inhibitory effect on p53 wild type cells (A549), and p53 null cells (H1299) (Figure S1). Canavanine and citrulline did not show significant inhibition up to 4 mM concentration, on any

of the 4 cell lines tested (Figures S2 and S3). The studies show promise for polyarginine and polyornithine on the p53 mutant aggregation associated cancer cell proliferation inhibition.

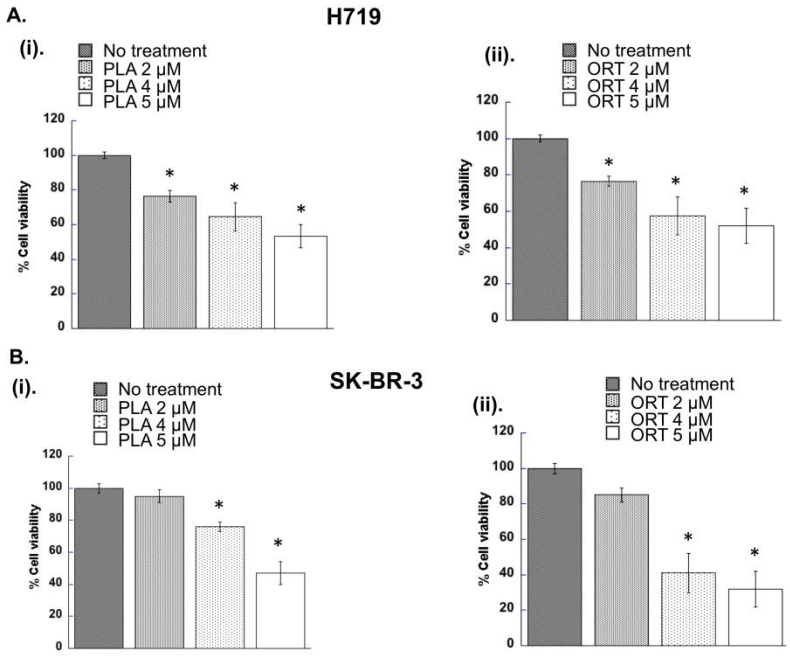

Figure 4.2. (A). Effect of H719 tumor cell proliferation in the presence of (i). PLA, (ii). ORT. **(B).** Effect of SK-BR-3 tumor cell proliferation in the presence of (i). PLA, (ii) ORT. * $p < 0.01$. Data are expressed as mean ± SEM.

We then tested the toxicity effects of these small molecules on normal cells. Human brain microvascular endothelial cells were used as normal cells for the study. Polyarginine and Polyornithine at concentration below 4 μM, and cananvanine below 4 mM did not exhibit significant toxicity (Figure 3). Citrulline did not exhibit any toxicity effect up to 5 mM concentration. This gives further encouragement of these molecules potential as candidates for cancer causing p53 aggregation inhibition.

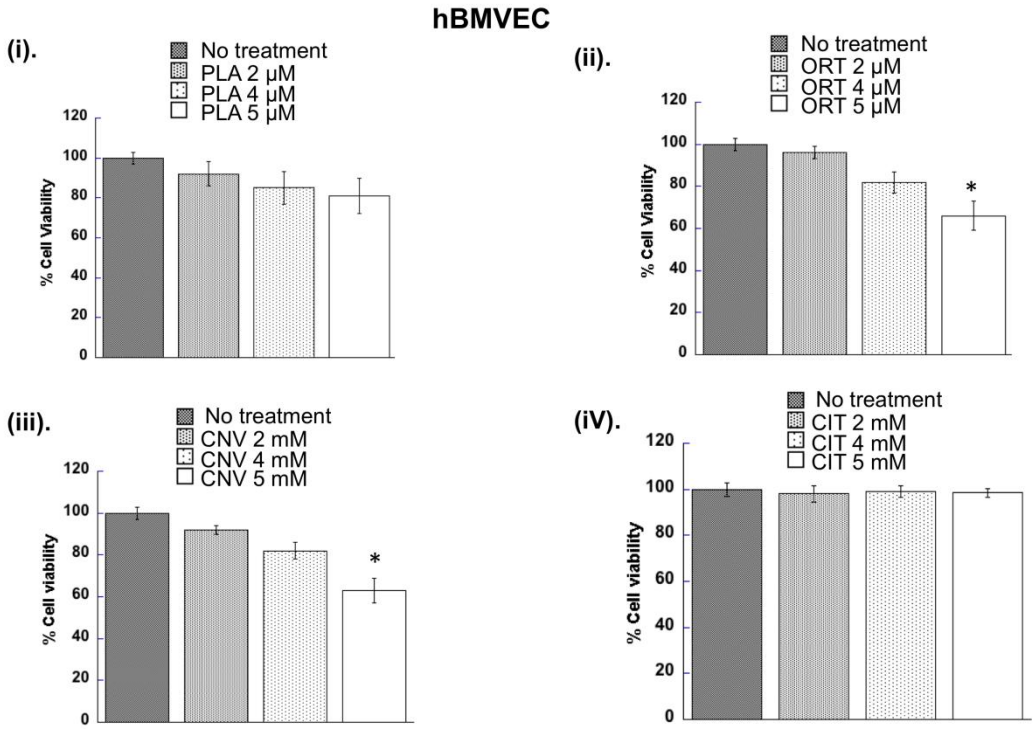

Figure 4.3. Effect of polyarginine, polyornithine, canavanine, and citrulline on normal cell toxicity. hBMVEC cells were treated with PLA (i), ORT (ii), CNV (iii), and CIT (iv), and cellular toxicity was assessed after 48 hours. * $p < 0.01$. Data are expressed as mean ± SEM.

Finally, we tested the treatment effect of polyarginine, and analogues on the p53 functionality. We did immunofluorescence staining of p21 expression, a marker for p53 functionality [18]. As can be seen from Figure 4, all three molecules show increase in p21 staining compared to cells without any treatment. Among them, polyornithine and canavanine showed significant increase in p21 positive cells. The results suggest that polyarginine and its analogues promote the p53 mediated p21 expression, and hence the p53 functionality.

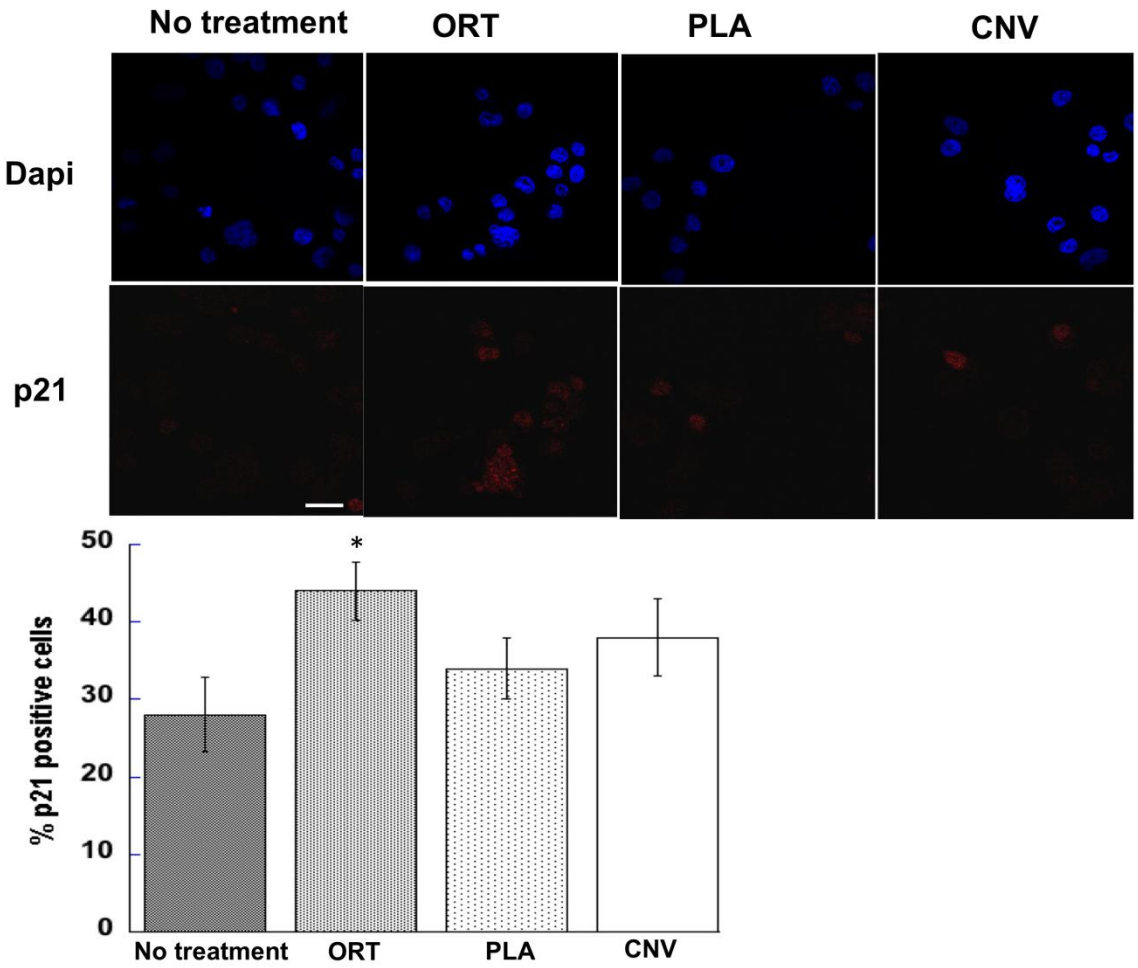

Figure 4.4. Effect of polyarginine, and its analogues on p21 expression, a p53 functional marker. Skbr3 cells were treated with PLA (4 μM), ORT (4 μM), and CNV (4 mM) for 48 hours, and then stained for p21. **(i).** Immunofluorescence images show, enhanced p21 expression in the presence of the inhibitory molecules. Scale bar 20 μm. **(ii).** Corresponding image analysis quantifying the percentage of p21 positive cells, averaged over five separate images. * $p < 0.05$.

4.5. Discussion

In this proof of principle study, we show that cationic polyarginine, and polyornithine have the potential to inhibit p53 mutant peptide aggregation, and p53 mutant dependent tumor cell growth *in vitro*, and hence could have therapeutic potential in treating the p53 mutant protein aggregation in cancer. The mechanism by which arginine molecules protect macromolecules is believed to be by the prevention of association of misfolded or partially folded proteins, by interacting with the protein residues [11, 19]. Hence it could be that the preferential hydration of p53 mutant peptide

by arginine and its analogues may have reduced the possibility of formation of aggregation and subsequent fibrillar structures of the peptide. The stabilization could have further strengthened by specific hydrophobic interaction between the arginine molecules and p53 peptide/protein that may have aid in the disruption of hydrogen bond formation between partially aggregated peptide monomers. Different arginine analogue molecules could differ in their ability to protect protein under mutation/misfolding, as can be seen from our results, where canavanine and citrulline did not show significant inhibitory potential. It is therefore necessary to identify the optimal inhibitory molecule or mixture of those by high-throughput screening methods and assays to develop novel synthetic multifunctional stress molecule analogs or chimeric molecules on protein aggregation inhibition to treat cancer due to p53 mutant aggregation. Lastly, the novel finding of p53 aggregation inhibition by arginine and its analogues will open new avenues to treat p53 aggregation associated cancer.

4.6. Acknowledgments

We would like to thank the University of Michigan Dearborn department of Mechanical engineering, and the College of Engineering and Computer Science for funding. We would like to thank Dr. Kalyan Kondapalli (University of Michigan-Dearborn) for providing human brain microvascular endothelial Cells. We would like to thank the University of Michigan Microscope and Imaging (MIL) facility for the use of TEM and confocal microscopes.

4.7. Supplementary Information

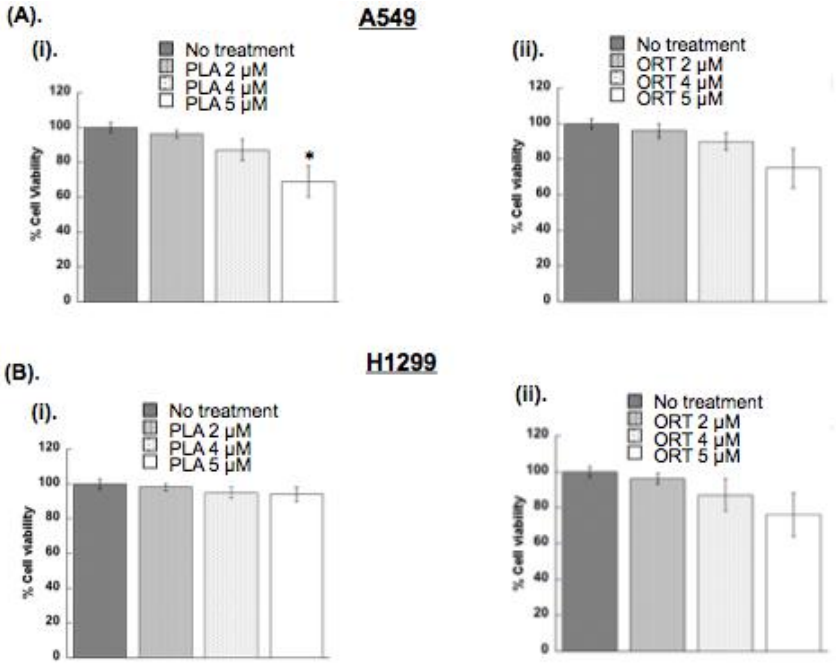

Figure 4.S1. (A). Effect of A549 tumor cell proliferation in the presence of (i). PLA, (ii). ORT. **(B).** Effect of H1299 tumor cell proliferation in the presence of (i). PLA, (ii) ORT. * $p < 0.01$. Data are expressed as mean ± SEM.

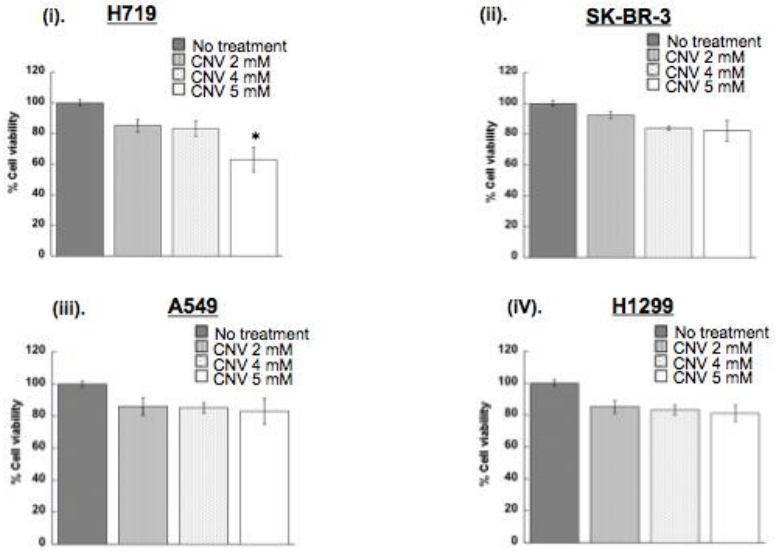

Figure 4.S2. Effect of canavanine (CNV) on tumor cell proliferation. (i). p53 mutant cell line H719 . (i). p53 mutant cell line SK-BR-3 (iii). p53 wild type cell line A549 (iv). p53 null cell line H1299. * $p < 0.01$. Data are expressed as mean ± SEM.

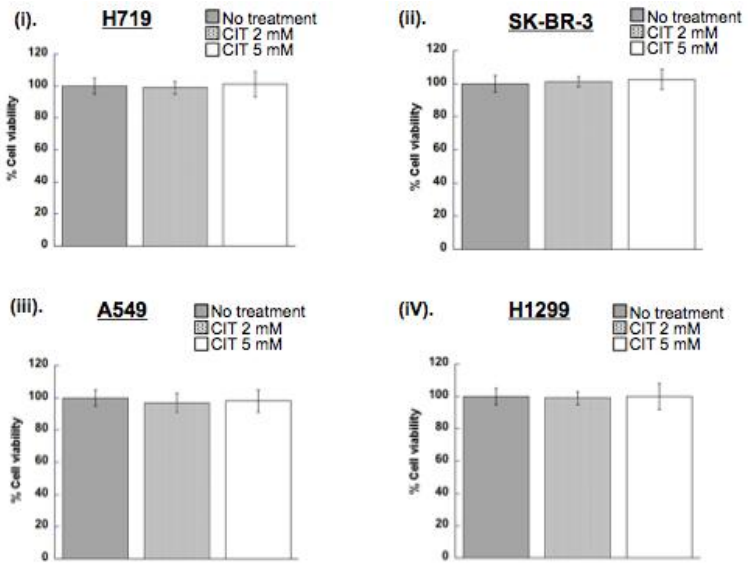

Figure 4.S3. Effect of citrulline (CIT) on tumor cell proliferation. (i). p53 mutant cell line H719 . (i). p53 mutant cell line SK-BR-3 (iii). p53 wild type cell line A549 (iv). p53 null cell line H1299. * $p < 0.01$. Data are expressed as mean ± SEM.

4.8. References

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Chapter 5

Anti- p53 Mutant Aggregation Small Molecules to Modulate Cisplatin Mediated Chemoresistance in p53 Mutant Cancer Cells

5.1. Introduction

Chemoresistance is a major hurdle encountered in cancer therapy. Platinum based chemotherapeutic drug cisplatin is one of the widely accepted treatment for cancer [1]. However, cisplatin exhibit chemoresistance, resulting in failure of about 70% of cancer therapies [2, 3]. Recently it was reported that, that p53 aggregation could be one of the factors that promote chemoresistance [4, 5]. It has been reported when p53 loses its function, the tumors are less responsive to chemo and radiotherapy [6, 7]. Natural small stress molecules are known to stabilize proteins and prevent protein misfolding and aggregation, thereby preserving the function [8, 9]. Further, our studies show (chapter 3 and 4) they have p53 anti-aggregation effects. However, the application of natural molecules to modulate chemoresistance hasn't been explored yet, and they could be potential drug candidates for sensitizing chemoresistance.

Here we tested the potential of p53-antiaggregation natural osmolyte drugs N-α-carbamoyl-L-glutamine 1-amide (cga), and betaine hydrochloride (BH) on their ability to overcome cisplatin mediated chemoresistance in two p53 mutant cancer cells. The p53-anti-aggregation properties of the small molecules were studied by thioflavin-T binding assay. We did a dose dependent

study of cga and BH on p53 mutant peptide aggregation inhibition monitored by ThT fluorescence. Then we tested their potential for chemosensitization. Cancer cells SKBR-3 (p53 mutant), H719 (p53 mutant), were used for the study. Cells were treated with cisplatin, with and without the small molecules cga, and BH and the cell viability was assessed by XTT assay. The results show greater potential for small molecule-platinum drug combination therapy for sensitizing chemoresistance.

5.2. Materials and Methods

All materials including cisplatin, and small stress molecules were purchased from Sigma Aldrich (St.Louis, MO), and used as obtained.

5.2.1. XTT assay

Effect of cellular toxicity was studied by XTT assay. SK-BR-3 and H719 are obtained from ATCC, and cultured according to standard protocol. Human microvascular brain endothelial cells were kindly provided by, Dr. Kalyan Kondapalli. For the experiment, cells were cultured in 96 well plates for 24 hours, and then treated with cisplatin of concentrations 5 uM, and 10 uM, with or without small molecules of concentrations ranging from 4-10 mM for 48 hr. Cell viability was measured by XTT assay.

5.2.2. ThT fluorescence

For the ThT measurement, similar procedures used in the previous studies will be used. Peptides of 0.7 mM concentration, were dissolved in 1 mM LiOH, 100 μM tris buffer, pH 7 and 50 μM ThT with or without cga ranging from 7 μM- 0.7 mM. ThT fluorescence was measured at

440/482 nm excitation/emission. The ThT binding kinetics was performed every few minutes for an hour at room temperature, and the experiments were repeated three times.

5.2.3. Statistical Analysis

Three or more replicates of data for each experiment were collected. The data are presented as mean ± standard error of the mean (SEM). P-values were determined from the 3-independent experimental results. Unpaired T-test was used for statistical significance.

5.3. Results and Discussion

First, we tested the p53 anti-aggregation effects of cga and BH by thioflavin-T. Thioflavin-T measurement show, both cga, and BH have significant aggregation inhibition of p53 mutant peptide QRPILTIITL (p53 248-257), bearing the R248Q mutant at equal molar concentrations (Figure 1).

Next we tested the effect of cisplatin dosage on p53 mutant cancer cells SK-BR-3, and H719. The mutant SK-BR-3 cells seems to be sensitive to chemo drug above 10 uM compared to p53 mutant cell line H719, which showed more resistance (Figure 2). Based on the chemoresistance, cisplatin concentrations of 5 uM for SK-BR-3, and 10 uM for H719 were chosen for the chemoresistance study with drug combination. We then tested the effect of small-molecule drugs/cisplatin combination for the chemoresistance study. From Figure 3, it can be seen, for SK-BR-3 cells treated with the cga or BH/cisplatin combination therapy, significant inhibition of proliferation is observed.

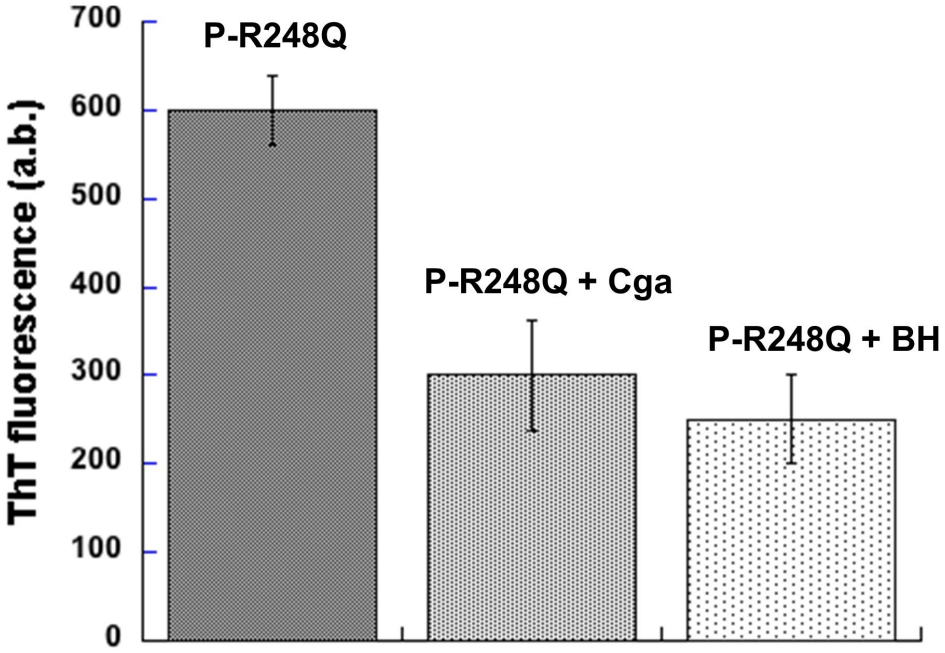

Figure 5.1. Thioflavin-T fluorescence of peptide R248Q treated Cga and BH. The peptides show less fluorescence in the presence of cga and BH indicating inhibition of aggregation.

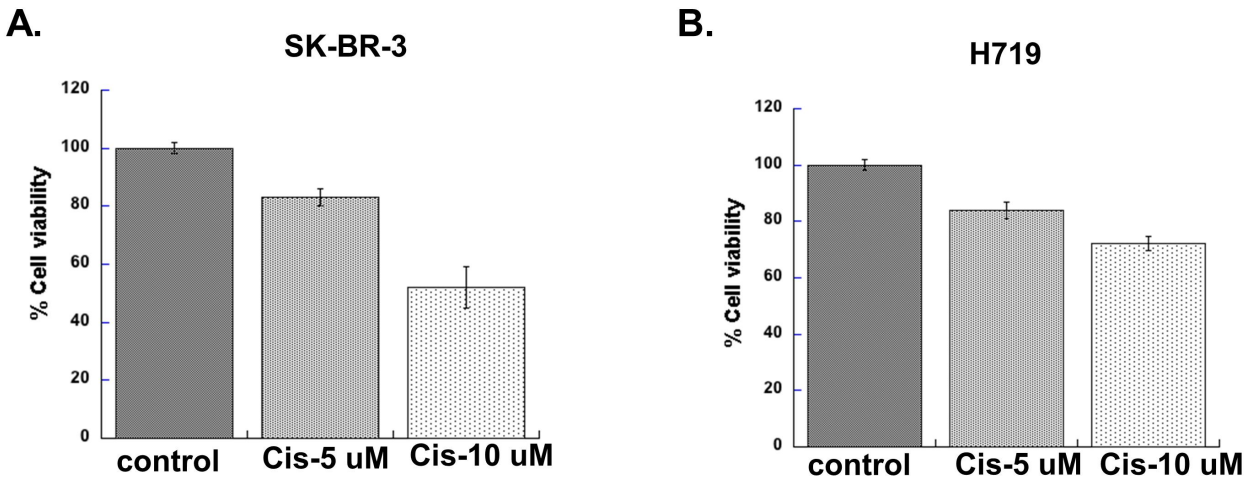

Figure 5.2. Cisplatin dose effect on **(A)** SK-BR-3 cells **(B)** H719 cells. H719 cells show significant resistance compared to SK-BR-3

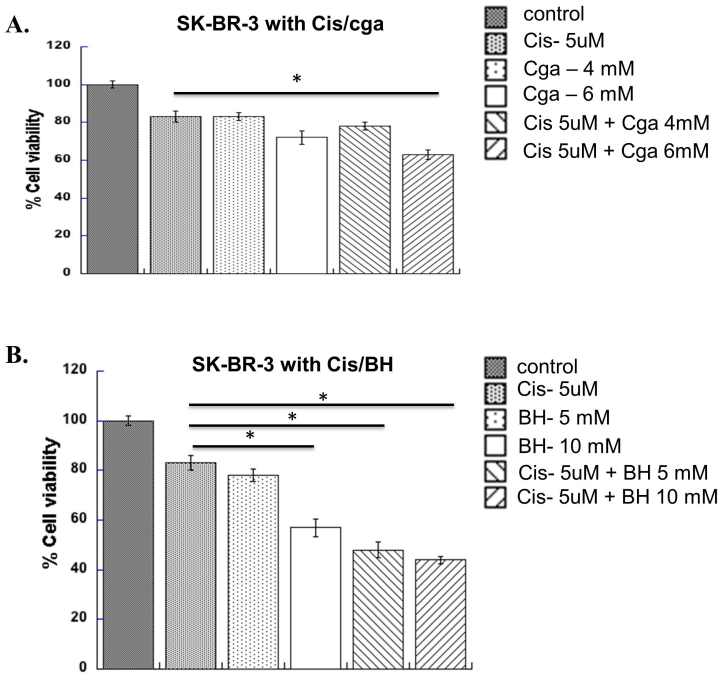

Figure 5.3: Effect of cisplatin/small molecule combination therapy on SK-BR-3 chemoresistance. **(A)** Cisplatin (5 uM) treated with cga, show significant chemoresistance when treated with cga of 6 mM concentration. **(B)** Betaine hydrochloride show significant chemosensitization effect.

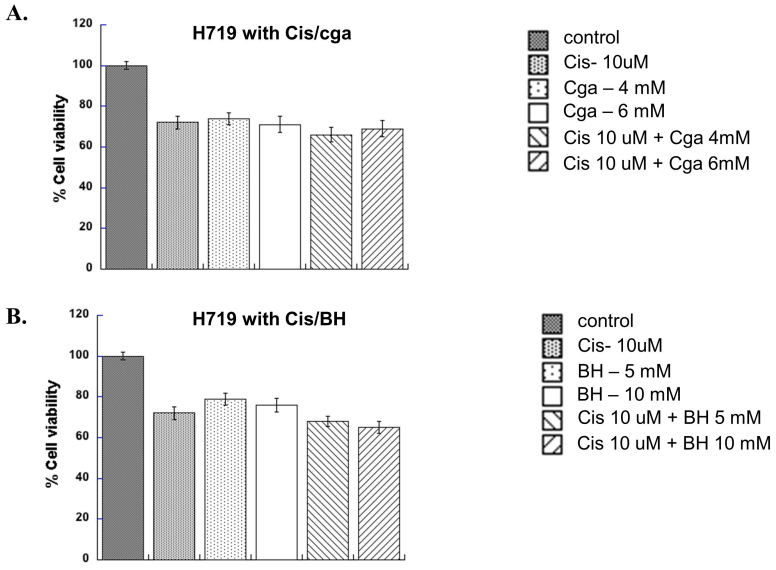

Figure 5.4. H719 cells show chemoresistance even in the presence of **(A)** cga **(B)** BH small molecule cisplatin drug combination.

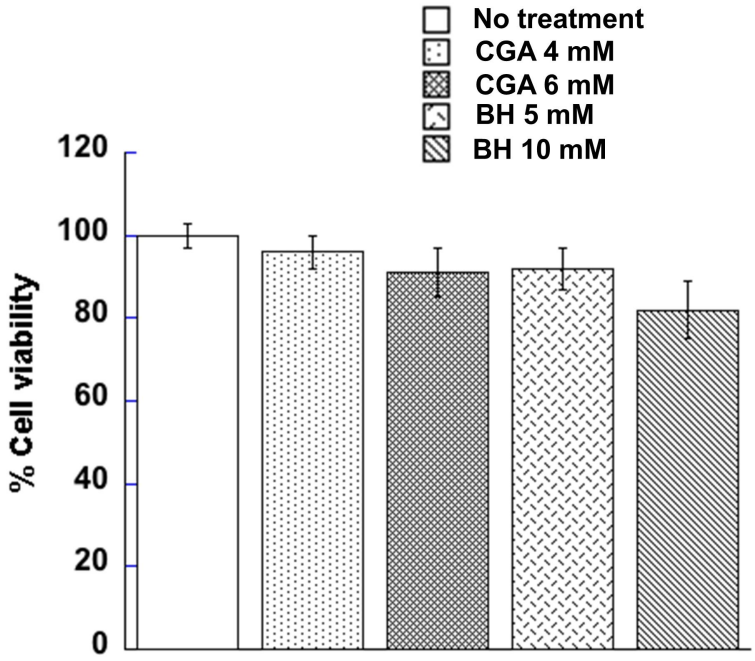

Figure 5.5. The toxicity effect of cga and BH was assessed in normal cells. Human microvascular brain endothelial cells were used for the study. Cga and BH at the concentrations used in the study show no significant toxicity.

However, the combination therapy was not significantly effective for the highly resistance H719 cell line (Figure 4). Finally, we tested the toxicity effects of these drugs in human microvascular endothelial cells. The molecules did not exhibit significant toxicity at the concentrations used for the study（Figure 5）.

The results show that small molecules have potential in inhibiting p53 mutant cancer cells chemoresistance. Hence could be potential therapeutic candidates for treating cancer chemoresistance associated with p53 aggregation. The proof of principle study, open a new therapeutic avenue for chemoresistance in p53 mutant cancer cells. A detail mechanistic study on how p53 aggregation plays a role in chemoresistance is need for developing successful therapies. Further, the effect of the small molecules could be further enhanced by nanoparticle medicated

small molecule delivery approach. In addition, nanoformulations are known to reduce toxicity, and increase therapeutic efficacy and hence could be clinically relevant.

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Chapter 6

Development of Anti-p53 Aggregation Drug Nanoformulation for Increased Therapeutic Efficacy

6.1. Introduction

Nanotechnology approaches have the advantage to remedy some of the problems faced by conventional systemic chemotherapeutic tumor drug delivery [1-3]. Nanoformulations, increase the drug solubility, stability, and can be tuned to deliver the cargo in a controlled release manner all of which increase the therapeutic efficacy of the drug [4, 5]. Hence, in this chapter work, a nanoparticle based small molecule formulation is developed to achieve high therapeutic index for the inhibition of p53 mutant aggregation associated cancer.

Several nanoformulations categories are utilized for drug delivery. Among them polymeric nanoparticles, and liposomal particles are of particular interest, due to the ease of multi-functional capabilities [5-7]. Drug molecules can be either encapsulated or coated on the particles. Further, tissue specific targeting moieties could be conjugated to the surface of the particles to render site-specific drug delivery. Here we are interested in developing liposomal particles for the anti-p53 drug delivery. Liposomes have the advantage of incorporating both hydrophilic and hydrophobic drugs, as well could be surface modified to selectively target tumor [8, 9]. Hence, it could be an ideal drug depot for p53-antiaggregation drugs.

The objective of this chapter study is to develop a liposomal p53 anti aggregation drug nanoformulation and test their efficacy in p53 mutant cancer cells growth inhibition compared to bare small molecule. For the small drug molecule N-α-carbamoyl-L-glutamine 1-amide (cga) is used in the study, it's anti-p53 aggregation properties were confirmed by ThT fluorescence binding kinetics.

6.2. Materials and Methods

Lipids were purchased from Avanti polar lipids. p53 mutant peptide was custom synthesized from Genscript. All other materials were obtained from Sigma Aldrich.

6.2.1. Liposome formulation

Liposomes were formed by extrusion method. Briefly, lipids were mixed with N-α-carbamoyl-L-glutamine 1-amide (cga) in methylene chloride. The lipids were then allowed to form emulsion in PBS buffer overnight. The following day, liposomes were formed by extrusion. Liposomes were then characterized by, dynamic light scattering, and atomic force microscopy for size and morphology. Dynamic light scattering measurements were performed with Malvern zetasizer in the lab. Atomic force microscopy images in tapping mode were obtained from Hitachi AFM in the mechanical engineering department. The encapsulation efficiency of cga was determined by utilizing fitc-conjugated cga, and then determined by M3 spectrophotometer.

6.2.2. XTT assay

Effect of cell growth and toxicity was studied by XTT assay. p53 mutant cell lines H719 (R248Q), and SK-BR-3 (R175H) were purchased from ATCC, and cultured according to the

manufacturer's protocol. Cells at a density of $2x10^4$ cells/well, were cultured in 96 well plates for 24 hours, and then treated with and without cga formulations for 48 hr. Cell viability was measured by XTT assay.

6.2.3. ThT fluorescence

For the ThT measurement, similar procedures used in the previous studies will be used. Peptides of 0.7 mM concentration, were dissolved in 1 mM LiOH, 100 μM tris buffer, pH 7 and 50 μM ThT with or without cga ranging from 7 μM- 0.7 mM. ThT fluorescence was measured at 440/482 nm excitation/emission. The ThT binding kinetics was performed every 2 minutes for an hour at room temperature, and the experiments were repeated three times.

6.3. Results and Discussion

First, the p53 anti-aggregation property of cga was confirmed by thioflavin-T. Two p53 mutant peptides used in the previous studies, WRPILTIITL (p53 248-257) (P-R248W), QRPILTIITL (p53 248-257) (P-R248Q) were aggregated with and without cga at two different molar ratios. The aggregation kinetics was monitored for 1 hour. The rate constant was determined by curve fitting using the equation $y = yo + \frac{a}{1+e^{\frac{-(x-xo)}{b}}}$ as previously described [10]. Here y is the fluorescence at time x, initial fluorescence value yo, time when fluorescence reaches 50% xo, maximum fluorescence at equilibrium phase a. The kinetic constant is calculated by 1/b. As can be seen from Figure 1A, and B, cga inhibits both peptides aggregation. The rate constant is several folds high compared to that of the cga treated samples.

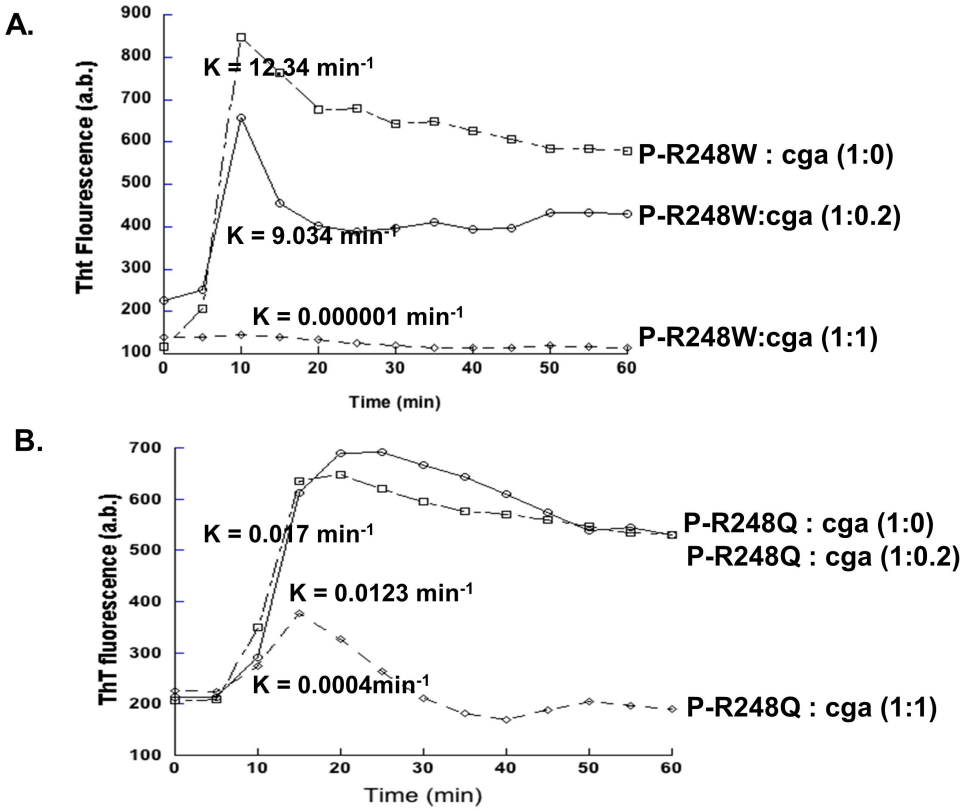

Figure 6.1. Thioflavin binding kinetics of p53 mutant peptides aggregation with and without cga. **(A).** P-R248Q, and **(B).** P-R248W

Next, the liposomes were formed by extrusion, and characterized by DLS, and AFM (Figure 2). The AFM images reveal the average size of the liposomes alone is around 200 nm, while with the cga encapsulation is around 300 nm. Similar observation was observed with DLS measurement showing zeta size of around 200 nm for liposomes alone, and about 350 nm for the cga encapsulated liposomes. Next, the encapsulation efficiency was determined by encapsulation of FITC fluorescently labelled cga, and subsequent measurement of fitc fluorescence with the M3 plate reader. Measurements reveal, about 1% encapsulation efficiency.

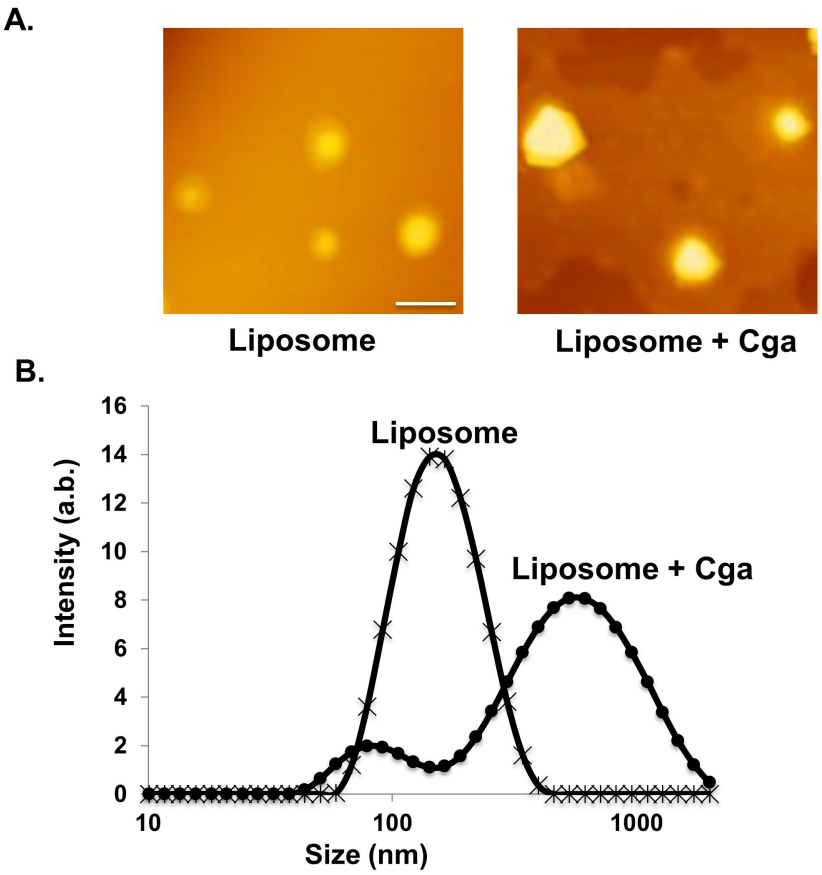

Figure 6.2. Liposome characterization. (A) The morphology of the liposome was characterized by AFM. Images show liposomes alone have a average size around 200 nm, while liposomes with cga show slightly larger size around 350 nm. Scale bar 200 nm.

Finally, we tested the efficacy of the nanoformulation in SKBR-3 and H719 cells. Results show, that the liposomal nanoformulation exhibit more than 25 fold efficacy compared to bare cga (Figure 3). Further, bare nanoformulation did not exhibit significant toxicity. The results indicate the potential of nanoformulation in increased therapeutic efficacy with minimal toxicity. This approach could be applied for p53 aggregation nanotherapeutics development and could be clinically relevant.

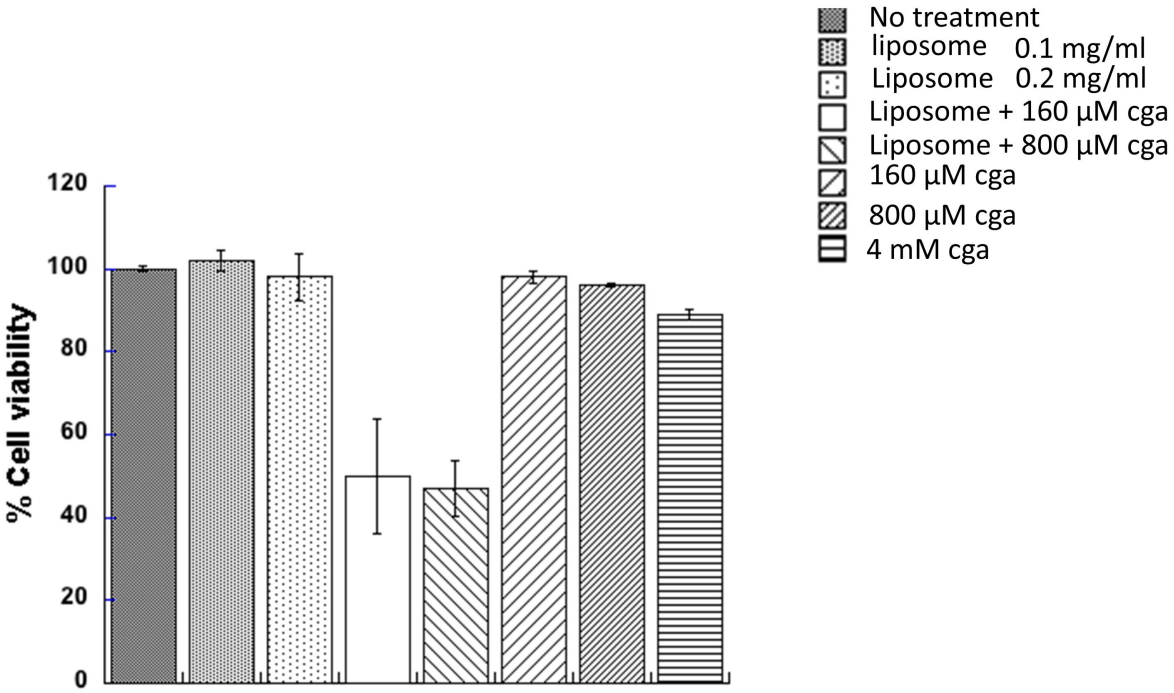

Figure 6.3. Therapeutic efficacy evaluation of liposome. Comparing with the liposome alone groups and cga alone groups, the therapeutic efficacy of cga-encapsulated liposome is significantly increased.

6.4. References:

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Chapter 7

7.1. General conclusions

Although several synthetic small molecules have been utilized for treating p53 mutant associated cancer, use of natural small molecules, in particular on the p53 aggregation inhibition, and subsequent suppression of tumor growth hasn't been explored. In this thesis proposal, small molecule osmolytes that are capable of inhibiting p53 mutant aggregation in vitro, and p53 mutant aggregation associated cancer has been identified. Thioflavin-T, congo red, TEM, AFM, DLS, and cellular characterization was used to corroborate the inhibitory effect of these molecules. Further, the molecules show potential in sensitizing chemoresistance of p53 mutant cancer cells. Finally, the therapeutic efficacy of the molecules could be increased more than twenty fold, with the help of nanoformulation. The anti-aggregate drug nanoformulation has the capability of decreasing toxicity and hence could have translational potential.

7.2. Future directions

While the thesis study show for the first time, the potential of small molecule drug candidates as therapeutics for p53 aggregation associated cancer, a detail research yet to be done. Although we have shown the inhibitory effects of these molecules, we haven't explored the mechanism of action of these molecules. Further since we are using segments of whole p53 to predict the aggregation in these studies, it won't reflect the actual full protein properties at all conditions.

Hence, future studies should focus on investigating full protein aggregation in addition to peptide segments. In addition, a detail functional effect on p53 by these small molecules has to be elucidated. Finally, for the molecules to be translational, the molecules need to be tested in animal models. In summary, the study paves way for further research on investigating the potential of these molecules in cancer, and new therapeutic interventions for p53 aggregation associated cancer.