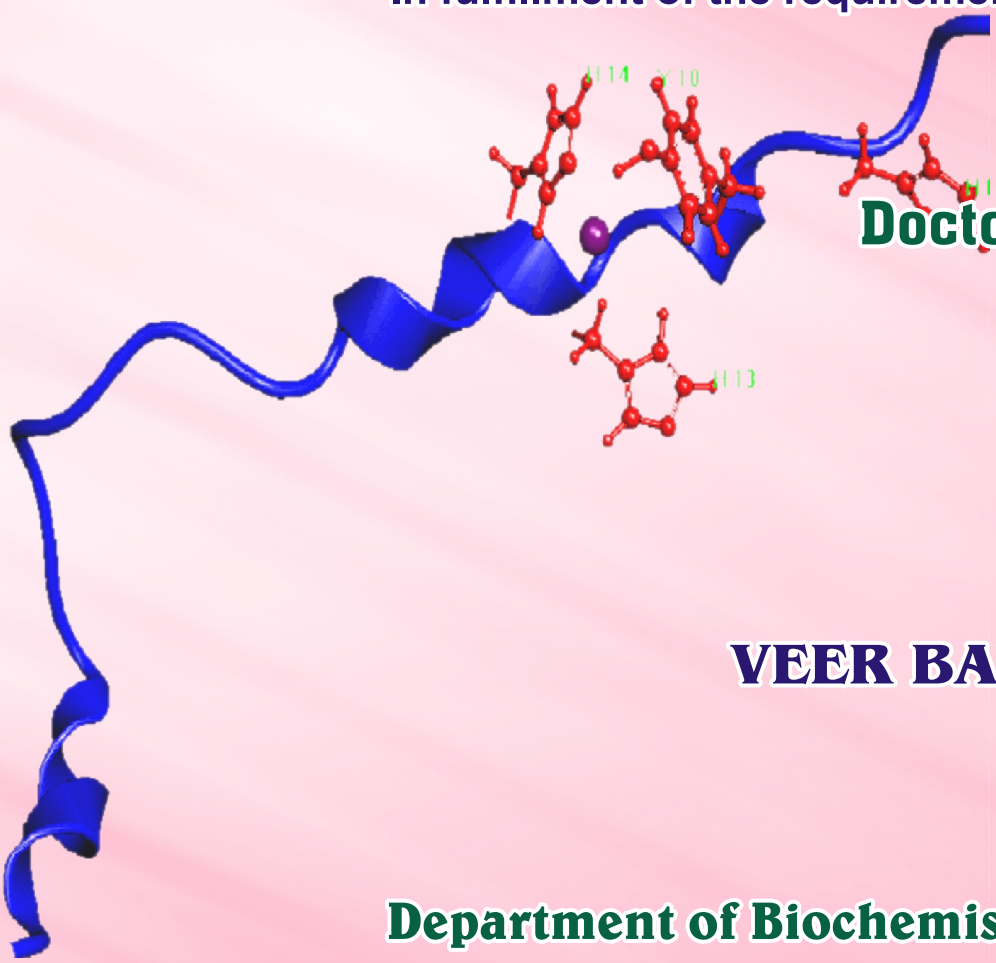
STUDIES ON
AMYLOID BETA PEPTIDE AGGREGATION AND ITS MODULATION BY GARLIC COMPONENTS: RELEVANCE TO ALZHEIMER'S DISEASE

A Thesis submitted to the
UNIVERSITY OF MYSORE
In fulfillment of the requirements for the degree of



Doctor of Philosophy
in
BIOCHEMISTRY

by

VEER BALA GUPTA, M.Sc.

Department of Biochemistry and Nutrition
CENTRAL FOOD TECHNOLOGICAL RESEARCH INSTITUTE
Mysore - 570020, India

December, 2007

STUDIES ON AMYLOID BETA PEPTIDE AGGREGATION AND ITS MODULATION BY GARLIC COMPONENTS: RELEVANCE TO ALZHEIMER'S DISEASE

A Thesis submitted to the

Department of Biochemistry

UNIVERSITY OF MYSORE

In fulfillment of the requirements for the degree of

Doctor of Philosophy
in
BIOCHEMISTRY

by

Veer Bala Gupta, ***M.Sc.***

Under the supervision of

Dr. K.S. Jagannatha Rao, *Ph.D.*

Department of Biochemistry and Nutrition
CENTRAL FOOD TECHNOLOGICAL RESEARCH INSTITUTE
Mysore - 570020, India

December 2007

.....Dedicated to my beloved parents

Acknowledgements

*All gratitude and praise to **The lord**, for the ocean of blessings, He has showered on me.*

*I wish to express my sincere gratitude to my research supervisor **Dr. K.S. Jagannatha Rao**, for suggesting the problem, able guidance, valuable insight, constant encouragement and support throughout the investigation.*

*My sincere thanks to **Dr. V. Prakash**, Director, CFTRI, Mysore, for providing the necessary facilities to work in the institute and permitting me to submit the results in the form of the thesis.*

*I am grateful to **Dr. P.V. Salimath**, Head, Biochemistry & Nutrition, for his constant help and encouragement. My grateful acknowledgements to **Dr. Lalitha R. Gowda** for her advice during the course of investigation.*

*I am obliged to **Prof K. Subba Rao**, University of Hyderabad, who extended openhanded milieu to carry out DNA strand break studies in his lab. I thank **Dr. Surolia**, Chairman, MBU, IISc, Bangalore, for allowing me to use Circular Dichroism Instrument. I thank **Mr. Govindarajulu** for all his help. I am grateful to **Dr. S.S. Indi**, IISc, for allowing me to carry out Electron microscopy studies.*

*I am indebted to all **scientists**, **staff members** in the department, both past and present, my **friends** and **well wishers** for creating an inspiring working atmosphere to complete my research work.*

*It is my profound duty to thank **ICMR**, New Delhi for the financial support in the form of fellowship.*

*Very special thanks to my brother, **Vivek**, who stood as a tower of strength during moments of frustration & motivated me towards the achievement of my goal.*

*No words suffice my gratefulness and how indebted i am to my brothers, **Jagveer** & **Vishal** without whose moral support and encouragement, i would have never been able to reach this stage of life.*

*Special thanks to my little nephew, **Shivam**, whose mere presence in my life inspired me to move ahead successfully.*

*I have saved the best compliments for my Father, **Sh. Jagmohan Gupta**, who I feel God sent to make me strong and took him away to make me stronger.*

*To the one person whom I try to convey my thanks, words fail me as she stood by me through the thick and thin in all my endeavors. At this juncture, "Dear **Mumma**, All I can say is Thank You."*

*I have been fortunate to have the best and most supporting **Mother-in-law** & **Father-in-law**. My gratitude to my **Sister-in-laws** for their unflagging love and care.*

*Lastly, but most importantly, i heartily thank my dear & loving husband, **Ravi**, whose patience, support and encouragement was instrumental in accomplishing this task.*

Knowledge is piling up facts, and wisdom is simplifying it. Thanks to the creators of google, entire web sources, journals, magazines, newspapers, for helping me in acquisition of thoughts and translation of scientific knowledge.

Ms. Veer Bala Gupta

DECLARATION

I hereby declare that this thesis entitled "**Studies on amyloid beta peptide aggregation and its modulation by garlic components: relevance to Alzheimer's disease**", submitted herewith, for the degree of Doctor of Philosophy in Biochemistry of the University of Mysore, Mysore, is the result of work done by me in the Department of Biochemistry and Nutrition, Central Food Technological Research Institute (CFTRI), Mysore, India, under the guidance and supervision of Dr. K.S. Jagannatha Rao, during the period of April, 2003 - December, 2007.

I further declare that the results of this work have not been previously submitted for any other degree or fellowship.

Mysore

Date: 17-12-2007 **VEER BALA GUPTA**

Guide

Head of the Department

Dr. K.S. Jagannatha Rao, M.Sc, Ph.D.
Scientist,
Department of Biochemistry and Nutrition
CFTRI, Mysore-570 020

CERTIFICATE

I hereby certify that this thesis entitled "**Studies on amyloid beta peptide aggregation and its modulation by garlic components: relevance to Alzheimer's disease**," submitted by Ms. Veer Bala Gupta to the University of Mysore, Mysore, for the degree of Doctor of Philosophy in Biochemistry is the result of research work carried out by her in the department of Biochemistry and Nutrition, Central Food Technological Research Institute (CFTRI), Mysore, under my guidance and supervision. This work has not been submitted either partially or fully for any other degree or fellowship.

Mysore (**Dr. K.S. Jagannatha Rao**)

Date: 17-12-2007 Guide

CONTENTS

LIST OF ABBREVIATIONS

^{3}[H]TMP	Tritium-labelled thymidine-5′-monophosphate
8OHG	8-hydroxyl-2-deoxygaunosine
Aβ	Amyloid beta
Aβ(1-40)D	Aβ(1-40) consisting of all D amino acids
Aβ(1-40)L	Aβ(1-40) consisting of all L amino acids
Aβ(40-1)L	Aβ(40-1) consisting of all L amino acids
AD	Alzheimer's disease
AIF	Apoptosis inducing factor
Al	Aluminium
ANS	1-anilinonaphthaleine-8-sulphonic acid
APOE	Apolipoprotein E
APP	Amyloid precursor protein
ATA	Aurin tricarboxylic acid
ATP	adenosine triphosphate
AU	arbitrary units
BGE	boiled garlic extract
bp	base pairs
BSA	Bovine serum albumin
Ca	calcium
CD	circular dichroism spectroscopy
CG	curcumin glucoside
CNS	central nervous system
CREB	cAMP-responsive element binding protein
CSF	cerebrospinal fluid
Cu	copper
DADS	di-allyl di-sulfide
dATP	deoxyadenylate triphosphate
dCTP	deoxycytidylate triphosphate
DEPC	diethyl pyrocarbonate

DFFB	DNA fragmentation factor subunit beta
DGTP	deoxyguanylate triphosphate
DNA	Deoxyribonucleic acid
DNAse	deoxyribonuclease
DPM	Disintegrations per minute
DSBs	Double strand breaks
dsc	double stranded circular
DTTP	deoxythymidylate triphosphate
EDTA	ethylenediamine tetra acetic acid
ER	endoplasmic reticulum
ERK	extracellular signal-regulated kinase
EtBr	ethidium bromide
Fe	iron
FGE	fresh garlic extract
GE	garlic extract
GF6	Glass Fiber filter
H_2O_2	hydrogen peroxide
HD	huntingtin disease
HEPES	4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid
HPLC	High profile liquid chromatography
KCl	Potassium chloride
MAD	moderate Alzheimer's disease
Mg	magnesium
$MgCl_2$	magnesium chloride
MMS	mini mental statement
MRI	magnetic resonance imaging
mRNA	messenger RNA
NaCl	sodium chloride
NaOH	sodium hydroxide
NFTs	neurofibrillary tangles
NMP	nuclear matrix protein

NMR	Nuclear magnetic resonance imaging
PAGE	polyacrylamide gel electrophoresis
PARP	Poly [ADP-ribose] polymerase 1
PD	parkinson's disease
PDB	protein data bank
PHFs	paired helical filaments
PS1	presenilin- 1
PS2	presenilin- 2
RNA	ribonucleic acid
RNAse	ribonuclease
ROS	reactive oxygen species
SAC	S- allyl L-cysteine
SAD	severe AD
SAMC	S-allyl mercapto L-cysteine
scDNA	supercoiled DNA
SDS	sodium dodecyl sulfate
SE	size exclusion
SPs	senile plaques
SSBs	single stranded breaks
ssc	single stranded circular
ssDBP	single stranded DNA binding protein
TAE	Tris acetate EDTA
TCA	Tricarboxylic acid
TEM	Transmission electron microscopy
Thio-T	Thioflavin-T
Tm	melting temperature
ZFP	zinc finger protein
Zn	zinc

List of Tables

LIST OF FIGURES

ABSTRACT

The conformational changes in the proteins namely Aβ play an important role in neurodegeneration in AD, however little is known about the DNA conformational alteration. Further, the aggregation properties of Aβ strongly depend upon the amino acid sequence and their stereospecificity. In this perspective, we used Aβ(1-40) all-L, all-D and reverse peptides to understand the role of amino acid stereospecificity in modulating Aβ aggregation. Our data suggested that the aggregation and folding parameters of Aβ are stereospecific. There was differential aggregation pattern of both Aβ(1-40) all-L and all-D. Both enantiomers formed insoluble aggregates as a function of time with different fibrillar morphology. Aβ(1-40) all-L had longer fibrillar morphology, while Aβ(1-40) all-D had favored short fibrils, but reverse sequences (40-1) did not form fibrils. Also, the L-enantiomer of Aβ (1-40) was more prone for aggregation when compared to that of D-enantiomer. This study clearly showed that the aggregation property of Aβ is coded in its sequence and depends upon the amino acid sequence and their stereospecificity.

Further, the relationship between altered DNA topology and neurodegeneration in AD is not yet established. We demonstrated that the two enantiomers of Aβ40 alter conformation of ScDNA differently. Aβ40L induced Ψ–DNA conformation, which was similar in conformation to Z-DNA and this conformation, was reported in hippocampal region of AD brain. Aβ40D induced altered B-DNA. In the present investigation, the role of amino acid stereospecificity in DNA nicking was also studied. The D-enantiomer of Aβ40 was more potential in nicking ScDNA when compared to L-enantiomer of Aβ40 in a time and concentration dependent manner. However, Aβ(40-1) did not nick the DNA. The mechanism of nicking was initially through SSBs, which accumulated as DSBs, indicating that DSBs were formed as a result of cumulative SSBs in ScDNA. Moreover, both Aβ40L and D showed enhanced DNA nicking activity in presence of Mg^{2+} and Ca^{2+} whereas Zn inhibited Aβ40L/D from nicking DNA. The nuclease inhibitors like ATA and DEPC abolished DNA nicking activity of Aβ peptides. Based on the above results, we hypothesized that Aβ may induce toxicity through an independent non-apoptotic mechanism by behaving like a nuclease.

The prevention of formation of Aβ oligomers/ toxic species by biomolecules is considered as one of the therapeutic approaches to AD. In this perspective, the efficacy of garlic and curcumin derivatives were evaluated. There was a dose-dependent inhibition of Aβ40 aggregation with increasing w/w ratio of garlic extract. Further, a component of garlic, SAC also showed inhibitory effect on Aβ aggregation and disaggregation of preformed fibrils. Hence, garlic could be used as an important bio-source for the development of potential anti-amyloidogenic agents. CG inhibited Cu^{2+} induced Aβ aggregation and also caused disaggregation of preformed fibrils. Thus, curcumin might not only function as an antioxidant and inhibitor of Aβ fibril formation directly but also indirectly by chelating metals such as Cu^{2+} to prevent Aβ aggregation.

The above study provided us an insight into the mechanism on the role of amino acid stereospecificity based aggregation pattern; non-apoptotic Aβ induced DNA nicking and neuro-neutraceutical property of garlic and curcumin derivatives. These findings provided us a molecular understanding of the neurodegeneration and neuroprotection in AD brain.

Introduction

Alzheimer's disease (AD) is a complex neurodegenerative disorder having multiple etiologies. It is a devastating dementia, correlated with a region specific neuronal cell loss. The complex neuropathology includes cortical atrophy, formation of neurofibrillary tangles, deposition of amyloid β peptide (Aβ) in senile plaques, synaptic loss, oxidative stress, metal deposition, and apoptosis and all these coupled events lead to the neuronal loss. Despite progress in uncovering many of the factors that contribute to the etiology of the disease, the mechanism of the nerve cell death still remains unknown. It is very well accepted that conformational changes in the proteins namely Aβ play an important role in neurodegeneration in AD, however little is known about the DNA conformational alteration. A few earlier studies reported the nuclear localization of Aβ immunoreactivity. Moreover, there appeared to be an independent non-apoptotic relationship between DNA binding, DNA damage and Aβ in AD. Taken together, the above findings strongly link the Aβ and DNA damage.

Further, the aggregation properties of Aβ strongly depend upon the amino acid sequence and their stereospecificity. Racemization of different aminoacids in Aβ has varied effects on its aggregation kinetics. Previous studies have shown that all D-enantiomers of Aβ (25-35) and Aβ (1-42) exhibit similar biological properties to their L-counterparts. However, there are no studies to understand the effect of complete racemization of amino acids comprising Aβ (1-40) on its DNA nicking and aggregation properties.

Further, preventing the formation of Aβ oligomers/ toxic species by biomolecules is considered as one of the therapeutic approaches to AD. In particular, dietary

components like garlic, S-allyl L-cysteine (SAC) and curcumin are known to have biological activities like anti-amyloidogenic, anti-inflammatory effects. There are limited studies on the role of garlic, its component SAC and curcumin in the prevention of Aβ aggregation. But the mechanism regarding the role of dietary components in modulating aggregation is not clearly understood. Hence, we studied the mechanism of inhibition of Aβ aggregation in presence of selected dietary components using *in vitro* fibrillation model.

We focused on the following objectives in the present investigation:

Objectives:

a) To study the role of amino acid racemization on aggregation kinetics of Aβ (1-40) [Chapter 2].

b) To understand the role of stereo-specificity and modified amino acids on Aβ (1- 40) interaction with DNA [Chapter 3].

c) To study the role of garlic active components and other selected dietary molecules on modulation of conformation and aggregation of Aβ (1-40) [Chapter 4: Part A, Part B].

The research work carried towards achieving these objectives makes the subject matter of the thesis. The thesis is divided in to six chapters.

Chapter 1: General Introduction

This chapter begins with a general account on AD, followed by an overview of current literature on symptoms, classification, criteria for clinical diagnosis of AD, genetic, environmental risk factors of AD and various existing hypothesis regarding cause of AD. The

role of Aβ in AD has been discussed in detail explaining different aspects of Aβ toxicity such as altered DNA topology in AD, Aβ induced DNA damage, intraneuronal and intranuclear localization of Aβ. The role of racemization of amino acids in Aβ aggregation and the current therapeutic strategies for AD focusing on dietary components have also been highlighted. The chapter also highlights the aims and scope of the present study.

The subsequent chapters have a general format of Introduction followed by sections on Materials and methods, Results and Discussion. Literature cited has been listed in an alphabetical order at the end as Bibliography under Chapter 6.

Chapter 2: Role of amino acid stereospecificity in Aβ aggregation

Amyloid fibril formation is a process during which soluble proteins misfold and aggregate into fibrillar structures. In addition to the proposed genetic mutations or a polymorphism of presenilins, APP or apolipoprotein E, the post translational modifications of Aβ such as isomerization/racemization are well speculated as enhancers for Aβ aggregation. Since racemization of amino acid residues in Aβ causes structural changes and functional inactivation or enhances the aggregation process in the modified proteins, this post-translational modification is suggested to be one of the progression factors in sporadic cases of AD. So far there are no studies to understand how changing the stereospecificity of all the amino acids comprising Aβ (1-40) influences the characteristics of Aβ aggregation parameters.

In this perspective, we used Aβ(1-40) all-L, all-D and reverse peptides to understand the role of amino acid stereospecificity in modulating Aβ aggregation. Our data suggested that the aggregation and folding parameters of Aβ are stereospecific. The

differential aggregation pattern of both Aβ(1-40) all-L and all-D was revealed by both Thio-T pattern and TEM. Both enantiomers formed insoluble aggregates as a function of time with different fibrillar morphology. Aβ(1-40) all-L had longer fibrillar morphology, while Aβ(1-40) all-D had favored short fibrils, but reverse sequences (40-1) did not form fibrils. The monitoring of intrinsic tyrosine fluorescence, acrylamide quenching, ANS binding studies demonstrated that L-enantiomer of Aβ (1-40) is more prone for aggregation when compared to that of D-enantiomer. This study clearly showed that the aggregation property of Aβ is coded in its sequence and depends upon the amino acid sequence and their stereospecificity.

Chapter3: Differential DNA nicking activity of L- and D- enantiomers of Aβ(1-40)

The relationship between altered DNA topology and neurodegeneration in AD is not yet established but Aβ could be a crucial candidate for modulating DNA conformation and integrity. A few studies have demonstrated the neuronal localization of Aβ immunoreactivity in apoptotic nuclei in the vicinity of DNA/chromatin of the hippocampal neurons in human AD brain. Further, i*n vitro* studies have shown that Aβ (1-42) and Aβ (1-16) fragments effectively bind to DNA and alter the conformation of DNA. Aβ(1-42) induced a B→Ψ DNA conformation, while Aβ(1-16) caused an altered B-DNA conformation. However, there are no mechanistic studies to examine the nature of the Aβ-DNA interactions and to determine whether DNA- nicking activity of Aβ exhibits stereospecificity.

We demonstrated in the present study, the two enantiomers of Aβ40 alter conformation of ScDNA differently. Aβ40L induced Ψ–DNA conformation, which was

similar in conformation to Z-DNA and this conformation, was reported in hippocampal region of AD brain. Aβ40D induced altered B-DNA. Previous studies indicated that Aβ42 nicks DNA like nuclease but the mechanism was not understood. In the present investigation, the role of amino acid stereospecificity in DNA nicking was studied. The D-enantiomer of Aβ40 was more potential in nicking ScDNA when compared to L-enantiomer of Aβ40 in a time and concentration dependent manner. However, Aβ(40-1) did not nick the DNA. The mechanism of nicking is initially through Single Strand Breaks (SSBs), which leads to Double Strand Breaks (DSBs), indicating that DSBs were formed as a result of cumulative SSBs in ScDNA. Moreover, both Aβ40L and D showed enhanced DNA nicking activity in presence of Mg^{2+} and Ca^{2+} whereas Zn inhibited Aβ40L/D from nicking DNA. The nuclease inhibitors like ATA (Aurin tricarboxylic acid) and DEPC (Diethyl pyrocarbonate) abolished DNA nicking activity of Aβ peptides. Based on the above results, we hypothesized that Aβ may induce toxicity through an independent non-apoptotic mechanism by behaving like a nuclease.

Chapter 4: Anti-amyloidogenic activity of Garlic, its components and curcumin derivative on Aβ(1-40)

Aβ deposition is a hallmark feature of AD pathogenesis. The disease modifying treatment of AD involves reduction in Aβ aggregation and toxicity. Therefore, many therapeutic efforts are targeted at reducing Aβ production, increasing Aβ clearance and inhibiting Aβ aggregation. A number of antioxidants have been reported to inhibit the formation and extension of Aβ fibrils, as well as to destabilize preformed fibrillar Aβ in vitro. Guided with this precept, a search for the natural inhibitors of fibrillogenesis such

as, bioavailable components of garlic and curcumin could be realistic to clinical practice. Garlic and its components were demonstrated to have anti-amyloidogenic, anti-inflammatory and anti-tangle effects in Alzheimer's transgenic model Tg2576. However, there are no mechanistic studies to show the effect of Garlic and its constituents on modulation of Aβ aggregation.

Further, severe dysregulation of metal ion homeostasis has been reported in AD. Increased concentrations of metals like Cu^{2+} are detected in senile plaques in AD, where it is known to bind and induce Aβ aggregation. Studies also suggested that curcumin can bind metal ions such as Cu^{2+} and Fe^{2+}. Moreover, curcumin is known to significantly reduce levels of brain Aβ, plaques and also inhibit the formation of Aβ oligomers and fibrils *in vitro*. In this perspective, we tried to explore the mechanism of curcumin glucoside, a water-soluble curcumin derivative binding to Aβ in presence of Cu^{2+}. There is an advantage of using water-soluble curcumin derivative, as organic solvents are known to induce conformational changes in Aβ.

Part A: The present study investigated the effects of aqueous Garlic extract (GE) and SAC on Aβ fibrillogenesis *in vitro.* There was a dose-dependent inhibition of Aβ40 aggregation with increasing w/w ratio of fresh GE. The inhibition of Aβ aggregation in presence of boiled GE eliminated the possibility of Aβ degradation by proteases present in garlic. The extract could even cause disaggregation of preformed fibrils in solution. Further, a component of garlic, SAC also showed inhibitory effect on Aβ aggregation and disaggregation of preformed fibrils. There is induction of partially folded conformation in Aβ on binding to SAC. Aβ-SAC binding was also studied using Size-exclusion

chromatography, Fluorescence quenching measurements. There was a gradual decrease in the fluorescence intensity on addition of SAC indicating burying of the tyrosine in the core of the newly formed structure. Taken together, our data indicated that garlic could be used as an important bio-source for the development of potential anti-amyloidogenic agents.

Part B: The results demonstrated that Curcumin Glucoside (CG) inhibits Cu^{2+} induced Aβ aggregation and also causes disaggregation of preformed fibrils. Transmission Electron Microscopy revealed the disappearance of Cu^{2+} induced large fibrillar networks of Aβ in presence of CG. It inhibited the formation of higher molecular weight assemblies and stabilizes the 4 kD soluble Aβ as shown by SDS-PAGE and SEC-HPLC. In presence of CG, majority of Aβ remained in random coil conformation as studied by circular dichroism. The 3D structure of Aβ-Cu^{2+}-CG complex was also predicted employing automated docking studies. Thus, curcumin might not only function as an antioxidant and inhibitor of Aβ fibril formation directly but also indirectly by chelating metals such as Cu^{2+} to prevent Aβ aggregation.

Chapter 5: General summary and conclusions

The thesis ends with a comprehensive summary and conclusions describing the key observations and salient aspects that emanated from the present study described in Chapters 2-4.

In a nut shell the study provides the following significant contributions,

- The aggregation parameters of Aβ are stereospecific; L-enantiomer of Aβ(1-40) is more prone for aggregation when compared to that of D-enantiomer.
- A new evidence on stereospecificity of DNA nicking activity of Aβ; D-enantiomer of Aβ40 is more potential in nicking ScDNA when compared to L-form; mechanism of DNA nicking is through the induction of SSBs.
- Aqueous GE prevents Aβ aggregation and also dissolves preformed fibrils through non-protease activity; Boiled GE only prevents aggregation and does not dissolve preformed fibrils.
- SAC has anti-amyloidogenic activity on Aβ; partially folded conformation is induced in Aβ on binding to SAC.
- CG acts as a dietary metal chelator by inhibiting Cu^{2+} induced Aβ aggregation.

Conclusion: The above study provided us an insight into the mechanism on the role of amino acid stereospecificity based aggregation pattern, non-apoptotic Aβ induced DNA nicking and neuro-neutraceutical property of garlic and curcumin derivatives. These findings provided an insight in molecular understanding of the neurodegeneration and neuroprotection in AD brain.

Chapter 6: Bibliography

(Veer Bala Gupta) (Dr. K.S. Jagannatha Rao)

Candidate Research Guide

CHAPTER 1

General Introduction

1.1. Introduction

Alzheimer's disease (AD) is a progressive, degenerative disease of the brain, which causes thinking and memory to become seriously impaired. It is the most common form of dementia. The major clinical symptoms include loss of memory, judgment and reasoning, and changes in mood, behavior and communication abilities. AD reduces the remaining life expectancy by almost half and raises the risk of death over five years threefold. The duration of AD, from onset to death, ranges from 2 to 20 years. Symptoms of AD will often become noticeable between the ages of 65 and 85, becoming more prevalent as the person grows older and the disease progresses. Although rare, AD can manifest as early as age 45, and is termed "early onset" AD when it occurs before 65. More than 4.5 million Americans are believed to have AD and by 2050, the number could increase to 13.2 million. Approximately 65,800 victims die and 350,000 new cases of AD are diagnosed each year. America is not alone in dealing with this terrible affliction. In every nation where life expectancy has increased, so has the incidence of AD. AD is becoming tragically common. It is estimated that there are currently 26 million people worldwide with AD. This figure is projected to grow to more than 106 million people by 2050. Previously, in India and South Asian nations, cultural traditions took the onset of senility as a natural process of aging. However, there is a greater recurrence of this disease now noticed in India, especially among the people of middle classes. Dr. Alois Alzheimer first identified the disease in the year 1906 (Alzheimer, 1907). Today, this degenerative brain disorder bears his name. Dr. Alzheimer noticed changes in the brain tissue of a woman who had died of an unusual mental illness. Upon her death, he performed an autopsy on her brain and described dense deposits outside and around the nerve cells, now called as senile plaques (SPs). Inside the nerve cells he noted the presence of twisted bands of fibers, now called as neurofibrillary tangles (NFTs). A molecular genetic and histologic analysis of the neuropsychiatric case described by Dr. Alzheimer has been reported (Graever et al., 1997). The observation of the SPs and NFTs at autopsy is still required to obtain a definitive diagnosis of AD because these are considered hallmarks of AD.

Divry (1927) succeeded in staining the SPs using the special dye Congo Red, which bound to a component called amyloid on the basis of its physicochemical properties resembling those of polysaccharides. In the middle of the 1980s, the core protein component of paired helical filaments (PHFs) was identified as microtubule-associated protein tau by the groups of Wisniewski in the U.S. and Brion in Belgium (Grunke-Iqbal et al., 1986; 1988; Brion et al., 1986). Close to the same time, Glenner and collaborators found that the amyloid deposits were composed of a 4-kDa peptide with a significant beta-sheet structure called beta amyloid (Glenner and Wong, 1984).

As AD progresses and affects different areas of the brain, various abilities become impaired. The result is changes in abilities and/or behaviour. At present, once ability is lost, it is not known to return. However, recent studies are now suggesting that some relearning may be possible. Those who develop the disorder later in life may die from other illnesses (such as heart disease) before AD reaches its final and most serious stage. The reaction of an individual to the illness, his or her capacity to cope with it, also varies and may depend on such factors as lifelong personality patterns and the nature and severity of stress in the immediate environment. Depression, severe uneasiness, and paranoia or delusions may accompany or result from the disease, and they can often be alleviated by appropriate treatments. Although there is no cure for AD, treatments are available to alleviate many symptoms that cause suffering. Histopathologic characteristic features seen in the brain of AD patients are the presence of SPs with Aβ accumulation, NFTs and cerebrovascular amyloid deposits (Selkoe, 1989; Terry, 1994). Figure 1.1 shows a comparison between the AD and non-AD brain tissue.

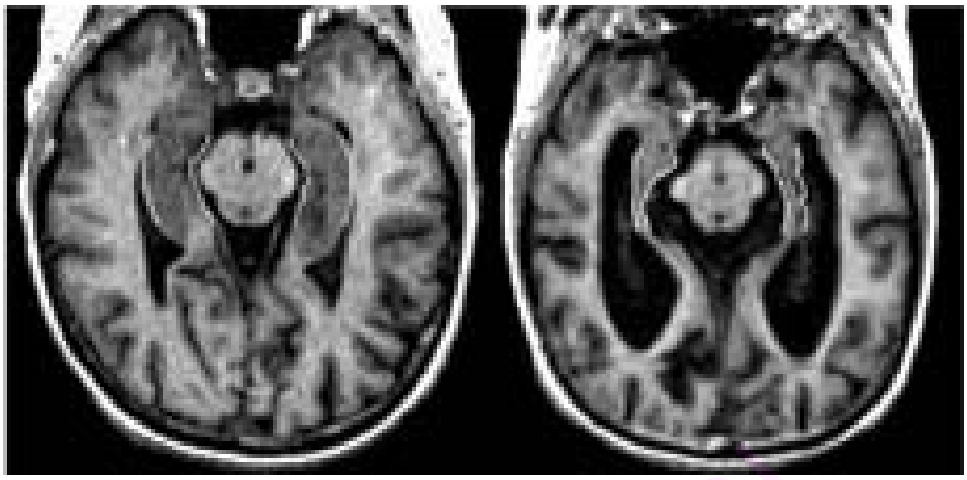


Figure 1.1: MRI showing difference between AD and non-AD cases: A person with AD has less brain tissue (right) than a person who does not have the disease (left). This shrinkage will continue over time, affecting all the brain functions. (Image credit: via Wikipedia).

1.2. Symptoms

At first, the main symptom may be mild forgetfulness, which can be confused with age-related memory change. Most people with mild forgetfulness do not have AD. In the early stages of AD, people may have trouble in remembering recent events, activities, or the names of familiar people or things. They may not be able to solve simple math problems. Such difficulties may bother, but usually they are not serious enough to cause alarm. However, as the disease progresses, symptoms are more easily noticed and become serious enough to cause people with AD. Forgetfulness begins to interfere with daily activities. People in the middle stages of AD may forget how to do simple tasks like brushing their teeth or combing their hair. They can no longer think clearly. They can fail to recognize familiar people and places. They begin to have problems in speaking, understanding, reading, or writing. Later on, people with AD may become anxious or aggressive, or wander away from home.

However, National Institute of Aging has declared seven warning signs of AD, which are following:

1. Asking the same question over and over again.

2. Repeating the same story, word for word, again and again.

3. Forgetting how to cook, or how to make repairs, or how to play cards, activities that were previously done with ease and regularity.

4. Losing one's ability to pay bills or balance one's checkbook.

5. Getting lost in familiar surroundings, or misplacing household objects.

6. Neglecting to bathe, or wearing the same clothes over and over again, while insisting that they have taken a bath or that their clothes are still clean.

7. Relying on someone else, such as a spouse, to make decisions or answer questions they previously would have handled themselves.

Efforts to increase knowledge about AD symptoms need to be expanded, with special attention to risk groups. Improved recognition of AD symptoms will promote adequate help-seeking behaviors and may increase early identification and treatment of AD (Werner et al., 2003).

1.3. Classification

AD is usually divided into early-onset (presenile) dementia accounting for 25% of cases and late-onset (senile) dementia using the age 65 yrs at onset as the cut off age (Terry and Katzman, 1983). It is also divided into familial and sporadic forms of disease according to family history (genetic background). Early-onset familial cases comprise 10% and late-onset familial cases 30% of all AD patients (Van Duijn et al., 1991). Most cases of AD are sporadic and familial AD patients only account for about 5% of all AD. Clinical and pathological findings are common to both sporadic and familial AD.

1.4. Diagnosis

AD is primarily a clinically diagnosed condition based on the presence of characteristic neurological features. Determination of neurological characteristics is made utilizing patient history, clinical observation, memory testing and assessment of intellectual functioning over a series of weeks or months. Supplemental physical testing, including blood tests and neuroimaging, is utilized to rule out other diagnoses. Psychological testing includes screening for depression and a mini mental state examination (MMSE). This can be helpful in establishing the presence and severity of

dementia, but does not readily allow for the distinction of AD from other forms of dementia. The MMSE or Folstein test is a brief 30-point questionnaire test that is used to assess cognition. It is commonly used in medicine to screen for dementia. In the time span of about 10 minutes, it evaluates various functions, including arithmetic, memory and orientation. It was introduced by Folstein et al., (1975), and is widely used with small modifications. The MMSE is a copyrighted test by Psychological Assessment Resources, Inc. Interviews with family members and/or caregivers are also utilized in the initial assessment of the disease, as a patient with AD may tend to minimize his or her symptoms, or may undergo evaluation at a time when his or her symptoms are less apparent, as quotidian fluctuations ("good days and bad days") are a common feature of the disease. Observations noting that a patient's good memory function decreases over time, play a critical role in the diagnosis of AD.

No medical tests are available to diagnose AD conclusively pre-mortem. However, the clinical diagnosis of AD is based on criteria defined in the Diagnostic and Statistical Manual of Mental Disorders (DSM III-R, American Psychiatric Association 1987) and criteria of the National Institute of Neurological and Communicative Disorders and Stroke and AD and Related Disorders Association Work Group (NINCDS-ADRDA) (Mc Khann et al., 1984). The NINCDS-ADRDA criteria provide guidelines for clinical diagnosis of AD. Definite AD can be confirmed in neuropatholigical examination of the brain tissue either from biopsy or autopsy material. The criteria of the Neurophathology Task Force of the Consortium to Establish a Registry for AD (Mirra et al., 1991) have been commonly used to set the neuropathologic diagnosis of AD. Expert clinicians who specialize in memory disorders can now diagnose AD with an accuracy of 85–90%, but a definitive diagnosis of AD must await microscopic examination of brain tissue which generally occurs at autopsy.

1.4.1. Criteria for clinical diagnosis of AD

The new criteria proposed by Waldemar et al., (2007) aims to define the clinical, biochemical, structural, and metabolic presence of AD. The key or cornerstone criterion proposed is episodic memory deficit that is isolated or in combination with other cognitive changes. Other features of episodic memory deficit include:

- Gradual and progressive change in memory function reported by patients or informants over more than six months.
- Objective evidence of significantly impaired episodic memory on testing.

Memory deficit should be combined with one or more of these supportive features:

- Presence of medial temporal lobe atrophy including volume loss of hippocampi, entorhinal cortex, and amygdala as determined by Magnetic resonance imaging (MRI).
- Low Aß42 concentrations, increased total tau concentrations/ increased phospho-tau concentrations or combinations of these three in cerebral spinal fluid (CSF).
- Reduced glucose metabolism in bilateral temporal parietal regions on functional MRI, or other "well validated ligands, including those that foreseeably will emerge such as Pittsburgh compound B or FDDNP (fluoroethyl](methyl)amino]-2-naphthyl]ethylidene) malononitrile."
- Proven AD autosomal dominant mutation within the immediate family.

AD can be excluded as a diagnosis if there is a sudden onset of memory deficit or early occurrence of gait disturbances, seizures, or behavioral changes and these are.

- Hemiparesis, sensory loss, visual field deficits.
- Major depression.
- Cerebrovascular disease.
- Toxic and/or metabolic abnormalities.
- MRI FLAIR or T2 signal abnormalities in the medial temporal lobe that are consistent with infectious or vascular insults.

1.5. Genetic risk factors of AD

The genetics of AD is proving to be complex and the number of genetic risk factors associated with this disorder are increasing steadily (Bertram and Tanzi, 2004; Cacabelos, 2004). The progress in genetic analysis has made it possible to identify three genes that cause familial AD, which are the genes for amyloid precursor protein (APP) on chromosome 21, presenilin-1 (PS1) on chromosome 14, and presenilin-2 (PS2) on chromosome 1 (Seherrington et al., 1995; Levy-Lahad et al., 1995a and b; Rogaev et al.,

1995; Goate et al., 1991, Lambert et al., 2007). Mutations in the APP gene on chromosome 21 can cause early onset disease. Mutations of PS1 lead to the most aggressive form of familial AD (Vetrivel et al., 2006). The presenilins have been identified as essential components of the proteolytic processing machinery that produces beta amyloid peptides through cleavage of APP. Inheritance of the ε4 allele of the ApoE gene is also regarded as a susceptible genetic risk factor both in sporadic and familial late-onset AD patients (Saunders et al., 1993).

1.5.1. Mutations in APP

APP is an integral membrane protein expressed in many tissues and concentrated in the synapses of neurons. Its primary function is not known, though it has been implicated as a regulator of synapse formation (Priller et al., 2006) and neural plasticity (Turner et al., 2003). APP is best known and most commonly studied as the precursor molecule whose proteolysis generates Aβ, a 39-42 amino acid peptide whose amyloid fibrillar form is the primary component of SPs found in the brains of AD patients. The APP gene is located on chromosome 21 (Goate et al., 1991) and contains at least 18 exons in 240 kilobases (Yoshikai et al., 1990; Lamb et al., 1993). Native APP is a transmembrane protein of 110 to 135 kDa, with a long extracellular N-terminal segment and a short intracellular C-terminal tail. The important cleavage product of the APP is a 39-43 amino acid peptide, the 4kDa Aβ (Glenner and Wong, 1984; Masters et al., 1985; Kang et al., 1987). Two thirds of sequence of the Aβ localizes to the N-terminal region of the APP and the remaining portion of the Aβ is contained within the transmembrane domain (Kang et al., 1987; Selkoe et al., 1988; Lemaire et al., 1989). APP undergoes extensive post-translational modification including glycosylation, phosphorylation, and tyrosine sulfation, as well as many types of proteolytic processing to generate peptide fragments (De Strooper et al., 2000). It is commonly cleaved by proteases in the secretase family; alpha secretase and beta secretase both remove nearly the entire extracellular domain to release membrane-anchored carboxy-terminal fragments that may be associated with apoptosis (Zheng et al., 2006). It is cleaved by gamma secretase within the membrane-spanning domain that generates the Aβ fragment. Depending upon the site of gamma secretase cleavage, the resultant peptides are typically either short (40 amino

acids) or long 42/43 amino acid). The longer form of Aβ is thought to be the most amyloidogenic and to be critical in the onset and progression of AD. Gamma secretase is a large multi-subunit complex whose components have not yet been fully characterized, but notably include presenilin, whose gene has been identified as a major genetic risk factor for AD (Chen et al., 2006). The amyloidogenic processing of APP has been linked to its presence in lipid rafts. When APP molecules occupy a lipid raft region of membrane, they are more accessible to and differentially cleaved by beta secretase, whereas APP molecules outside a raft are differentially cleaved by the non-amyloidogenic alpha secretase (Ehehalt et al., 2003). Gamma secretase activity has also been associated with lipid rafts (Vetrivel et al., 2004). The role of cholesterol in lipid raft maintenance has been cited as a likely explanation for observations that high cholesterol and apolipoprotein E (Apo E) genotype are major risk factors for AD (Riddell et al., 2001).

Several alternative splicing isoforms of APP have been observed in humans, ranging in length from 365 to 770 amino acids, with certain isoforms preferentially expressed in neurons. The changes in the neuronal ratio of these isoforms have been associated with AD (Matsui et al., 2007). A total of six mutations have been described so far, all of which lead to AD. The APP gene mutations are responsible for about 2% of all published cases of familial AD (Tanzi et al., 1996; Campion et al., 1996). The first clue pointing to the involvement of chromosome 21 in AD came from the observation that individuals with Down's syndrome, who have chromosome 21 trisomy, invariably develop clinical and pathological features of AD if they live over 30yrs (Mann et al., 1986). This supported a theory that chromosome 21 can underlie AD phenotype (Wisniewski et al., 1985; Mann, 1985). Mutations in APP gene instigate overproduction of Aβ, an elevated synthesis of Aβ (1-42) compared to Aβ (1-40) or the synthesis of peptides containing single amino acid substitution. Studies have shown that this substitution triggers an accelerated protofibril formation, which represents an observation that links enhanced formation of neurotoxic assemblies with disease etiology (Lahiri and Greig, 2004).

1.5.2. Mutations in Presenilins genes

Presenilins are a family of related multi-pass transmembrane proteins that function as a part of the gamma-secretase protease complex. Vertebrates have two presenilin genes, called PSEN1 (located on chromosome 14 in humans) that encodes presenilin 1 (PS-1) and PSEN2 (on chromosome 1 in humans) that codes for presenilin 2 (PS-2). The genes for the presenilins were found through linkage studies using mutations present in familial AD cases in 1995 (Sherrington et al., 1995). Mutations in the presenilin proteins are known to cause early onset AD.

Mutations of PS1 are the most common finding in patients with familial AD. At least 41 different mutations have been found in the PS-1 gene (Hardy, 1997). PS-1 mutations account for 30-50% of presenile AD families (Hutton et al., 1996). The PS-2 mutations (2% of all early-onset familial AD) are less common and cause early-onset familial AD than mutations in the PS-1. In familial AD families with the PS-1 gene mutation, the mean age of onset is earlier (45 yrs; range 29 to 62 yrs) than in families with the PS-2 gene mutation (52 yrs, range 40 to 88 yrs) and in the APP gene mutation linked families (50 yrs; range 43 to 62 yrs) (Lendon et al., 1997). The mechanism of action of the PS1 and PS2 and their familial AD- associated mutations in AD are not yet known. However, a number of functions have been proposed for presenilins, including direct involvement as a g-secretase-like protease in the cleavage of membranous proteins such as APP and Notch, a role in intracellular trafficking and modulation of calcium homeostasis. Presenilins are also implicated in the processing of notch, an important developmental protein. Mice that have the PS1 gene knocked out die early during development from developmental abnormalities similar to those found when notch protein is disrupted (Jie Shen et al., 1997). An important part of the disease process in AD is the accumulation of Aβ protein. To form Aβ, APP must be cut by two enzymes, beta secretases and gamma secretase. Presenilin is the sub-component of gamma secretase that is responsible for the cutting of APP by gamma secretase. Gamma secretase can cut APP at several points within a small region of the protein which results in Aβ of various lengths. The lengths associated with AD are 40 and 42 amino acids long. Aβ 42 is more likely to aggregate to form plaques in the brain than Aβ 40. Presenilin mutations

lead to an increase in the ratio of Aβ 42 produced compared to Aβ 40, although the total quantity of Aβ produced remains constant (Citron et al., 1997). This can come about by various effects of the mutations upon gamma secretase (Bentahir et al., 2006). Further, PS1 interacts with Glycogen synthase kinase, one of the critical proteins involved in tau phoshphorylation. Thus PS1 mutations in some familial AD cases would increase tau hyperphosphorylation at sites that transform tau into protein lacking the ability to associate with the cytoskeleton and generate PHF.

1.5.3. Polymorphism in ApoE

ApoE, a major lipoprotein in brain is a lipid and cholesterol transport protein. ApoE is involved in the growth and regeneration of both peripheral and central nervous tissues during development and following an injury in the central nervous system (CNS). Additionally, apoE is believed to play a pivotal role in the redistribution of lipid and cholesterol during membrane repair and has been postulated to be important for maintaining synaptic plasticity, especially after neuronal injury (Guillaume et al., 1996). In humans, apoE is a single gene with three major allelic variants (e2, e3 and e4) encoding three protein isoforms (Weisgraber and Mahley, 1996).

The gene for apoE is located on the proximal arm of chromosome 19, in fact, in the very same region where a gene for late-onset familial AD is located (Pericak-Vance et al., 1991). These alleles determine apoE polymorphism, resulting in six possible phenotypes e 2/2, 2/3, 2/4, 3/3, 3/4, and 4/4. ApoE e 4 allele is recognized as a risk factor for late-onset familial (Strittmatter et al., 1993) and sporadic AD (Saunders et al., 1993) in a dose-dependent manner. The greater the number of copies of this allele in a person, the greater their chance of developing AD. However, recent work with African populations has shown that this rule is not universal (Gureje et al, 2006). The risk increases with an increasing number of e 4 alleles (Corder et al., 1993). In contrast, e2 allele appears to have a protective effect for AD. The risk associated with e4 may lose its significance after a certain age and may no longer be a risk among the oldest old (Hyman et al. 1995). A low frequency of ApoE allele consistent with low prevalence of AD has been reported in India (Chandak et al., 2002). The exact role of apoE in the pathogenesis of AD is unknown, but isoform-specific differences have been identified in the binding of

apoE to Aβ. It is proposed that apoE e2 and e3 stabilize the structures, whereas e4 is a susceptibility factor leading to increased vulnerability or a cause of pathologic alterations (Strittmatter and Roses, 1995). Synaptosomes from mice with human apoE4 have shown to be significantly more vulnerable to the oxidative stress associated with Aβ (1-42) than synaptosomes from apoE3 and apoE2 mice. Besides AD, subjects carrying the e 4 allele also have higher levels of total and low-density-lipoprotein cholesterol, a higher risk for myocardial infarct and coronary heart disease.

1.6. Environmental risk factors

The etiology of AD has not yet been fully understood. It is not one single cause, but several factors that affect each person differently. The finding that monozygotic twins may not both develop AD (Rapport et al., 1991) suggests that environmental factors also play major role in the development of AD.

1.6.1. Aging

Age is the most important known risk factor as AD is an age dependent disorder and its prevalence increases with advancing age. The number of people with the disease doubles every 5 years beyond age 65 yrs. Recent finding has identified 2 potential mechanisms related to aging that may contribute to the development of the disease. One concept is that the reactive oxygen species (ROS) produced during cellular respiration may play important role in the process of aging and in the onset of AD. Another possible mechanism related to aging is messenger RNA. A novel type of mutations in 'vulnerable' dinucleotide repeats in messenger RNA has been discovered in AD patients (van Leeuwen et al., 1998). In this type of mutation, a mutated transcript is produced from a correct DNA sequence by a process called "Molecular misreading". Molecular misreading can be regarded as a genetic biological source of transcript errors that may be involved in cellular derangement in numerous age-related pathological conditions apart from AD. The resulting '+1 proteins' are prominent neuropathological hallmarks of AD and they are present in most elderly non-demented people also. This suggests that the dinucleotide deletions in the transcript could be one of the earliest events in the neuropathogenesis of AD and a factor in neuronal aging (van Leeuwen et al., 1998).

1.6.2. Diet

It is crucial to investigate the environment and diet as primary risk factors in AD pathology (Dosunmu et al., 2007). A high-fat diet during early and mid-adulthood may be associated with an increased risk of developing AD, especially in people with a marker called the ApoE-e4 allele. ApoE-e4 carriers aged 60 and over who consumed a similar high-fat diet had a 12-fold higher risk of developing AD than those who ate a high-fat diet and did not have the e4. In people aged 20-39, the combination of ApoE-e4 and a diet with more than 40 percent of calories from fat raised the risk of AD by almost 23 times compared with those with high fat diets and no ApoE-e4. Fat may be subject to a process called oxidation, which results in the formation of damaging chemicals called free oxygen radicals that can cause tissue injury. Antioxidant nutrients, as well as other chemicals called flavonoids and carotenoids, can act as defenses against these molecular flamethrowers.

A high fat, low carbohydrate diet improves AD in mice. A study by Auwera et al., (2005) showed that ketogenic diet reduces Aβ40 and 42 in a mouse model of AD. If carbohydrates are high, dietary fat is not oxidized and is instead stored as body fat." When carbohydrates are very low and fat is high, compounds called ketone bodies are generated (ketosis) and these compounds may play a role in the observed reduction in Aβ.

1.6.3. Smoking

There is conflicting research but there are indications that nicotine is a protector against AD. However, smoking is also known to cause cardiovascular and respiratory diseases that are AD risk factors.

1.6.4. Lack of exercise

An increasing amount of research indicates that moderate regular exercise helps to reduce risk for AD.

1.6.5. Alcohol

Low alcohol intake can be beneficial to your health. However high alcohol intake is known to increase your risk of dementia significantly.

1.6.6. Intelligence, education risk factor for AD

A higher standard of education has been identified as offering some protection against AD. Smaller brains and head size leading to fewer connections within the brain have also been suggested in causation of AD. It is likely that poor education may mask other factors such as poverty which may well help the development of AD.

1.6.7. Severe head trauma and previous depression

These may also increase the risk of AD (Mortimer et al., 1991; Van Duijn et al., 1991; Jorm et al., 1991). Head trauma or traumatic brain injury is also associated with a predilection to the development of AD accounting for between 2% of AD cases (Mortimer et al., 1991). The presence of cerebral infarcts, even if small and scarce, raised the risk of dementia by as much as 20 times for those with AD-type lesions. This may be the most common mechanism by which vascular injuries contribute to dementia, since infarcts in the absence of AD-type lesions had few cognitive effects. Thus the vigorous treatment of hypertension and other vascular risk factors and the promotion of healthy diet and exercise could potentially reduce the incidence of dementia. The presence of *low folate levels* is also considered modifiable risk factor of AD (Katzman, 2004).

1.6.8. Estrogen

Several epidemiological studies have shown that women on estrogen replacement therapy may be less likely to be diagnosed with AD (Henderson, 1997; Henderson, 2004), and a few small trials claim improvement in female patients with AD who are taking estrogen. The wide spread effects of estrogen in the brain make these results plausible, although the mechanisms have not been fully elucidated. Menopausal transition is a critical phase of women's life where the occurrence of an unfavorable biological milieu would predispose to an increased risk of neurodegeneration (Bonomo et al., 2007).

1.6.9. Metal Toxicity

There has been growing interest in the area of metals and their implication in AD. A recent review summarizes studies that implicate a role for several metals in contributing to or causing AD (Shcherbatykh et al., 2007). Evidences suggest that trace metal homeostasis plays a crucial role in the normal functioning of the brain and any

disturbance in it, can exacerbate events associated with AD. Epidemiological evidence suggests that exposure to aluminium (Al) from drinking water is associated with increased incidence of AD (Martyn et al., 1989; McLachlan et al., 1996). Inoculation of Al into rabbit brain was demonstrated to produce NFT's resembling the NFT's of AD (Savory et al., 1999). Further Al exerts several neurotoxic effects which mimic that of AD (Gupta et al., 2005). A significant piece of circumstantial evidence linking Al neurotoxicity to AD can be derived from promising clinical trials involving the treatment of AD patients with Al ion chelator, desferrioxamine B (McLachlan et al., 1991). Al concentration was also shown to be elevated in NFTs and SPs. However for various reasons the role of Al in AD has become a matter of controversy. It was shown that Al has low DNA binding affinity while it has higher affinity towards RNA (Schuurmans et al., 1990). They also found low Al levels in the nucleus. However, Lukiw et al. (1991) have shown increased amounts of Al in chromatin and they detected high concentration of Al (885.4 mg/g DNA) in DNA isolated from the neuronal nuclei of AD. Recent studies from our lab showed that Al not only strongly binds to DNA but also causes helicity change in DNA (Latha et al., 2002; Champion et al., 1998; Rajan et al., 1996).

Large body of evidence indicates that the homeostasis of zinc (Zn), copper (Cu) and iron (Fe) and their respective binding proteins, are significantly altered in the AD brain (Atwood et al., 1998; Doraiswamy and Finefrock, 2004). There are two generic reactions of metals with reference to neurodegenerative diseases. Firstly, a metal-protein association leading to protein aggregation and secondly, metal-catalyzed protein oxidation leading to protein damage and denaturation. Aβ, a metalloprotein binds to metal ions Zn^{2+}, Cu^{2+} and Fe^{2+}. Zn^{2+} binding precipitates Aβ and also it may have a protective effect by displacing Cu^{2+} and Fe^{2+} enriched in Aβ plaques in AD. On the other hand, there is an evidence that Zn^{2+} can initiate plaque formation by its ability to bind to Aβ under non-acidic conditions and by creating the inflammation, which leads to acidity. Under acidic conditions, which exist in inflamed tissue, Cu^{2+} displaces Zn^{2+} (Cuajungco and Faget, 2003). Cu^{2+} enhances β-sheet formation of Aβ fibrils and this enhancement is potentiated by apoE (Huang et al., 1999), which in turn promotes the aggregation of Aβ

that deposits as SPs. Also binding of redox active Cu^{2+} and Fe^{2+} to Aβ produces H_2O_2 that involves the reduction of these metal ions.

Mercury is also elevated in the AD brain. Mercury can bind to tubulin, the primary protein constituent of microtubules, thereby interfering with microtubule assembly. Zinc & selenium may protect against mercury neurotoxicity. The figure below summarizes the different factors involved in AD pathogenesis (Figure 1.2).

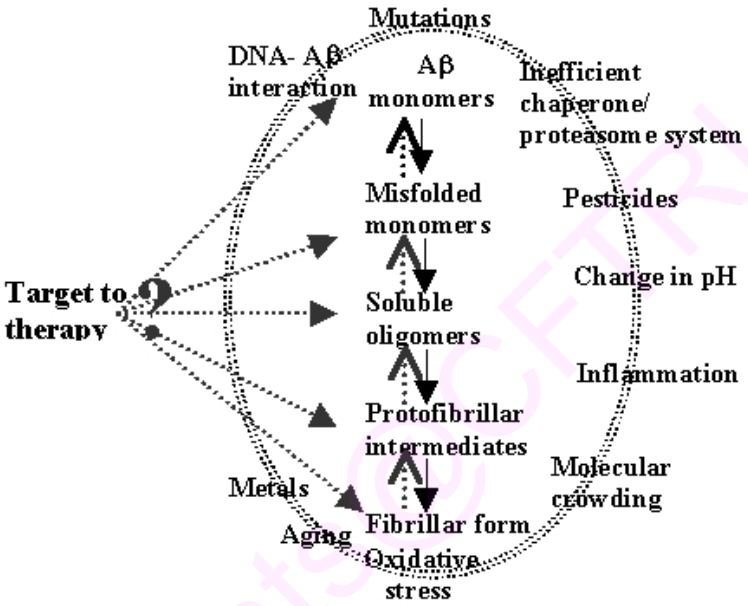


Figure 1.2: Different factors involved in regulating Aβ toxic pathway: There is a lot of controversy regarding which form of Aβ should be targeted for therapy (as indicated by dotted arrows above). We highlighted that the predominant resultant form obtained after therapy should be non-toxic to the neurons.

1.7. Neuropathology of AD

The clinical heterogeneity of AD is a result of variation in the distribution, quality and severity of pathological changes in the brain. This diversity has even led to the assumption that AD might be considered as a convergent syndrome rather than a single disease. In brief, currently AD is considered to be a multifactorial disease, with a combination of aging, genetic aberration and/or environmental factors triggering the pathological cascade: accumulation of hallmarks of AD pathology, followed or accompanied by cytoskeletal and mitochondrial abnormalities, loss of neurons and

synaptic connections, impaired cellular homeostasis, inflammatory reaction, and gliosis, which eventually lead to the clinical presentation of the disease to take place. Although cerebral atrophy is a typical manifestation of AD, it does not distinguish from AD accurately enough to be diagnostic; this applies to neuroimaging as well as gross inspection at post mortem. However, microscopic examination reveals the critical features of the disease- a cerebral cortex peppered with SPs and NFTs: the classic pathological changes considered as hallmarks of AD (Yankner, 1996).

1.7.1. Amyloid plaques or senile plaques

AD is now identified as a protein misfolding disease due to the accumulation of abnormally folded Aβ protein in the brains of AD patients (Hashimoto et al., 2003). Aβ monomers are soluble and contain short regions of beta sheet and polyproline II helix secondary structures in solution, (Danielsson et al., 2006) though they are largely α-helical in membranes (Tomaselli et al., 2006). However, Aβ at sufficiently high concentration, undergoes a dramatic conformational change to form a β sheet-rich tertiary structure that aggregates to form amyloid fibrils (Ohnishi et al., 2004). These fibrils deposit outside neurons in dense formations known as SPs, in less dense aggregates as diffuse plaques. These also deposit sometimes on the walls of small blood vessels in the brain in a process called amyloid angiopathy or congophilic angiopathy.

SPs are complex and consist of extracellular deposits of amyloid material and are associated with swollen, distorted neuronal process called dystrophic neurites. The term 'amyloidosis' was coined by Virchow (1854) being derived from amylose or amylon and means 'starch-like'. Amyloid is a generic term for the primarily extracellular accumulation of fibrillar protein deposits, which have unique tinctorial and structural properties. At least 20 unrelated normally nonfibrillar proteins are known precursors of amyloid (Westermark, 1997). In AD, Aβ peptide aggregates and accumulates in the brain as diffuse and compact plaques. Diffuse plaques are not associated with degenerative changes; where as compact plaques composed of Aβ fibrils are associated with pathological changes in the surrounding brain parenchyma. The basis of this differential neuronal response is unclear but may relate to the different physical states of Aβ in

diffuse and compact plaques (Yankner et al., 1990). An imbalance between Aβ production and Aβ clearance is the basis for the formation of SPs (Kowalska, 2004).

Aβ, a self- aggregating peptide, is derived by proteolytic cleavage of APP. While it was originally thought that Aβ represents an abnormal cleavage product, but now Aβ has been established as a normal product of neuronal APP metabolism, found in the CSF and serum of healthy individuals (Haass et al. 1992; Shoji et al., 1992). APP and its isoforms are distributed evenly in neuronal cell bodies and their axons and dendrites. The APP –positive neuronal processes showed mesh-like networks and in AD mesh-like networks are generally decreased (Shoji et al., 2000). Differential activity between three different secretase, α, β and γ at their specific cleavage sites yields a number of different products, including Aβ(1–40) and Aβ(1–42) (Sisodia, 1992). While Aβ (1–40) is the predominant product of this proteolytic pathway, Aβ(1–42) is far more fibrillogenic *in vitro* and is the major Aβ species present in the core of SP (both AD and non-AD related) (Burdick et al.,1992; Jarrett and Lansbury, 1993). The deposition of Aβ(1–40) and Aβ(1–42) into SP begins with the nucleation of soluble Aβ(1–42) into fibrils followed by accumulation of normally soluble Aβ(1–40) (Jarrett and Lansbury, 1993). Micro-environmental changes in the brain, such as pH, metal ion (Cu, Fe and Al) availability and oxidants, likely impact upon Aβ structural conformation and its deposition as amyloid plaques (Atwood et al., 1998; Smith et al., 1997). As of late a great deal of attention has also been focused on the fact that soluble forms of amyloid that are pre-fibrillar may also be involved in AD pathogenesis (Lambert et al., 1998). Nevertheless, soluble forms of Aβ, including oligomers, correlate quantitatively with the number of SPs (Teller et al., 1996).

The destructive nature of Aβ is evident from a close examination of histological preparations in the immediate vicinity of SPs revealing degenerative dendritic processes surrounding and infiltrating the plaques (Geddes et al., 1986). Additionally, regions severely affected by disease, including the hippocampus and frontotemporal cortices, show colocalization between Aβ plaques and neuronal cell death (Rogers and Morrison, 1985). This lead investigators to explore whether Aβ is toxic to neurons in both *in vitro*

culture assays and in the intact brain of animals. The results of these experiments at first seemed extremely contradictory, stemming from lot-to-lot variability in the peptide and the lack of proper control over whether Aβ was aggregated into fibrils of β-sheet conformation (Cotman et al., 1992). But, it is now established that fibrillation of Aβ is required to obtain neurotoxic effects (Lorenzo and Yankner, 1996; Pike et al., 1993) and that it is inherently toxic to neurons and clonal cell lines in culture (Pike et al., 1991; Yankner et al., 1990). Toxicity of the peptide has been shown to reside in between amino acids 25 and 35 (Pike et al., 1995). The neurotoxicity of the peptide in vivo was likewise assessed by infusion of the peptide in a variety of animal models. Notably, intracortical injection of Aβ(1–42) or Aβ(25–35) fragments into aged rats or primates produced lesions reminiscent to those seen in AD patients (Kowall et al.,1992). In vivo injection of Aβ into the brain also led to neurodegeneration and dystrophic neurites in hippocampal neurons (Pike et al., 1992).

The source of Aβ toxicity has yet to be established, however, a number of theories have been advanced. The possibility that Aβ may act through a cell surface receptor and thereby trigger an intracellular signaling cascade, while not being eliminated as a possibility, is supported by little experimental evidence. Numerous studies have since supported the idea that an oxidative event is critical for Aβ toxicity (Mattson and Goodman, 1995). It is thought that the peptide is capable of generating ROS, which is supported by the fact that Aβ peptides can induce the generation of H_2O_2 (Hensley et al., 1994; Huang et al., 1999), can stimulate inflammatory cells to produce ROS (Akama et al., 1998; Butterfield et al., 1996; Meda et al., 1995) and that neurotoxicity can be attenuated by administration of anti-oxidants and free radical scavengers, such as Vitamin E (Behl et al., 1992). The prooxidant potential of Aβ is also supported by in vivo evidence where deposits are associated with oxidative damage (Atwood et al., 2001; Smith et al., 1994) and such damage is, like Aβ deposition (Selkoe, 1996), viewed as an extremely proximal event in disease pathogenesis (Nunomura et al., 1999 and 2000). However, while it is clear that Aβ, either directly or indirectly, promotes oxidative stress and that toxicity can be attenuated by anti-oxidants, the precise mechanism by which

amyloid deposition leads to increased oxidative stress remains elusive. Indeed, while studies have suggested that the neurotoxicity of aggregated Aβ is mediated by its ability to induce oxidative stress via the spontaneous generation of free radicals and ROS (Hensley et al., 1994), this proposition has been questioned on theoretical and methodological grounds (Dikalov et al., 1999; Turnbull et al., 2001). Instead, it now appears that the oxidant effects of Aβ are mediated by its interaction with redox-active metals such as Fe and Cu since metal chelation treatment of Aβ significantly attenuates toxicity (Rottkamp et al., 2000). Significantly, Aβ has an unusually high affinity for both Fe and Cu (Atwood et al., 1998; Cuajungco et al., 2000) and is capable of reducing these metals with subsequent production of H_2O_2 and oxidized amyloid (Huang et al., 1999). The relevance of this mechanism to disease pathogenesis is highlighted by the association of redox active metals with SPs in AD (Sayre et al., 2000; Smith et al., 1997). In addition, the deposition of this normally soluble cellular protein promotes a chronic inflammatory response of the AD brain, whereby activated microglia release ROS as part of the respiratory burst (Atwood et al., 2001). Therefore, Aβ likely has much to answer for with regards to the oxidative damage observed in the AD brain. Aβ(1-42) and Aβ(25-35) were also shown to cause marked oxidative damage to mitochondrial DNA (Pappolla et al., 1999). Further, studies also revealed increased mitochondrial DNA oxidation in AD (Mecocci et al., 1994).

1.7.2. Neurofibrillary Tangles

AD is also considered a tauopathy due to abnormal aggregation of the tau protein, a microtubule-associated protein expressed in neurons that normally acts to stabilize microtubules in the cell cytoskeleton. Like most microtubule-associated proteins, tau is normally regulated by phosphorylation; however, in AD patients, hyperphosphorylated tau accumulates as paired helical filaments (Goedert et al., 2006) that in turn aggregate into masses inside nerve cell bodies known as NFTs and as dystrophic neurites associated with SPs. NFT is the most consistent post-mortem characteristic of AD, consisting of phosphorylated fibrillary proteins aggregated within the neuronal cytoplasm. Tau protein (consisting of 441 amino acids) incorporation into PHFs is a pathognomonic sign of AD. In 1986, Brion and coworkers showed that antibodies against the cytoskeletal protein tau

labeled NFTs; these studies were complemented by findings in several laboratories that tau is the main component of PHF (Kosik et al., 1986). NFTs composed of arrays of PHFs are present mainly in the hippocampus, entorrhinal cortex, and amygdala. PHFs are anomalous structures generated by self-aggregation of hyperphsophorylated forms of tau protein that form a compact filamentous network (Maccioni and Cambiazo, 1995). Tau, a member of the microtubules associated protein family, is a phosphoprotein with multiple phosphorylation sites and widely expressed in brain. The primary function of tau is to maintain microtubule stability (Geula et al., 1998), which plays an important role in axonal transport and morphogenesis.

The neurofibrillary changes in AD may present as NFTs in nerve cell bodies and apical dendrites, and neuropil threads in remote dendrites and in the abnormal nerve endings associated with SPs. Nerve cells with NFTs and synapses within the region covered by SPs are believed to undergo degeneration, become dysfunctional and result in AD. The NFTs developed within the soma of the neuron, get converted into extraneuronal structures, and are finally engulfed and degraded by astrocytes, after degeneration of the parent cell (Braak and Braak, 1991). The development of tangles is a major and possibly the main mechanism of neuronal death in AD (Gomez- Isla et al., 1998). Some groups of neurons are preferentially affected by tangles in AD. For example NFTs frequently occur in areas of the hippocampus that are involved in processing experience prior to storage as permanent memories. This correlates with the clinical deficits observed in the early stages of AD in learning and in the creation of new memories, as well as with the relative preservation of established memories. The neurons at the basal forebrain that provide most of the cholinergic innervations to the cortex are also permanently affected resulting in the cholinergic neurotransmitter deficits.

The discovery of tau as the major subunit of PHFs in AD has markedly increased interest in understanding the structure and function of this protein. In a normal neuron, tau is localized in the axons and neuronal soma (Binder et al., 1985). The tau and tangle hypothesis argue that in AD, the normal role of tau in stabilizing microtubules is impaired and indeed, in diseased neurons microtubules are gradually replaced by tangles. At present much of what is known regarding tau hyperphosphorylation and cytoskeletal

degeneration has come from studies aimed at understanding the neurotoxic effects of Aβ (Yankner, 1990). Understanding how tau is produced and processed is important to researchers as they attempt to understand how AD unfolds in the human brain. Tau processing also may serve as a target for the development of new therapies to treat the disease. In AD, aggregated tau is hyperphosphorylated and phosphorylation of tau reduces its ability to bind microtubules (Frank et al., 2003). It is the incorporation of excess phosphate groups i.e. hyperphosphorylation, which lead to the formation of PHF/tau. Currently promising efforts are underway to establish phosphorylated tau in CSF as a putative disease specific biological marker of AD (Lu and Kosik, 2001; Rachakonda et al., 2004; Olsson et al., 2004). In normal brain, equilibrium between phosphorylation and dephosphorylation of tau modulates the stability of the cytoskeleton and consequently axonal morphology. Amyloid fibril formation has been shown to be one factor associated with tau protein phosphorylation in AD (Busciglio et al., 1995). Further, the pattern of tau phosphorylation correlates with a loss of neuronal integrity and with the severity of dementia (Augustinack et al., 2002). NFTs account for between 2.2 and 17.2% of neuronal loss in AD (Kril et al., 2004).

Immunohistochemical experiments show that tau exists in nuclei of various cell lines, such as human neuroblastoma and human retinoblastoma. In the nucleus, as detected by Tau-1 monoclonal antibody, tau localizes to the nucleolar organizer regions (NORs) (Loomis et al., 1990; Haque et al., 1999; Greenwood et al., 1995). However, whether or not tau is associated with DNA in mature brains *in vivo* needs further study. Further, studies have shown that tau can bind both eukaryotic (calf thymus, plasmid DNA) and phage DNA and synthetic polynucleotides of arbitrary nucleotide sequence when they were double stranded and single stranded DNA did not bind to tau. Hence tau appears to bind to double stranded DNA and induced DNA conformational bending (Corces et al., 1980; Hua and He, 2000; Hua and He, 2002 a and b), however the function of tau in the neuronal nuclei is unknown. Microtubule-associated proteins have been shown to have higher affinity for DNA than for microtubules and their removal from the microtubules by DNA causes microtubule breakdown (Corces et al., 1980). Separation of

tau from chromatin fraction has indicated the direct or indirect association of nuclear tau with DNA (Greenwood et al., 1995).

1.8. Hypotheses regarding the cause of AD

Although the gross histological features of AD in the brain are well characterized, three major hypotheses have been advanced regarding the primary cause. The oldest hypothesis suggests that deficiency in cholinergic signaling initiates the progression of the disease. Two alternative misfolding hypotheses instead suggest that either tau protein or Aβ initiates the cascade. While researchers have not identified a clear causative pathway originating from any of the three molecular hypotheses to explain the gross anatomical changes observed in advanced AD, variants of the amyloid beta hypothesis of molecular initiation have become dominant among the three possibilities.

1.8.1. Cholinergic hypothesis

The oldest hypothesis is the "cholinergic hypothesis". It states that AD begins as a deficiency in the production of acetylcholine, a vital neurotransmitter. Much early therapeutic research was based on this hypothesis, including restoration of the "cholinergic nuclei". The possibility of cell-replacement therapy was investigated on the basis of this hypothesis. All of the first-generation anti-Alzheimer's medications are based on this hypothesis and work to preserve acetylcholine by inhibiting acetylcholinesterases (enzymes that break down acetylcholine). These medications, though sometimes beneficial, have not led to a cure. In all cases, they have served to only treat symptoms of the disease and have neither halted nor reversed it. These results and other research have led to the conclusion that acetylcholine deficiencies may not be directly causal, but are a result of widespread brain tissue damage, damage so widespread that cell-replacement therapies are likely to be impractical. More recently, cholinergic effects have been proposed as a potential causative agent for the formation of plaques and tangles (Shen, 2004) leading to generalized neuroinflammation (Wenk, 2003).

More recent hypotheses center on the effects of the misfolded and aggregated proteins, Aβ and tau. The two positions are lightheartedly described as "ba-ptist" and "tau-ist" viewpoints in scientific publications by AD researchers. "Tau-ists" believe that

the tau protein abnormalities initiate the disease cascade, while "ba-ptists" believe that Aβ deposits are the causative factor in the disease (Mudher and Lovestone, 2002).

1.8.2. Tau hypothesis

The hypothesis that tau is the primary causative factor has long been grounded in the observation that deposition of amyloid plaques does not correlate well with neuron loss (Schmitz et al., 2004). A mechanism for neurotoxicity has been proposed based on the loss of microtubule-stabilizing tau protein that leads to the degradation of the cytoskeleton (Gray et al., 1987). However, consensus has not been reached on whether tau hyperphosphorylation precedes or is caused by the formation of the abnormal helical filament aggregates (Mudher and Lovestone, 2002). Support for the tau hypothesis also derives from the existence of other diseases known as tauopathies in which the same protein is identifiably misfolded (Williams, 2006). However, a majority of researchers support the alternative hypothesis that amyloid is the primary causative agent (Mudher and Lovestone, 2002).

1.8.3. Amyloid hypothesis

The amyloid hypothesis is initially compelling because the gene for the amyloid beta precursor APP is located on chromosome 21, and patients with trisomy 21 - better known as Down syndrome - who thus have an extra gene copy almost universally exhibit AD-like disorders by 40 years of age (Nistor et al., 2006; Lott and Head, 2005). The traditional formulation of the amyloid hypothesis points to the cytotoxicity of mature aggregated amyloid fibrils, which are believed to be the toxic form of the protein responsible for disrupting the cell's calcium ion homeostasis and thus inducing apoptosis (Yankner et al., 1990). This hypothesis is supported by the observation that higher levels of a variant of the Aβ protein known to form fibrils faster in vitro correlate with earlier onset and greater cognitive impairment in mouse models (Iijima et al., 2004) and with AD diagnosis in humans (Gregory and Halliday, 2005). However, mechanisms for the induced calcium influx, or proposals for alternative cytotoxic mechanisms, by mature fibrils are not obvious.

A more recent and broadly supported variation of the amyloid hypothesis identifies the cytotoxic species as an intermediate misfolded form of Aβ, neither a soluble

monomer nor a mature aggregated polymer but an oligomeric species, possibly toroidal or star-shaped with a central channel (Blanchard, 2000) that may induce apoptosis by physically piercing the cell membrane (Abramov et al., 2004). A related alternative suggests that a globular oligomer localized to dendritic processes and axons in neurons is the cytotoxic species (Barghorn et al., 2005; Kokubo et al., 2005). Recently Lesne et al., (2006) reported a significant observation on the existence of a specific Aβ protein assembly in the brain, termed as Aβ*56, which is involved in memory impairment. They showed that young Tg2576 mice, have normal memory and lack any neuropathology. However, middle aged mice develop memory deficits along with extracellular accumulation of Aβ*56 without neuronal loss. The aged mice developed memory loss with abundant neuritic plaques containing Aβ. Further, they showed Aβ*56 could able to induce memory loss when injected into young mice. They also demonstrated the presence of lesser quantities of nonamers along with the Aβ*56 which is a dodecamer of Aβ42. They proposed that Aβ*56 impaired memory independently of plaques.

Relevantly, the cytotoxic-fibril hypothesis presented a clear target for drug development: inhibit the fibrillization process. Much early development work on lead compounds has focused on this inhibition (Blanchard et al., 2004; Porat et al., 2006; Kanapathipillai et al., 2005); most are also reported to reduce neurotoxicity, but the toxic-oligomer theory would imply that prevention of oligomeric assembly is the more important process (Lee et al., 2005) or that a better target lies upstream, for example in the inhibition of APP processing to amyloid beta (Espeseth et al., 2005).

1.9. Oxidative Stress in AD

The term ‘oxidative stress’ is used when the body’s natural defense mechanisms are exceeded by the production of deleterious ROS, resulting in damage to susceptible cell components such as DNA, proteins and lipids. Classically oxidative stress is described as an imbalance between generation and elimination of ROS. These reactive species were originally considered to be exclusively detrimental to cells (Scandalios et al., 2002). It is now recognized that redox regulation involving ROS, is key to the modulation of critical cellular functions, ion transport, calcium mobilization, and

apoptosis program activation. When oxidative stress occurs, cells functions to counteract the resulting oxidant effect and to restore the redox balance. Oxidative stress has been implicated as an important mechanism in AD. The detrimental effects of oxidative stress in the brain and nervous system are primarily a result of the diminished capacity of the CNS to prevent ongoing oxidative damage (Reynolds et al., 2007). A balance between the production of ROS and antioxidant defenses is essential to avoid oxidative stress. Under normal conditions damage by oxygen radicals is kept in check by an array of antioxidant systems which display extensive redundancy (e.g: the simultaneous metabolism of H_2O_2 by catalase and glutathione peroxidase). However, the oxidant versus antioxidant balance is altered during degenerative conditions, as it is associated with ageing either primarily or secondarily. But fortunately the cells have a variety of mechanisms designed to compensate during periods of increased oxidative stress. In AD, evidence suggests that susceptible neurons recruit multiple regulatory mechanisms that involve a complex interplay between proteins, DNA and metals. Hence, it is highly important to maintain a balance between them. Antioxidant therapies might prove promising avenue to certain extent for treatment. However, if the treatments have to be successful the initial source or the triggering factor for this has to be characterized. Neither the upregulation, nor subsequent deposition of Aβ, nor the protein modifications found in NFT seem to be the cause of the oxidative stress. But ROS is termed to be the initial trigger and the major contributing factor for the oxidative imbalance, including the redox active transition metals and abnormalities in the mitochondrial metabolism.

The rationale to examine oxidative stress in aging brain is based on the following aspects a). Brain has high content of easily peroxidizable unsaturated fatty acids (especially high in 20:4 and 20:6 fatty acids); b). Brain requires very high amounts of oxygen per unit weight (about 20% of the total amount used in humans; c). Brain has a high content of both Fe and ascorbate (i.e. they are key ingredients in carrying out membrane lipid peroxidation) and d). Brain is not highly enriched in antioxidant protective defenses and this then adds to its otherwise readily poised potential for oxidative damage. While most of the radicals are sequestered in the mitochondria, oxidative insult is exacerbated by age, metabolic demand, and disease conditions such as

AD. Free radicals produced during oxidative stress are speculated to be important in AD and other neurodegenerative diseases (Smith et al., 1995). Free radicals produce tissue damage through multiple mechanisms, including excito-toxicity, metabolic dysfunction, and disturbance of intracellular homeostasis of calcium. Over the past decade, modification to virtually all classes of biomacromolecules indicative of oxidative stress has been described in association with the susceptible neurons of AD: a). DNA and RNA oxidation is marked by increased levels of 8-hydroxyl-2-deoxygaunosine (8OHG). DNA repair deficiency is also noted in AD since higher levels of DNA breaks, DNA nicking and fragmentation are observed in AD patients (Mecocci et al., 1994; Mecocci et al., 1997; Nunomura et al., 1999; Kadioglu et al., 2004). b).Oxidative modification of proteins is marked by significantly elevated levels of protein carbonyl and widespread nitration of tyrosine residues (Smith et al., 1997). Some specifically oxidized proteins have recently been identified by proteomics (Castenga et al., 2003) and it is notable that many are either enzymes that are related to ATP generation or enzymes involved in glycolysis. Therefore, oxidative modification may lead to metabolic impairment in AD. Moreover, crosslinking of proteins, by oxidative process, may lead to the resistance of the lesions to intracellular and extracellular removal even though they are extensively ubiquitinated (and this resistance of NFTs to proteolysis might play an important role in the progression of AD (Crass et al., 1995). c). Lipid peroxidation is marked by higher levels of thiobarbituric acid reactive substances, malondialdehyde, 4-hydroxy-2-transnonenal and isoprostane and altered phospholipid composition (Butterfield et al., 2001) and d). Modification to sugars is marked by increased glycation and glycoxidation (Castellani et al., 2001; Vitek et al., 1994).

Increasing evidence suggests that the very earliest neuronal and pathological changes characteristic of AD show indication of oxidative damage (Perry et al., 1998; Pratico et al., 2001) indicating that oxidative stress represents a very early contributor to the disease. A systematic examination of the spatiotemporal relationship between the presence of oxidative modification and the hallmark AD lesions at early AD stages suggest that many markers of oxidative damage are present in susceptible neurons even without neurofibrillary pathology (Nunomura et al., 1999). Further, there is marked

accumulation of 8OHG and nitrotyrosine in the cytoplasm of cerebral neurons from Down syndrome cases in their teens and twenties, which temporally precedes Aβ deposition by decades (Nunomura et al., 2000). Some specific mechanisms proposed for generation of ROS in AD include direct generation by Aβ, activation of microglia by Aβ and possibly electron transport chain defects (Mattson and Goodman, 1995). In AD, ROS have been proposed to interact with amyloid protein to create SPs and amyloid may generate oxygen radicals within the membrane and damage membrane lipids and other proteins (Mattson and Goodmann, 1995).

1.10. Apoptosis in AD

Apoptosis, a programmed cell death by intrinsic mechanism to regulate cell population, characterized by cell shrinkage and nuclear condensation and fragmentation, has been shown to occur extensively in brains from patients with AD. In addition to NFTs and SPs, abundant apoptotic neuronal and glial cells are another pathological hallmark of AD (Su et al., 1994; Cotman and Anderson, 1995; Smale et al., 1995; Lucassen et al., 1997). An aberration in apoptosis regulation is implicated in the pathogenesis of neurodegeneration, a multifaceted process that leads to various chronic disease states in addition to AD such as Parkinson's (PD), Huntington's (HD) diseases, amyotrophic lateral sclerosis, spinal muscular atrophy, and diabetic encephalopathy (Okouchi et al., 2007).

Increasing evidence points towards a role for apoptosis in AD. This evidence comes from both *invitro* and *invivo* studies (Smith et al., 1996; Sugaya et al., 1997; Su et al., 1996; Anderson et al., 1995). Two families of proteins have been shown to play a major role in initiating and regulating apoptosis. The first is a family of cysteine proteases, the caspases, which cleave substrates following the aspartic residue and bring about cell death through proteolysis of proteins vital to cellular homeostasis (Cohen, 1997, Nicholson and Thornberry, 1997). The second, is the Bcl-2 family of proteins (Barinaga, 1994; Clearly et al., 1986; Martin et al., 1994) which comprises both antiapoptotic and proapoptotic members and plays an important role in regulating caspases activation (Jacobsen, 1996). The Bcl-2 onco-protein has a protective effect

against apoptotic and necrotic cell death (Clearly et al., 1986; Garcia et al., 1992; Hockenbery, 1995; Reed, 1995; Stellar, 1995).

Bcl-x is also a member of the Bcl-2 family of protein (Barinaga, 1994; Frankowski et al., 1995). It has two spliced forms, Bcl-x_s and Bcl-x_I (Minn et al., 1995), while Bcl-x_s promotes apoptosis, Bcl-x_I has a similar apoptosis sparing effect to Bcl-2 (Barinaga, 1994; Minn et al., 1996). Another member of the family is the Bax protein, which is believed to promote apoptosis. The four members of the family are known to hetero and homodimerize with known inhibiting each other's action. Therefore in a cell that expresses various amounts of these proteins, the fate of the cell may be dependent upon the ratio of these proteins (Barinaga, 1994; Oltvai et al., 1993). The absence of the apoptosis-sparing Bcl-2 protein from neurons in the hippocampus of AD patients may indicate that these neurons are vulnerable to apoptotic stimuli. Although the Bcl-x protein expression did not seem to be clearly linked to any individual pathological feature in AD. Strong positive signal for Bcl-x was never detected in controls and overall labeling intensity showed an increase with accumulation of AD-related pathology. It was shown that Bcl-x related phenomenon might possibly be involved in the death or survival of the neurons in AD-affected regions of the brain. The presence of this apoptosis promoting protein in these structures and the absence of Bcl-2 or Bax which are capable of hetero-dimerizing with Bax and counteracting its effect, indicate that the cells with Bax-positive processes in neuritic plaques are likely to be prone to cell death. These results strengthen the evidence that apoptosis-related phenomena are related to cell death in AD and related disorders. However, the pathway involved in triggering apoptotic cell death may be different in different cells and may also differ from one patient to another suggesting that these phenomenon are heterogenous. NFTs formation, Aβ deposits, high concentration of APP, caspase-3, the PS 1 and 2 gene and nitric oxide are considered to be important triggers of neuronal and glial apoptosis (Ugolini et al., 1997). It was shown that the APP gene is upregulated in dying motorneurons deprived of trophic support (Barnes et al., 1998), and that the production of Aβ is increased by approx. 2-4 folds in human primary neuronal cultures induced to undergo apoptosis by serum deprivation (LeBlanc et al., 1995). Under *in vitro* conditions, Aβ (1-42) is reported to be capable of inducing

apoptosis in cultured cortical neurons (Loo et al., 1993; Cotman et al., 1994). Synthetic Aβ peptides are shown to trigger the degeneration of cultured neurons through activation of an apoptotic pathway (Huang et al., 2000). Neuronal cells treated with Aβ exhibit morphological and biochemical characteristics of apoptosis, including membrane blebbing, compaction of nuclear chromatin and internucleosomal DNA fragmentation. Esposito et al., (2004) reported that Aβ, especially intracellular Aβ counteracts the antiapoptotic function of its precursor protein and primes proapoptotic pathways. Interestingly, studies by Baron et al., (2001) suggested that Aβ exert its toxic effect via activation of transcription factors. The authors reported that Aβ (25-35) induced the expression of the growth arrest and DNA damage-inducible gene (gadd 45) implicated in the DNA excision-repair process (Baron et al., 2001). In particular, they observed strong up regulation of gadd45, indicating DNA damage in Aβ cytotoxicity suggesting that DNA strand breaks occurred rapidly in cells exposed to Aβ which may be a critical event in Aβ neurotoxicity (Baron et al., 2001). Furthermore, aurintricarboxylic acid a nuclease inhibitor was shown to prevent apoptotic DNA fragmentation and delays cell death caused by Aβ (Loo et al., 1993). Moreover DNA damage suggestive of apoptosis was increased 30 fold in neurons, oligodendrocytes, and microglia of AD brains (Lassmann et al., 1995).

There appears to be a strong link between Aβ deposition and neuronal apoptosis (Earnshaw et al., 1999). According to the study by Gervais et al., 1999, during apoptosis in the brain APP gets proficiently cleaved by members of the apoptosis- associated family of proteases known as caspases resulting in increased formation of Aβ peptide. On the other hand, in neurons associated with amyloid deposits in the brains of AD, there was observed increased DNA damage and caspase activity with alterations in expression of apoptosis- related genes such as Bcl-2 family members, Par-4, and DNA damage-response genes (Su et al., 1994). The involvement of apoptosis in AD is further suggested by the fact that APP is a substrate for caspase-3 which results in the release of a C-terminal peptide namely C31, a potent inducer of apoptosis (Gervais et al., 1999). Figure 1.3 highlights the different mechanisms leading directly or indirectly to apoptosis in brain.

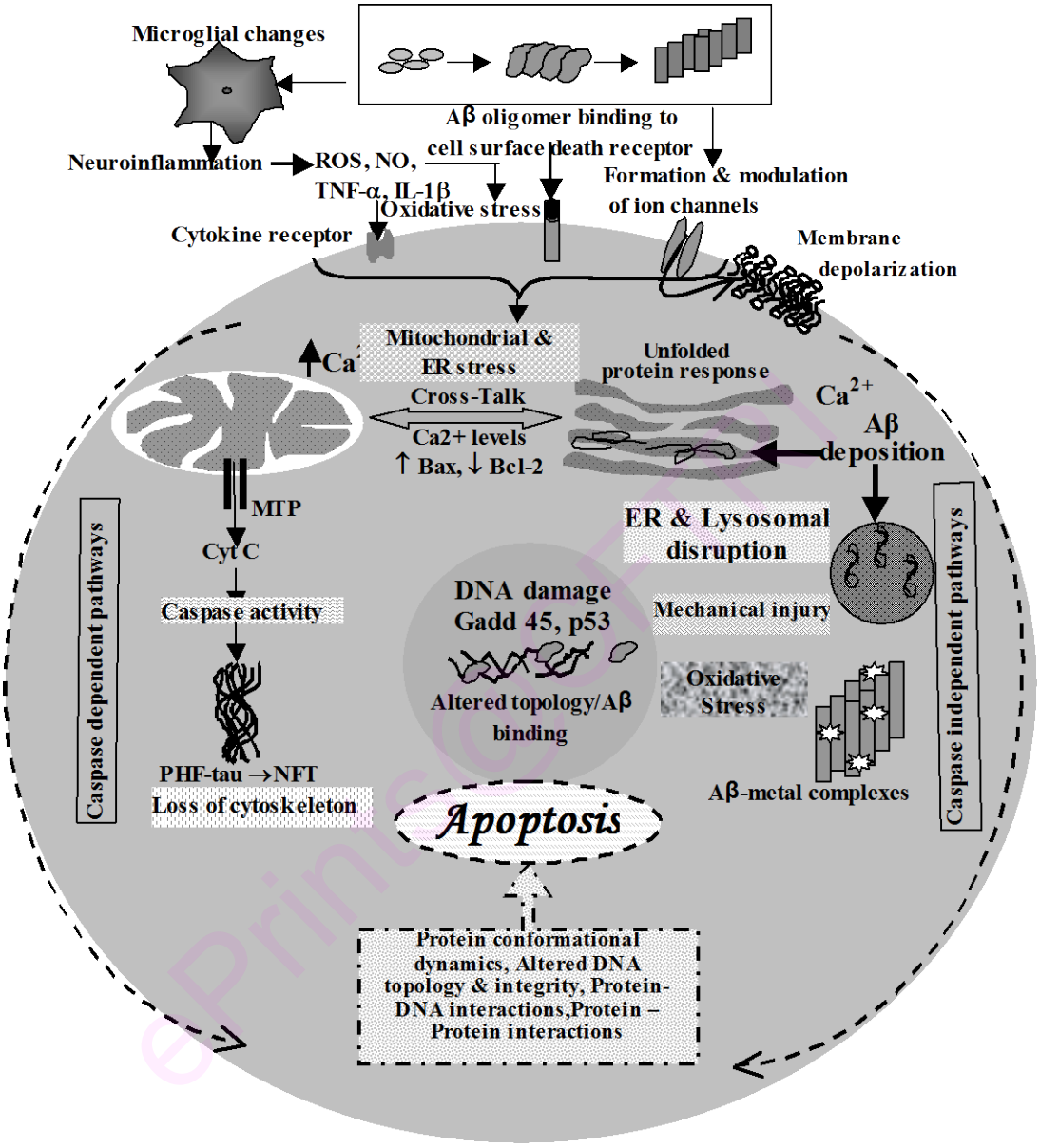


Figure 1.3: Different mechanisms leading directly or indirectly to apoptosis in brain: The stimuli for the cell death can be intrinsic as well as extrinsic ultimately causing mitochondrial and ER leading to increased Ca^{2+} concentration, hence activating caspase cascade, leading to apoptosis- DNA damage. DNA damage also may occur by direct binding of these amyloidogenic proteins or may activate certain DNA damage inducing genes, hence giving rise to excess DNA damage observed in these disorders.

1.11. DNA damage and altered DNA topology in AD

The etiopathogenesis of AD is poorly understood. One of the mechanisms responsible for cellular death in AD might be an accumulation of DNA damage. Mullaart et al., (1989) found that at least a twofold higher level of DNA damage in cortex from individuals with AD as compared to control. The appearance of apoptotic cells in AD brains was analyzed mainly by the method of deoxynucleotidyl transferase dUTP end labeling (TUNEL) staining which indicates cells with fragmented DNA (a phenomenon resulting from DNA fragmentation in apoptotic cells). Several reports show that AD brains exhibit more abundant TUNEL staining than age-matched control brains and that in neurons, the TUNEL signal colocalizes with intracellular Aβ (Su et al., 1994; Anderson et al., 1995). DNA fragmentation was found frequently in cell bodies containing NFTs, as observed using either the anti-tau antibody or the anti-NFT antibody. There was tendency for more strongly anti-tau stained cells to exhibit DNA fragmentation more frequently (Sugaya et al., 1997). Damage to nucleic acids caused by ROS includes base modification, SSBs, and DSBs. The predominant marker of oxidative damage is the hydroxylated nucleoside 8OHdG. A relatively high basal level of 8OHdG is detected in the brain of control and AD subjects (Gabbita et al., 2001; Lyras et al., 1997, Mecocci et al., 1994). There are conflicting reports as to whether 8OHdG is elevated in nuclear DNA in any AD brain region (Gabbita et al., 2001) but an increase is detected in mitochondrial DNA (Mecocci et al., 1994). Elevated levels of oxidative DNA damage were also found in lymphocytes from patients with AD (Morocz et al., 2002; Kadioglu et al., 2004). In addition increased oxidative damage to RNA in neurons throughout the brain of AD subjects is observed (Nunomura et al., 1999). Evidence for widespread SSBs and DSBs in AD brains has been provided by in situ labeling methods (Lassmann et al., 1995; Stadelmann et al., 1998; Su et al., 1994). The relative contribution of free radical mediated DNA cleavage, ongoing or incomplete DNA repair process or endonuclease cleavage as part of an apoptotic cascade to the generation of DNA strand breaks is not known. Whether DNA fragmentation accumulation over the course of disease or occurs within the perimortem period is yet to be established. A correlation between DNA fragmentation and Aβ deposition was made in cortex and

midfrontal gyrus from patients with AD implicating Aβ as one of the factors of cell injury (Colurso et al., 2003).

Brain cells are very complex and have high level of gene expression, 3-4 times more than that found in liver and kidney. In brain cells, where the level of gene expression is very high, accumulated DNA damage may have serious consequences in terms of altering genomic integrity consequently affecting the transcriptional fidelity and ultimately the survival of the cell (Mandavilli and Rao, 1996). It was speculated that accumulated DNA damage in the brain might result in conformational changes in the DNA making it ineffective during transcription. Information on the conformational manifestation of eukaryotic DNA or their biological significance with reference to gene regulation and expression is limited. This is of considerable interest as biological function is often correlated with structure at molecular level. Under various conditions, non B-type conformations also occur in biological systems like Z-DNA, triple helix, tetraplexes, hairpin, cruciform etc., other than the right-handed B-form which is the most predominant conformation (Rich et al., 1993). It has been proposed that both the sequence and the DNA topology have a crucial role in DNA functional aspects like replication, transcription, and susceptibility of DNA for oxidative damage and mutations. Extensive studies on handedness and various high ordered structures were studied for the oligonucleotides (Latha et al., 2003; Cooke et al., 2004) but little is known about genomic DNA topology with respect to non Watson-Crick right-handed B-DNA forms, their functional ability and possible implications in the pathogenic features in the brain.

Our laboratory has for the first time evidenced the presence of left-handed Z-DNA conformation in the severely affected AD brain hippocampus and a B-Z intermediate DNA in moderate AD. The B-Z intermediate form observed in MAD (Moderate AD) and Z-DNA in SAD (Severe AD) reveal that the topological transition is AD progression dependent (Suram et al., 2002). In particular interest with AD, it is observed from the Human Genome Sequence that the Z-DNA conforming GC rich sequences are observed in 5´ regions of AD specific genes like PS1, PS2, and apoE (Rogaev et al., 1997). It is interesting to mention that some of these genes have been over expressed in AD and have significant role in AD pathogenesis. It was reported that Z-DNA formation excludes

nucleosome formation and could affect the placement of nucleosome as well as organization of chromosomes (Garner et al., 1987). It was also suggested that a distinct class of human genes that may potentially be transcriptionally regulated by a mechanism that couples Z-DNA with nuclear factor (Champ et al., 2004).

DNA fragmentation could be one of the crucial steps in B→Z DNA topological transition since the fragmentation reduces the high activation energy barrier required to induce this conformation. Studies have shown that radiation induced DNA damage i.e. strand breaks can lead to B → Z conformational transition assisted by metals. The smaller metal ion concentration could induce this conversion in DNA with strand breaks than in the absence of strand breaks. It is attributed to the reduction in the high activation energy barrier in the metal ion driven B → Z conformation (Trumbore et al., 1994). It also provides a clue as to why SAD-DNA could go to Z-form from B-form, probably due to DNA fragmentation and assisted by the metals like Zn, Cu, Fe, and Al, which are elevated in AD brain (Rao et al., 1999). Further our lab also proposed a new hypothesis on the relation between DNA damage and DNA topology in relevance to AD (Anitha et al., 2001).

Recently it was shown that lysine rich proteins favor the conversion of B→Z DNA transition and further it was found that methylated DNA could be easily converted from B to Z-DNA by the lysine rich proteins (Takeuchi et al., 1994). The altered DNA structure in the hippocampus, whether reflects the pathogenic event or represents an adaptation to cell death cannot be ascertained now. Some DNA-binding proteins have been found to have certain amino acid sequences that favor Z-DNA, hence it was hypothesized that AD specific proteins like Aβ and phosphorylated tau are lysine rich and probably have a role in inducing B→Z transition in AD brain and (Suram et al., 2002). It was proposed that Aβ, tau and Al interaction with DNA cause formation of intermediate structure, found in MAD and oxidative stress, altered metal homeostasis and cell shrinkage might be responsible for further change of DNA conformation from intermediate forms to the Z-DNA, observed in the SAD (Suram et al., 2002).

In vitro studies have further shown that Aβ(1-42) and Aβ(1-16) fragments bind to DNA and they also alter the conformation of DNA. Aβ(1-42) induced a B→Ψ DNA

transition in conformation, while Aβ(1-16) caused an altered B-DNA conformation in Supercoiled ScDNA (Hegde et al.,2004). It is interesting to correlate the Aβ induced conformational change in DNA to the B →Z DNA conformational transition observed in AD hippocampus (Suram et al., 2002). The Ψ DNA conformation induced by Aβ (1-42) has been found to be structurally similar to Z -DNA. Studies by Thomas and Thomas (Thomas and Thomas, 1989) clearly showed that Ψ DNA, an ordered, twisted, tight packing arrangement of the double helix, is immunologically related to Z- DNA family. Also, Ψ-DNA is left-handed in conformation like Z-DNA.

A possible complex role of Aβ and metals in modulating DNA helicity with reference to AD pathology has been proposed by our lab (Anitha et al., 2001; Hegde et al., 2004). It was proposed that Aβ and its fragments along with metals like Al, Cu, Zn etc. induce complex intermediate conformational changes (Ψ DNA, B →A, B →C or B-C-A, altered B) in genomic DNA in the initial phase of the AD pathology. In the later phase, secondary factors implicated in AD such as oxidative stress, cell shrinkage, ionic imbalance and polyamines are likely to play a role in converting these intermediary complex conformation to rigid, left-handed Z- DNA. Recent results showed that Aβ (1-42) has DNA nicking activity similar to nucleases. This finding of DNA nicking activity of Aβ peptides has biological significance in terms of causing direct DNA damage (Suram et al., 2007).

1.12. Intraneuronal Aβ toxicity

A new school of thought has emerged in the recent past, principally focusing on the importance of the intraneuronal Aβ besides the abundant deposits of extraneuronal Aβ. The earlier amyloid hypothesis was focused on the toxicity of aggregates or plaques of Aβ, which are predominately deposited in the extracellular milieu. The extracellular deposition of Aβ first came into light because it is a detectable neuronal alteration in AD. While some scientists earlier believed the relation between Aβ and AD simply involved the amyloid plaque, many have known for some time that it is much more complex. This is surely why the problem of the causes and treatment of AD is so difficult to solve.

Hence, this concept of SPs occupying a central stage, failed to exclusively explain majority of the pathological events in AD. Therefore, the subsequent debate with in the AD community had focused on whether fibrillar or soluble oligomers of Aβ are active species of the peptide that ultimately cause the synaptic loss and dementia associated with AD (Walsh et al., 2004). The native conformation of the protein in response to various environmental toxins, mutations, metals etc. might undergo misfolding. This misfolded form-monomer, acts as a seed and leads to the accumulation of oligomers, soluble which in turn leads to the formation of more detectable species i.e. Protofibrils, pathologically more distinct forms and subsequently to fibrils (Lambert et al., 1998). Even though, the molecular form associated with the neurodegeneration process had been identified, much success has not been achieved in understanding origin of Aβ toxicity in AD where oligomers of Aβ may be targeted for therapy. Hence, there was a definite indication that the toxicity also depends on the site of localization of Aβ. Therefore, the intracellular presence of potentially amyloidogenic fragments of Aβ became a subject of intensive investigation. Infact, the major reason why controversial amyloid hypothesis prevailed is that a full consideration to the localization of Aβ with regard to its toxicity was not given. Previously, SPs were known to be solely formed from the aggregation of the secreted Aβ from the neuronal cells extracellularly. Now it has been shown from histopathological studies that a portion of plaques seen in the AD brain, are actually derived from intracellular Aβ aggregation (Ginsberg et al., 1999). Nuclear remnants are found at the center of many developing SPs. Ginsberg et al., (1999) could also demonstrate the presence of neuron specific mRNAs in senile plaques, hence providing another clue for the intraneuronal origin of SPs. The death of the neurons just releases pre-aggregated Aβ, which later acts as a seed for the aggregation of extracellular Aβ into SPs. These studies suggested that Aβ-dependent toxicity could occur before significant extracellular accumulation. This may be due to some kind of perturbation in the intracellular Aβ metabolism which is responsible for promoting neurodegeneration in the initial stage. Figure 1.4 represents the contribution of different conformational forms of Aβ towards neurodegeneration in separate cellular compartments.

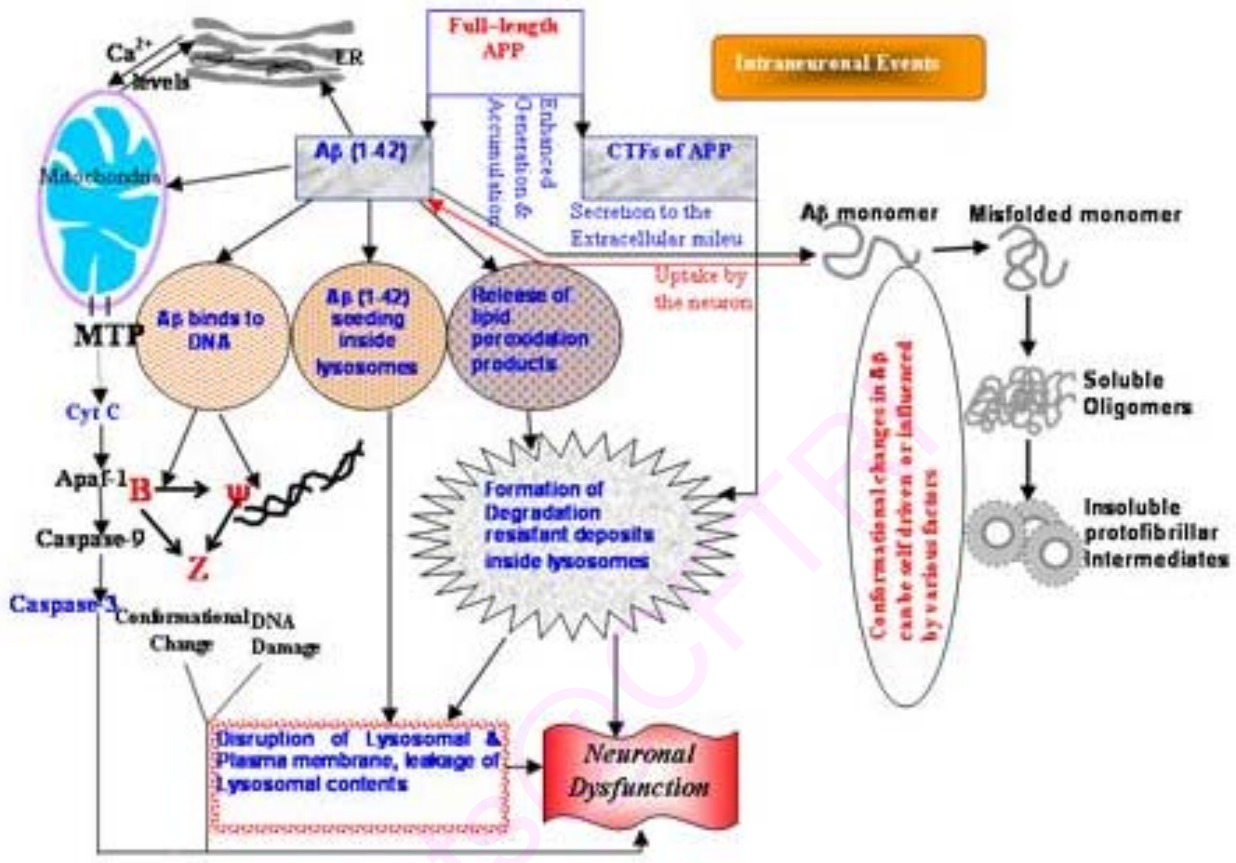


Figure 1.4: An overview of intraneuronal localization of Aβ and pathological mechanisms of Aβ toxicity: Aβ gets deposited in various organelles in the form of heterogenous degradation resistant deposits and leads to the disruption of vital cellular functions, resulting in neuronal dysfunction.

Hence, it can be hypothesized that Aβ protein is present in the brain for a good purpose but while serving the brain, it contributes to the toxicity depending upon the change in its molecular form, concentration and of course, its site of accumulation.

The existence of intracellular Aβ cannot be doubted upon because Aβ is generated intracellularly from APP cleavage mainly in the Golgi apparatus and endoplasmic reticulum (ER). It is certainly possible that some amount of Aβ42 accumulates inside the cells during this process. It has been reported that Aβ40 comprises 90% of secreted Aβ and Aβ42 comprises 10% of secreted Aβ, hence indicating that

length of the peptide is crucial for intraneuronal deposition of Aβ. It is known that Aβ42 forms water-soluble dimers that can form the building blocks of insoluble Aβ deposits (Kuo et al., 1996). Different sub-cellular locations are involved in the production of Aβ intracellularly, which includes the ER (Cook et al., 1997), the trans-Golgi network (TGN) (Xu et al., 1997) and the endosomal-lysosomal system (Koo et al., 1994). There are evidences on APP to be localized in the multivesicular bodies where Aβ may also be generated. Studies on primary neuronal cultures has shown that Aβ redistributes from the outer membrane of multivesicular bodies to the inner membranes of endosomes and microtubules as it aggregates from mono- to oligomeric state (Takahashi et al., 2002). There are reports on the presence of Aβ in the nuclear membrane and in the vicinity of DNA (Hegde et al., 2004, Grant et al., 2000). Aβ is known to get synthesized in the ER, from where it is transported to the Golgi and TGN where most of it is packaged in the post-TGN secretory vesicles. From this, it is eventually transported to the cell surface to be secreted extracellularly. But Aβ42 appears to be secreted less efficiently than Aβ40. Aβ42 is mainly generated in ER, nuclear envelope and ER pool of Aβ, and is known to be less secreted than that of Golgi, where Aβ40/42 are generated (Tienari et al., 1997). Cell surface Aβ can be later reinternalized via clathrin coated pit-mediated endocytosis into the endosomal-lysosomal system (Koo et al., 1994). Studies carried out to understand the regulation of APP processing showed that secreted Aβ and intracellular Aβ are generated by different mechanisms and cell- signaling events controlling the production of these two pools of Aβ are also different (Hartmann et al., 1997). The existence of dual pathways for the generation of intra and extraneuronal Aβ raises further question whether intraneuronal toxicity of Aβ is the primary mechanism of its action in AD. It is also interesting to mention that intraneuronal generation of Aβ is unique to neurons, because nonneuronal cells produce significant amounts of Aβ40 and Aβ42 only at the cell surface (Tienari et al., 1997). Inspite of its generation in intracellular membrane compartments, the extraneuronal Aβ drew much of our attention because of the fact that neurons secrete Aβ.

Masters et al., (1985) quoted that the “Amyloid is first deposited in the neuron

and later in the extracellular space". Wertkin et al., (1993) later demonstrated its presence intraneuronally. Reports suggest that neurons accumulate Aβ42 inside them with aging (Kawarabayashi et al., 2001) and also in Down's syndrome and AD (Masters et al., 1985). Mutations in APP or presenilin genes are also known to increase the production of intracellular Aβ42 (Holcomb et al., 1998). Decline in synaptic, behavioral and physiological functions of transgenic mice before the appearance of Aβ plaques indicates that the role of plaques in the pathogenesis of AD is not primary (Kawarabayashi et al., 2001). In addition, cytoplasmic proteins e.g. cathepsin D have also been noted in the space occupied by SPs (D'Andrea et al., 2001). Sheng et al., (2000) even observed inflammatory changes in the AD brain prior to plaque formation. The role of plaque associated Aβ became unclear with the postmortem studies in human brain where it was shown that soluble Aβ correlates better than insoluble Aβ or extracellular Aβ plaques with cognitive dysfunction in patients (Mclean et al., 1999). This gives us an indication that soluble intraneuronal Aβ42 protofibrils might be having a primary hand in the pathogenesis (Mclean et al., 1999). Studies with neuronal cell culture have also demonstrated that Aβ can accumulate intracellularly (Wertkin et al., 1993). Walsh et al., (2002) evidenced in cells derived from human brain that oligomerization of Aβ protein begins intracellularly. Neuronal cell culture studies also reveal the abundancy of intracellular Aβ42 in a time dependent manner (Skovronsky et al., 1998). Animal studies involving transgenic mice having a mutation in PS1 showed intraneuronal Aβ42 accumulation and neurodegeneration (Holcomb et al., 1998). Geula et al., (1998) results provide another very strong support in favor of soluble intraneuronal Aβ toxicity. They found that the micromolar concentrations of aggregated Aβ required for toxicity *in vitro* is in the range of Aβ levels in AD cortex where as single plaque-equivalent dose is required inside the cell for Aβ toxicity (Geula et al., 1998). In an attempt to understand the initial molecular triggers for the onset of AD-related cognitive decline, recently Billings et al., (2005) carried out studies on 3xTg-AD mice to show that intraneuronal Aβ is responsible for the onset of cognitive dysfunction. They also reported that clearance of the intraneuronal Aβ pathology by immunotherapy prevents the early cognitive deficits.

This study made it very clear that plaque/ tangle pathology does not contribute to cognitive pathology dysfunction at early stage.

Further, Bahr et al., (1998) have shown that neurons are also capable of internalizing Aβ, hence intracellular Aβ can also be derived from already present extracellular Aβ. They also demonstrated using hippocampal slice cultures that exogenous Aβ42 can be internalized within neurons and hence leads to the build up of endogenous neuronal βCTF which is an amyloidogenic precursor to Aβ. Knauer et al., (1992) further demonstrated that this internalization could be prevented under conditions that prevent endocytosis. This property of internalization is also not uniform for all the neurons. It was revealed that there is a selective uptake of Aβ42 by CA1 neurons whereas CA3 and dentate gyrus neurons are almost unaffected (Bahr et al., 1998). Hence, it can be said that extracellular secretion of Aβ42 by neurons can in turn increase the levels of intracellular Aβ42 and induce neuronal death. However, the trigging force behind the re-translocation of Aβ from extraneuronal to intraneuronal milieu is yet to be defined. We propose that the elevation of divalent metals like Cu and Zn in AD brain, could be one of the events to modulate Aβ homeostasis, as it has a high affinity for these metals. All these data unequivocally support the concept of intracellular existence, and toxicity of Aβ. In addition to it, we have also reviewed the events that take place intracellularly which lead to subsequent neuronal pathology.

Intracellular accumulation of APP toxic fragments has always been associated with neuronal degeneration and finally cell death. Studies have shown that apoptotic biochemical cascades like caspase activation, mitochondrial membrane depolarization, dysregulation of the cytosolic and mitochondrial calcium homeostasis get activated in AD brain after exposure to Aβ. There are reports of Aβ being directly toxic to isolated mitochondria (Casley et al., 2002). Aβ induced cell death involves the participation of mainly two subcellular organelles- mitochondria and ER through independent pathways. Further, an apoptotic cross-talk has also been identified between mitochondria and ER upon Aβ exposure (Takuma et al., 2005). Aβ is known to exert its effect through ligation of death receptors like the tumor necrosis factor receptor-1 and the Fas receptor causing the activation of procaspase-8. Caspase-8 can act further in two ways: It can activate

directly caspase-3 or cleave the pro-apoptotic Bcl-2 homology 3-only protein Bid, which then leads to the translocation, oligomerization and insertion of Bax into mitochondrial outer membrane and induces cytochrome *c* release from mitochondria, which complexes with apoptosis activating factor-1 and procaspase-9. Caspase-9 finally activates caspase-3 (Takuma et al., 2005). Mitochondria are considered to be the main target of Aβ induced cell death. Cytochrome *c* oxidase deficiency has also been reported in many cases indicating mitochondrial dysfunction in AD (Cottrell et al., 2001). Studies by Pereira et al., (1998) have shown that Aβ peptide significantly disrupts the activity of mitochondrial respiratory complexes-I, II-III, and IV leading to a decrease in mitochondrial oxygen consumption, depolarization of mitochondrial membrane and depletion of ATP levels. In addition to mitochondria, ER mediated apoptotic pathways are also activated upon exposure to Aβ via ER localized caspase-12. Localized Ca^{2+} release from ER activates calcium binding protein- calpain which cleaves and activates caspase-12, which activates caspase-9 to activate finally caspase-3. Alterations in ER Ca^{2+} homeostasis can also activate mitochondrial apoptotic pathway by translocating Bax or dephosphorylated Bad into the mitochondrial outer membrane (Takuma et al., 2005). In addition, mutations in PS-1 and PS-2, the two membrane proteins residing in the ER are known to weaken the stress response and enhance susceptibility by varying APP processing. PS-1 mutations make neurons more vulnerable for DNA damage, hence promoting ER mediated activation of calpains and caspase-12 (Chan et al., 2002). PS mutations are known to increase production of total Aβ and Aβ1-42, Aβ directly mediates ER stress responses and apoptosis, because Aβ is synthesized and accumulates in the ER (Takuma et al., 2005). It is also well known that proteins, which fold incorrectly and accumulate intracellularly have potential to induce cellular damage. Therefore, accumulation of Aβ in ER may also activate apoptotic mechanisms through the unfolded protein response (Takuma et al., 2005). Increase in cytosolic calcium concentration is known to specifically induce the production of large amounts of intraneuronal Aβ42. It is reported that calcium release from ER is not sufficient to induce the production of intraneuronal Aβ, which also requires influx of extracellular calcium (Pierrot et al., 2004).

It has also been proposed that any defect in autophagy which functions to degrade toxic misfolded proteins in neurodegenerative diseases is also known to increase cytosolic accumulation of Aβ (Hara et al., 2006). We are focused over pathological mechanisms of intraneuronal Aβ42 because it is more pathological when compared to its shorter counterparts. Aβ42 is known to form much more stable fibrils than Aβ(1-40) and shorter amyloid peptides (Barrow et al., 1991). The two C-terminal aminoacids, which are not present in the less"sticky" Aβ40, allow the formation of a β hairpin which then serves as the core of the fibrillar structure. Henderson et al., (1988) demonstrated that aggregates formed by Aβ42 are resistant to SDS when compared to the shorter Aβ peptides. Aβ42 when internalized is resistant to degradation and accumulates in late endosomes or lysosomes unlike Aβ40 and other shorter peptides which are rapidly degraded (Knauer et al., 1992). D-aminoacids and isopeptide bonds have been reported in amyloid deposits, characteristics of long-lived proteins, self explains why Aβ42 is resistant to degradation (Shapira et al., 1988). Yang et al., (1998) explains the mechanism that there is the leakage of the lysosomal contents into the cytoplasm due to the disruption of lysosomal membrane upon Aβ42 and other amyloidogenic fragment accumulation. No such leakage of lysosomal contents was observed on treatment of cells with non-accumulating Aβ1-40. The reason behind disruption of lysosomal and plasma membrane is the release of lipid peroxidation products due to Aβ42 accumulation inside the cell. Now scientists are of the view that it is not only insoluble Aβ42 which accumulates inside the cell but it is a heterogenous mixture of insoluble Aβ, APP and amyloidogenic fragments of APP. Inside the cell, soluble Aβ42 dimers or monomers are continuously produced and degraded, like any other protein but once the process of nucleation starts, the aggregate of Aβ42 becomes resistant to degradation. This nucleation can happen anywhere e.g. if inside the lysosomes, it would definitely alter the catabolism of APP, hence resulting in the accumulation of harmful amyloidogenic carboxyl-terminal fragments of APP (Yang et al., 1995). Just like Aβ42, these amyloidogenic C-terminal fragments of APP are also known to be toxic to cells by a mechanism which is perhaps similar to Aβ42 toxicity (Suh et al., 1997). Haass et al.,

(1992) suggested that the APP which is normally degraded in lysosomes, gives rise to a series of membrane-bound harmful amyloidogenic fragments containing the Aβ sequence. Under normal conditions, these fragments turn over rapidly and do not accumulate. But these amyloidogenic fragments of APP become very stable in the presence of Aβ42 aggregates and thus accumulate inside the cell (Yang et al., 1995).

It was understood from Yang et al., (1995) study that the accumulation of these amyloidogenic fragments is not due to an inhibition of lysosomal hydrolase activity because the N-terminal fragments turn over normally in the lysosome. Moreover, it is only the degradation resistant Aβ42 that makes this accumulation of amyloidogenic fragments of APP prominent but not the shorter amyloid fragments like Aβ1-28. If we analyze the mechanism of formation of degradation resistant heterogenous Aβ42 aggregates, it has been understood that it is the continuous addition of the amyloidogenic fragments over the Aβ42 core. This core acts as a seed for the assembly to grow and it propagates by the addition of further amyloidogenic fragments. It has been hypothesized that amyloidogenic fragments of APP containing Aβ sequence preferentially accumulate over Aβ42 core because of similar conformational domain as that of Aβ. Aβ42 is known to accumulate as the first Aβ species intraneuronally in AD (Selkoe et al., 2001). Thus, intraneuronally, it is the Aβ42 form of amyloid peptide which is considered more important in the pathogenesis of the disease, even though Aβ40 is more abundantly generated.

The other pathway in which Aβ42 accumulation can be pathological is the disruption of signaling pathways leading to neuronal dysfunction even before the integrity of neuron is lost. Echeverria et al., (2004) has thrown light on the role of Aβ inside the neurons and how its pathological increase inside the neurons dysregulates different signaling pathways. At physiological levels, Aβ is known to play a role in synaptic plasticity via the activation of CREB (cAMP-responsive element binding protein) directed gene expression. At pathological levels of Aβ, it causes abnormal phosphorylation patterns that end up dysregulating these pathways. Increased Aβ causes an upregulation of ERK (extracellular signal-regulated kinase), which then

phosphorylates a number of proteins, including tau. Increase in Aβ levels in the rats led to the diminished CREB phosphorylation, hence uncoupling it from its normal function in regulating the expression of certain genes. This was further explored in PC12 cell lines and found that the CREB-driven gene expression depends upon Aβ levels. It is recently identified by Cuello (2005) that the Rap 1/ B-raf / MEK/ ERK2 pathway is a key to Aβ function, and it can become dysregulated by high neuronal concentrations of Aβ. There is a clear linkage between the presence of excess Aβ and the accumulation of early stage phospho- tau variants. The above explanation makes it obvious that reducing Aβ may slow down the rate of tau filament formation. The onset of the above pathological pathways sets up much faster if there is a slight overproduction of Aβ42 as observed in many familial AD cases. The above understanding of the Aβ42 pathological pathways taking place internally due to accumulation of Aβ42 and other amyloidogenic fragments of APP tells us that this may be the primary mechanism of pathogenesis in AD. Another mechanism has been put forward where Aβ oligomers impair the functioning of neurons by interfering with NMDA type glutamate receptors and Ca^{2+} flow. Shankar et al., (2007) reported that Aβ dimers and trimers cause long-term depression and loss of dendritic spines by decreasing NMDAR mediated Ca^{2+} influx. In contrary to this mechanism, De Felice et al., (2007) reported Aβ mediated increase in Ca^{2+} influx leading to the generation of ROS species. Shankar et al., (2007) also found that low molecular weight Aβ oligomers i.e. primarily dimers and trimers cause progressive loss of spines in organotypic hippocampal slices. They further investigated the possibility of Aβ oligomers toxicity mediating via receptor signaling and found the involvement of NMDA type glutamate receptors. Moreover, our understanding of Aβ monomers forming oligomers and further fibrillation has been challenged by the recent discovery of Necula et al., (2007) where they indicated that the two assembly pathways namely oligomerization and fibrillation coexist independently and distinctly. Therefore, if we understand the initial steps in the pathogenesis of AD, it would be easier to understand further steps in the pathogenesis of the disease, which probably occur extracellularly. Table 1.1 highlights the parallel pathology in various neurodegenerative disorders.

Neurodegenerative Disorder	Proteins implicated	DNA Damage	Apoptosis	DNA-binding ability
Alzheimer's disease	β-Amyloid protein	+	+	+
Parkinson's disease	α-synuclein	+	+	+
Huntington's disease	Huntingtin	+	+	? Not Reported
Amyotrophic lateral sclerosis	Superoxide desmutase	+	+	? Not Reported
Prion protein disease	Prions	+	+?	+

Table 1.1 Parallel pathology in various neurodegenerative disorders: Apoptosis, DNA damage and the DNA binding ability of the various proteins implicated in these disorders.

1.13. Intranuclear localization of Aβ

The intranuclear localization of Aβ is still insufficiently confirmed, and controversial. The controversy comes from the fact that Aβ normally cannot become localized in the nucleus. The Aβ peptide is released either extracellularly (if the cleavage events occur while APP is at the plasma membrane) or within a membrane-bounded organelle (ER, Golgi, endosome, or secretory vesicle if APP is intracellular). However, the intra-nuclear localization of Aβ is reported in literature. However, it could be of interest to understand it, very critically and necessary to establish whether the presence of Aβ in the nucleus is an artifact or a reality. Protein aggregate formation is known inside the nucleus like polyglutamine repeat-containing proteins such as nuclear protein ataxin in spinocerebellar ataxia (Matilla et al., 1997; Skinner et al., 1997) and huntingtin protein in HD (Davies et al., 1997; DiFiglia et al., 1997) nuclear localization of Aβ is a debatable concept and yet less established result.

Based on the various reports which insight that Aβ reaches nucleus and localize in the nuclear membrane and in the vicinity of DNA, this issue cannot be ignored (Hegde et al., (2004); Grant et al., (2000); Kimberly et al., (2001); Johnstone et al., (1996); Buckig

et al., (2002). Ohyagi et al., (2005) proposed that intranuclear Aβ might have a role in inducing apoptosis but mechanism is not yet clear. Aβ is theoretically understood to form a β- hairpin shape followed by a helix-turn-helix motif (Durell et al, 1994), which is found to be an essential constituent of a DNA binding domain of heat-shock transcription factors (Wu et al., 1995). There is a probability that Aβ42 might directly bind to the p53 promoter as the p53 promoter contains heat shock elements (Sun et al., 1995). They indicated that an overload of oxidative stress results in oxidative DNA damage or overproduction of Aβ42. This may be due to FAD gene mutation which may induce Aβ42 accumulation in cytosol and then later in the nucleus activating p53 cascade resulting in enhancement of neuronal apoptosis in AD. Further, they studied the relative populations of cytosolic and nuclear Aβ42, and found that there are numerous number of cytosolic Aβ42 positive neurons but just a small number of both nuclear and cytosolic Aβ42 positive neurons. They also reported that 4kD soluble Aβ42 in nucleus activates the p53 promoter in a sequence specific manner.

Inspite of these observations, relevance of nuclear Aβ in AD pathogenesis is still considered as a debatable issue. However, it is positively definite that Aβ enters nucleus. Based on the above interpretations, it can be concluded that intracellular Aβ both in cytosol and nuclei can be treated as an important target in the pathogenesis and therapeutics for both familial and sporadic AD. However, it is important to mention here that many researchers in the AD field still question whether the intra-nuclearly detected Aβ is indeed Aβ, and/or whether it is the result of a normal process. All the above studies cannot just be ignored and needs investigative approach. Figure 1.5 highlights various factors and conditions that translocate Aβ to the nucleus. However, unless mechanisms of Aβ entry into nucleus are proposed, it is difficult to establish its intranuclear existence. It may be possible that Aβ diffuses freely inside the nucleus and then remains inside the nucleus because of its aggregation propensity or there could be selective uptake of Aβ via some unidentified mechanism. Once Aβ accumulates inside the nucleus, it could alter certain nuclear events leading to neuronal death.

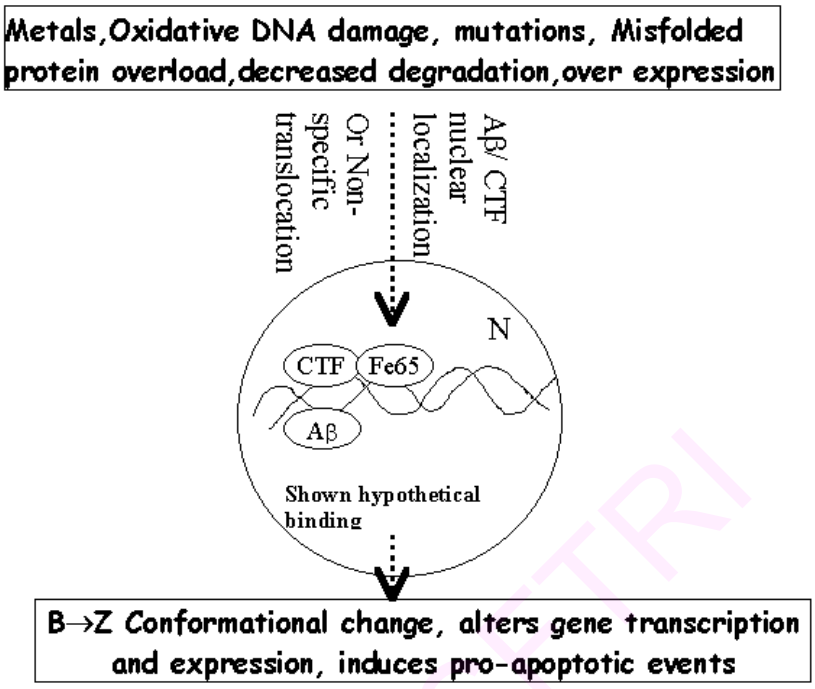


Figure 1.5: A proposed hypothesis indicating the factors that can translocate into the nucleus: This leads to the induction of conformational changes in the DNA or altering gene expression and hence taking part in certain pro-apoptotic events. (N: nucleus).

1.14. Racemization of aminoacids in Aβ

Several factors modulate the conformation and physical properties of Aβ, which inturn affects its biological functions. Age dependent changes in the stereospecificity of the amino acids comprising Aβ is one such factors (Kubo et al., 2003). A change in the stereospecificity of the amino acids comprising Aβ also influences the characteristics of Aβ aggregation parameters (Roher et al., 1993; Shapira et al., 1988). Further, it appears that the aggregation properties of Aβ depend upon the aminoacid sequence and their stereospecificity. In AD brain, there are reports of presence of racemized forms of aminoacids, in particular D-Asp, D-Glu, D-Ser in core amyloid plaques (Lukiw et al., 1997). Racemization of different aminoacids in Aβ has varied effects on its aggregation kinetics. Racemization at Asp23 accelerated Aβ aggregation whereas racemization at Asp7 slowed down the process of aggregation and Aβ in which both the Asp residues at 7

and 23 were racemized, led to a moderate aggregation (Tomiyama et al., 1994). Cribbs et al., (1997) reported that all D-enantiomers of Aβ(25-35) and Aβ42 exhibit similar biological properties to their L- counterparts. A better understanding of the affect of racemization of amino acids in Aβ sequence probably helps us to better know the mechanism of neurodegeneration. There are no mechanistic studies to show that how the complete amino acid racemization influences Aβ aggregation.

1.15. Therapeutic strategies

AD is a neurodegenerative disorder with an enormous unmet medical need. The search for novel therapeutic approaches targeting the presumed underlying pathogenic mechanisms has been a major focus of research and it is expected that novel medications with disease-modifying properties will emerge from these efforts in the future (Klafki et al., 2006). There probably is not one single cause, but several factors are important to describe the etiology of disease. Therefore, combination of compounds, which act as more than one target site, could be useful for the treatment. Owing to the beneficial effects of of α-tocopherol and ascorbic acid in reducing the free radical load in hippocampus and cerebral cortex (Bano and Parihar, 1997), a considerable interest in the use of these vitamins for treatment of AD has been made. Both α-tocopherol and ascorbic acid have been shown to slow the progression of the disease and reduce the risk for AD (Reiter, 1997). Research has shown that vitamin E slows the progress of some consequences of AD by about 7 months. Scientists now are studying vitamin E to learn whether it can prevent or delay AD in patients with cognitive dysfunction. Relatively long term antioxidant treatment may delay the onset of disease. Up-regulation of multiple ROS-scavenging enzyme capacities, which may be accomplished with various neurotrophins (Spina et al., 1992) may provide a mechanism for the prevention of neurotoxicity. Cholinergic drugs are routinely used in the treatment of AD to improve cognitive functions. These drugs together with multiple antioxidants have been proposed to be more effective in the treatment of AD than the individual agents alone (Prasad, 2000).

Preventions of mitochondrial oxidative damage and mitochondrial mutations have received considerable interest for the treatment of neurodegenerative disorders (Byrne, 2002). Infusion of nerve growth factor in aged animals upregulates cholinergic activity in the basal forebrain and striatum and improves learning and memory as well as motor performance (Mendoza-Ramirez et al., 1995). Estrogens have been shown to modulate neurotransmission, acting as a free radical scavenger and activating nuclear estrogen receptor in intracellular signaling (Behl and Holsboer, 1999). It prevents the formation of Aβ by promoting the non-amyloidogenic α-secretase processing of APP (Xu et al., 1998). Another hormone insulin which has been shown to regulate phosphorylation of tau protein that underlies neurofibrillary lesions in the brains of AD patients and also affects the metabolism of Aβ (Gasparini et al., 2002) highlighted new potential target for therapeutic intervention. Nonsteroidal anti-inflammation drugs, which inhibit the synthesis of prostaglandins, reduce the deterioration of cognitive functions in patients with advanced AD. Natural agents of food supplements that could have multiple properties (e.g., anti-inflammatory, antioxidant, improving mitochondrial energetics, etc.) and cross blood-brain barrier may apparently prevent or delay or maintain the individuals at their higher level of functioning. There is a growing interest in the use of polyphenolic antioxidants to reverse age-related decline in neuronal signal transduction and in cognitive and motor behavior deficits. Extracts of Ginkgo biloba has been shown to have beneficial effects on cognitive function. Recent research suggests that ginkgo biloba may be of some help in treating AD symptoms. There is no evidence that ginkgo will cure or prevent AD. Scientists now are trying to find out whether ginkgo biloba can delay or prevent dementia in older people. Neuroprotective properties of extracts of *Asparagus racemosus*, *Convolvulus pleuricauas* and *Withania somnifera* against free radicals induced damage in different brain regions in experimental animals have been investigated (Parihar and Hemnani, 2003, 2004). However, the mechanisms underlying their neuroprotective effect are not fully understood. Clinical efficacy and potential toxicity of active compounds in trials require further assessment.

Blocking the proteolytic machinery that produces Aβ42 is the preferred strategy. This could be accomplished either by reducing formation of APP or by inhibiting

proteolysis of APP to Aβ42. Inhibition of β- and γ- secretase activities and stimulation of α-secretase activity have been suggested to be the most promising strategy for neuroprotection (Citron, 2000). Complete inhibition of either β or γ- secretase alone should be sufficient to block the Aβ production completely. The generation of secretase specific protease inhibitors that penetrate the blood-brain barrier is a challenge for drug discoveries. However, the drug that blocks the function of γ- secretase might not be effective in patients developing AD by factors other than Aβ42 accumulation. The toxic forms of Aβ might be soluble oligomers. Therefore, agents that prevent Aβ nucleation could be more effective than those that merely block Aβ deposition (Wolfe, 2002). The anti-Aβ immunization either active or passive holds great promise in future for treating or even preventing AD (Schenk 2002; Hock et al., 2002).

However, for some people in the early and middle stages of the disease, the drugs tacrine (Cognex), donepezil (Aricept), rivastigmine (Exelon), or galantamine (Reminyl) may help prevent some symptoms from becoming worse for a limited time. Another drug, memantine (Namenda), has been approved for treatment of moderate to severe AD. Now, researchers are studying the NSAIDs celecoxib (Celebrex) and naproxen to find out if they can slow the onset of the disease. Treating these symptoms often makes patients more comfortable and makes their care easier for caregivers. Developing new treatments for AD is an active area of research.

Various compounds have been explored as inhibitors of Aβ aggregation such as; curcumin (Ono et al., 1998; Yang et al., 2005), ferulic acid (Ono et al., 2005), nicotine (Solomon et al., 1996; Ono et al., 2002), melatonin (Pappolla et al., 1998), laminin (Monji et al., 1999; Castillo et al., 2000), tetracycline (Forloni et al., 2001), sulfonated dyes (Pollack et al., 1995), the rifampicin series (Tomiyama et al., 1996), porphyrins (Howlett et al., 1997), benzofurans (Howlett et al., 1999), monoclonal antibodies that target pathological assemblies of Aβ (Lambert et al., 2007), peptide fragments homologous to the hydrophobic region of Aβ (Ghanta et al., 1996; Soto et al., 1996; Tjernberg et al., 2002; Findeis et al., 1999). Some of these compounds have also been reported to inhibit the Aβ fibril formation, by their propensity to bind to specific sites of Aβ (Solomon et al., 1997; Ono et al., 2002). It was also showed that a flavanoid baicalein

at a low micromolar concentration not only inhibited the Aβ, but also disaggregated pre-formed Aβ □fibrils(Zhu et al., 2004).

Guided with this precept, a search for the natural inhibitors of fibrillogenesis such as, bioavailable components of garlic and curcumin could be realistic to clinical practice. However, there is currently no wonder drug available that promises to cure AD in a week. In fact, curing AD in later stages may never become possible due to the difficulties to revert to neurodegeneration, once it is established. The goal of AD research for the oncoming years will be, to develop therapeutic strategies that treat the disease as early as possible. If drug safety permits, this should apply even before clinical symptoms become evident, providing the basis for these first generation drugs and pro-drugs that target the cause of the disease, and not the symptoms.

1.16. Aim and Scope of the study

The genomic integrity is essential for the living organism, as any damage or structural change of the genome will affect the transcriptional fidelity and ultimately the survival of the organism. There are various reports indicating Aβ binding to DNA could be causative factor for the DNA damage observed in various neurodegenerative disorders (Ahn et al., 2000; Hegde et al., 2004; Nandi et al., 2002). It was speculated that accumulated DNA damage in the brain might result in conformational changes in the DNA making it ineffective during transcription. But there exist no mechanistic studies on Aβ binding to DNA and biological significance of DNA-Aβ interactions. Moreover, in neurodegenerative brain, the amount of racemized forms of aminoacids, e.g. D-Asp, D-Glu, D-Ser are relatively in large proportions in core senile plaques and NFTs (Fisher et al., 1991). In the current study, we have utilized all- L- and all- D- aminoacid enantiomers of Aβ40 peptide as model peptides to elucidate the mechanism of DNA binding and nicking activity of Aβ40. The role of amino acid stereospecificity in Aβ aggregation was also evaluated. This is of considerable interest as biological function is often correlated with structure at molecular levels.

Further, the disease modifying treatment of AD involves reduction in Aβ aggregation and toxicity. Compounds that hinder the aggregation of Aβ into multimeric

species (oligomers, protofibrils, and fibrils) are most effective as they stop the protein polymerization prior to the formation of the primary cytotoxic moieties. Guided with this precept, a search for the natural inhibitors of fibrillogenesis such as, bioavailable components of garlic and curcumin could be realistic to clinical practice.

The following objectives have been undertaken for the study.

a) To study the role of amino acid racemization on aggregation kinetics of Aβ (1-40)

b) To understand the role of stereo-specificity and modified aminoacids on Aβ (1- 40) interaction with DNA.

c) To study the role of garlic active components and other selected dietary molecules on modulation of conformation and aggregation of Aβ (1-40).

The results of these studies are presented in the following chapters.

CHAPTER 2

Role of amino acid steeospecificity in Amyloid beta aggregation

2.1. Introduction

AD, vascular dementia, and hereditary cerebral hemorrhage with the Dutch type are neurological diseases that share an invariant pathological feature, namely the accumulation of an amyloidogenic peptide into insoluble fibrillar extracellular deposits (Harrison et al., 2008). Amyloid fibril formation is a process during which soluble proteins misfold and aggregate into fibrillar structures. The amyloid fibrils formed from different amyloidogenic proteins possess many common structural features (Rochet and Lansbury, 2000). It is well known that the biological activity of Aβ is dependent on its conformation. The formation of plaques, an age-dependent process is the most detectable neuronal alteration in AD. Proteins and peptides are susceptible to a variety of chemical modifications that can affect their structure and biological functions in brain. In addition to the proposed genetic mutations or a polymorphism of presenilins, APP or Apo E, the post- translational modifications of Aβ by oxygen radicals, truncations, or isomerization/racemization are well speculated as enhancers for Aβ aggregation (Shimizu et al., 2000). Biochemical studies revealed that Aβ peptides isolated from AD brains were post-translationally modified by isomerization, racemization, pyroglutamylation and truncation (Roher et al., 1993). As racemization of amino acid residues in Aβ causes structural changes and functional or enhances the aggregation process in the modified proteins, this post-translational modification is suggested to be one of the progression factors in sporadic cases of AD. Oligomeric and fibrillar Aβ may be toxic in AD, especially after post-translational modification cumulative over time. The racemization reaction proceeds spontaneously under physiological conditions through a five-membered succinimide ring intermediate. This intermediate is formed by the nucleophillic attack of the peptide bond nitrogen atom of the following residue on the side chain carbonyl group, resulting in dehydration of aspartic acid or deamination of asparagines (Shimizu et al., 2005). The L-succinimidyl intermediate then undergoes a relatively rapid hydrolysis either at the α- or β- carbonyl group to generate L-isoaspartate and normal L-aspartate in a ratio of approximately 3:1 in a variety of substrates. The formation of L-succinimide is accompanied by enhanced racemization at the α-carbon to generate a mixture of D-succinimidyl, D-aspartyl, and D-isoaspartyl forms (Shimizu et al., 2005). A similar series

of reactions would occur in glutamine and glutamate residues, generating D- and L-isoglutamate and glutamate residues (Shimizu et al., 2005).

Further, Kaneko et al., (2001) also highlighted the toxicity mechanisms of racemized aminoacids in Aβ1-40. The soluble [D-Ser^{26}] Aβ1-40, as well as Aβ1-40, in the brain is supposed to be digested into fragments by rich sources of endo- and exopeptidase in the brain. Chymotrypsin-like enzymes including lysosomal cathepsin G can degrade Aβ and also can be inhibited by α- antichymotrypsin which is accumulated in SPs. Aminopeptidases are one of the most dominant exo-type proteinases and found to play important role in the degradation of neuropeptides in the brain (Kaneko et al., 2001). All the chymotrypsin-digested fragments of Aβ1-40 get degraded to amino acids. While [D-Ser^{26}]Aβ25-35, [D-Ser^{26}]-Aβ21-40, one of the early degradation products by chymotrypsin or cathepsin G treatment, get converted into [D-Ser^{26}]Aβ25-40 by the aminopeptidase treatment. Aminopeptides do not cleave N-terminal amino acids when the first or second amino acid is substituted by a D-amino acid (Pert et al., 1976). Kaneko et al., (2001) also indicated that non-toxic [D-Ser^{26}]Aβ1-40 gets converted *in vitro* to a potent neurotoxic peptide, [D-Ser^{26}]Aβ25-35 or [D-Ser^{26}]Aβ25-40, by chymotrypsin-like enzymes and aminopeptidases.

The racemization of Ser and Asp residues of Aβ in SPs occurs as an age-dependent process (Lowenson et al., 1999). Kubo et al., (2003) provided an insight about the lag between the senile plaque formation and neurodegeneration in AD. Hence, it becomes important to find out the pathological implications of protein racemization in neurodegeneration in AD. Moreover, there are enough reports in literature citing the effect of racemization of individual amino acids on Aβ conformation and aggregation pattern. Kubo et al., (2002) reported that Aβ1-40 racemized at Ser26 becomes soluble and susceptible to proteolysis releasing toxic D-Ser26 Aβ (25-35) fragment *in vitro* and *in vivo*. Mori et al., (1994) have also reported that racemization at Asp23 of Aβ1-35 and Aβ1-40 accelerated peptide aggregation and fibril formation. Further, *in vivo* racemization of amino acids of Aβ also suggests one of the probable mechanisms of Aβ induced neurotoxicity in AD. There are considerable reports where they have shown the presence of racemized amino acids especially D-Ser and D-Asp in Aβ in the senile

plaques from AD brains (Roher et al., 1993; Shapira et al., 1988). Our study is an attempt to understand how changing the stereospecificity of the amino acids comprising Aβ influences the characteristics of Aβ aggregation parameters. Infact, changing the chirality of the amino acids comprising Aβ is not only useful to study the Aβ stereoisomer-specific ligand receptor interactions but also the aggregation parameters. In this study, we have used Aβ(1-40) all-L and Aβ(1-40) all-D enantiomers and compared their biophysical properties to characterize the stereospecific-based aggregation state, folding pattern and morphology of aggregates.

2.2. Materials and Methods

2.2.1. Chemicals

Aβ(1-40) all-L and Aβ(1-40) all-D and Aβ(40-1) all-L were purchased from Sigma chemical Co., USA. Copper grids (300-mesh size) were purchased from Sigma chemicals. Uranyl acetate was purchased from B.D.H laboratory chemicals division. All other chemicals thioflavin-T, 1-anilinonaphthaleine-8-sulphonic acid (ANS), acrylamide, L-tyrosine, D-tyrosine, sodium chloride (NaCl), Tris (hydroxymethyl aminomethane), sodium hydroxide (NaOH), glycine were of analytical grade and were purchased from Sisco Research Labs, Mumbai, India.

2.2.2. Preparation of Aβ40 Stock and Fibril formation

Lyophilized Aβ40 peptides at 200-μM concentration were dissolved in 0.01M Tris-Cl, pH 7.4, and for fibril formation, it was incubated at 37^0C without agitation/ stirring in glass vials.

2.2.3. Circular Dichroism Studies

The CD spectra (190-260nm) were recorded for Aβ 1-40 all-L and Aβ 1-40 all-D (20×10^{-6} M) in 0.01M Tris-Cl buffer (pH 7.4) on a JASCO-J 700 spectropolarimeter with a 0.1-cm path-length quartz cuvette. Each resulting point ($[\theta]_{obs}$, degree) was converted to mean residue ellipticity ([θ], degree cm^2 $dmol^{-1}$). Each spectrum was the average of three repetitions and all spectra were corrected by subtracting the buffer baseline. All measurements were performed at room temperature.

2.2.4. Thioflavin-T Assay for Monitoring Aβ Aggregation

The degree of Aβ aggregation was determined using the fluorescent dye thioflavin-T (Thio-T) (LeVine, 1993). Thio-T is a benzothiazole dye that undergoes a characteristic enhancement in fluorescence emission intensity on binding to amyloid fibrils in solution. It does not bind to amorphous, non-fibrillar aggregates and monomeric Aβ. Aβ enantiomers were used at the concentration of 2 μM in a total volume of 1,000μl. An aliquot of 10 μl from 200 μM stock peptide solution was drawn at interval of every 1 h for 144 h, treated with 100 μM Thio-T, and adjusted to a final volume of 1,000μl with 50 mM glycine NaOH buffer, pH 8.5. Each test sample was shaken for 10 s before each measurement. Measurements were carried out every 1-h at an excitation wavelength of 446nm and an emission of 482nm, wavelengths that result in the optimum detection of bound Thio-T. The relative degree of Aβ aggregation was assessed in terms of fluorescence intensity, which was measured at 37^0C using a model F4500 Hitachi fluorescence spectrophotometer. To account for background fluorescence, the fluorescence intensity measured from each control solution without Aβ was subtracted from that of each solution containing Aβ.

2.2.5. Transmission Electron Microscopy of Aβ(1-40) all-L and Aβ(1-40) all-D and Aβ(40-1) all-L Peptides:

Aβ(1-40) all-L and Aβ(1-40) all-D and Aβ(40-1) all-L peptides in both soluble and aggregated forms were observed under JOEL 1010 transmission electron microscope (TEM). Carbon- coated copper grids (300-mesh size) were glow discharged for 1-2 min (~150 milli Torr: discharge current, 2-3mA), dipped in absolute alcohol for 1 s and were air-dried. A drop (5 μl) of 200 μM stock solution was placed on the grid and was allowed to dry in air for 30 min. A second drop was applied after blotting the first drop with filter paper. The sample was then wicked off with lens paper, washed with distilled water, and then negative stained by transferring the grid face down to a droplet of 2% (w/v) uranyl acetate for 5-10 min before wicking off the solution and air drying. Uranyl acetate solutions were filtered through a 0.2-μM sterile syringe filter before use. Four individual experiments were carried out for each sample. The grids were completely dried so as to avoid moisture for EM examination (Thomas and Bloomfield, 1985).

2.2.6. Intrinsic Tyrosine Fluorescence

Intrinsic tyrosine fluorescence spectra were collected on a HITACHI 2000 spectrofluorimeter in a rectangular semimicro quartz cuvette with a 1-cm excitation light path. The light source was a 150-W xenon lamp. For tyrosine intrinsic fluorescence, Aβ-containing solutions were excited at 280 nm and emission monitored in the range from 295 to 375 nm. The concentration of Aβ peptides for intrinsic tyrosine fluorescence measurements were kept at 10 μM. Tyrosine emission spectra were acquired after every 24 h for 144 h. Aβ(1-40) has one tyrosine residue at the 10^{th} position but contains no tryptophan. Hence, the single tyrosine fluorescence has been used to monitor folding of Aβ. In the present investigation, the tyrosine-intrinsic fluorescence was used to compare the self-folding pattern of the two complete enantiomers of Aβ(1-40). Four independent experiments were carried out for each sample. Fluorescence data are expressed in arbitrary fluorescence units (AU).

2.2.7. Acrylamide Fluorescence Quenching

Information on the relative solvent exposure of tyrosine residues (there are no tryptophans in Aβ) can be obtained from analysis of the effect of quencher molecules such as acrylamide. The aging induced changes in the environment of tyrosine residues in Aβ(1-40) enantiomers have been studied by acrylamide induced tyrosine fluorescence quenching. Steady state acrylamide fluorescence quenching experiments were performed to study the exposure of the single tyrosine moiety of both the enantiomers of Aβ (1-40) in different assembly states.

Aliquotes of the stock-quenching solutions (5M) were added into a rectangular 1-cm semi-micro quartz cuvette containing 10 μM of Aβ peptide solution. Fluorescence intensities were corrected for dilution effects. Fluorescence quenching data were analyzed using the general form of the Stern-Volmer equation:

$I_o/I = 1+K_{sv}[Q]$

where, I_o and I are the fluorescence intensities in the absence and presence of quencher [Q] and K_{sv} is the Stern-Volmer quenching constant.

2.2.8. 1-Anilinonaphthaleine-8-Sulphonic Acid Binding

ANS is frequently used to demonstrate the presence of partially folded conformations of proteins, characterized by the presence of solvent-exposed hydrophobic clusters. This is because ANS binds to solvent exposed hydrophobic clusters, resulting in a considerable increase in the ANS fluorescence intensity and in a pronounced blue shift of the fluorescence emission maximum. We measured the change of ANS fluorescence to monitor the gain of structure in Aβ(1-40) in terms of solvent exposed hydrophobic clusters upon aging. A stock solution of 1mM ANS was prepared in distilled water. The [ANS]/[Aβ] ratio in all experiments was kept equal to 5. Fluorescence measurements were performed at 25^0C in 1 ml semi-micro quartz cuvette. Emission spectra were recorded from 400 to 600 nm with excitation at 350 nm. The protein concentration was 10 μM.

2.3. Results

2.3.1. Circular Dichroism Studies

The individual CD spectra of Aβ(1-40) all-L and Aβ(1-40) all-D were studied. The CD spectrum of native Aβ(1-40) all-L had a strong negative CD band at the 198 nm region, indicative of random coil conformation (Figure 2.1A). As circular dichroism is the differential absorption of right and left circularly polarized light by an optically active chromophore and when the two molecules are mirror images of each other, obviously CD spectra would be mirror images. Hence, it was found that the CD spectra of Aβ(1-40) all-D was a mirror image of Aβ(1-40) all-L spectra (Figure 2.1B). Schematic representation of L and D- amino acid is shown in the Figure 2.2A and 2.2B, respectively.

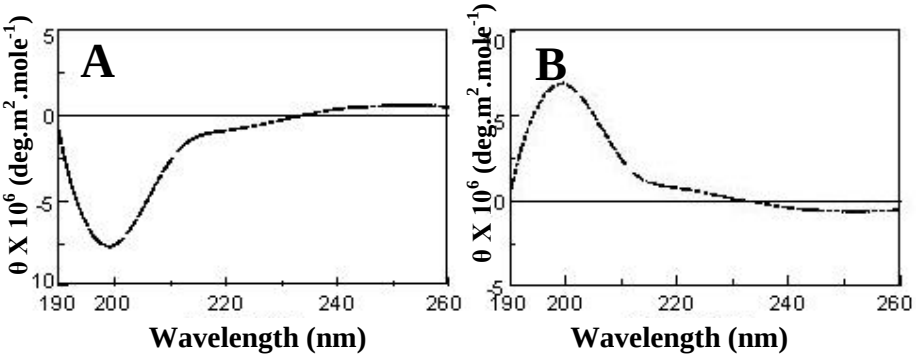


Figure 2.1: Circular Dichroism spectra of Aβ (1-40) enantiomers: A) 20μM Aβ (1-40) all-L, B) and 20μM Aβ (1-40) all-D.

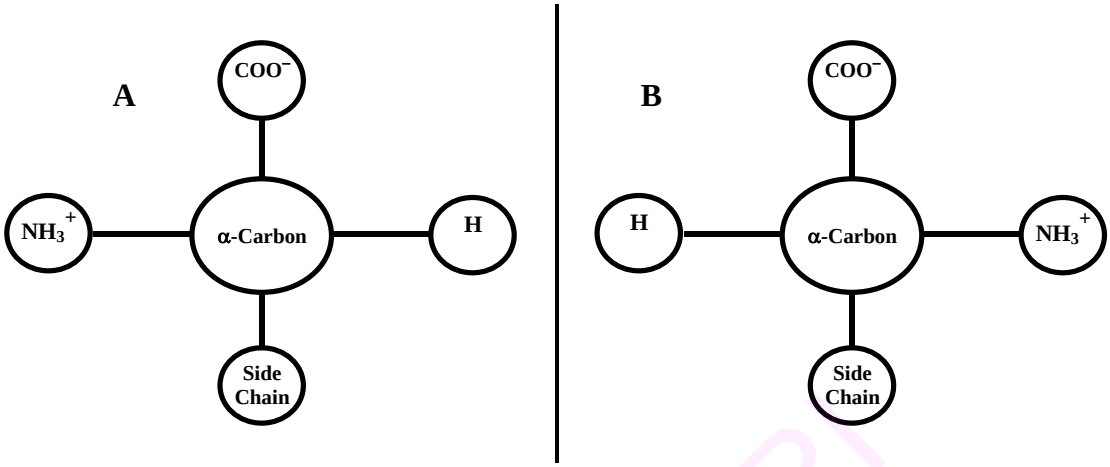


Figure 2.2: Schematic representations of L (A) and D (B) forms of amino acid.

2.3.2. Thioflavin-T Assay

Figure 2.3 represents the time kinetics of fibrillization of Aβ enantiomers. Thio-T fluorometric assay for monitoring aggregation of both Aβ(1-40) all-L and Aβ(1-40) all-D gave an interesting pattern. The initial (0 h) Thio-T binding of Aβ(1-40) all-D was lower when compared to the Aβ(1-40) all-L. In case of both Aβ(1-40) all-L and Aβ(1-40) all-D, it can be observed that there was a sharp increase in Thio-T binding from 24 h onwards till 3 days and then the gradual increase leads to flattening of the curve. In case of Aβ(1-40) all-L, there appears to be a gradual increase in log phase, whereas, comparatively, log phase of Aβ(1-40) all-D shows much sharper increase. Aβ(1-40) all-L, although having a higher initial Thio-T binding because of the presence of non-specific aggregates, showed lower Thio-T values at the time of log phase when compared to Aβ(1-40) all-D. Both initial and final Thio-T values are high in case of Aβ(1-40) all-L. In addition, Aβ (40-1) all-L had almost the same Thio-T value throughout with a negligible increase after 3 days. Aβ (40-1) all-L is resistant for aggregation as indicated by a very long lag phase followed by a short increase in Thio-T binding.

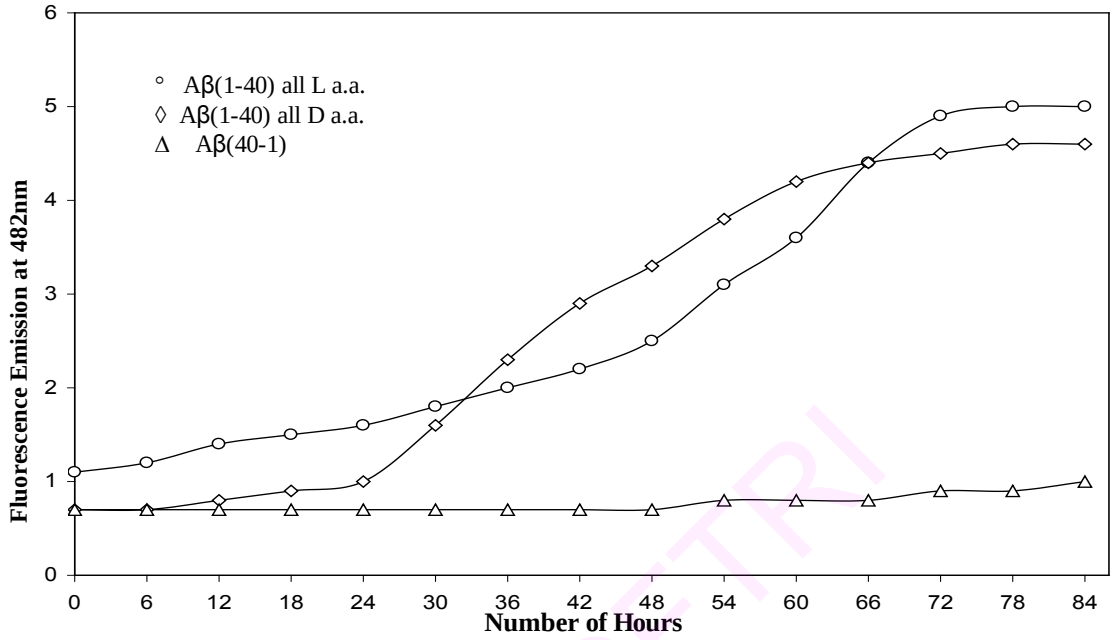


Figure 2.3: Thioflavin-T fluorescence assay for Aβ(1-40) enantiomers aggregation: 100 μM Aβ peptides was incubated at 37^0C in Tris-Cl. Aliquotes were drawn at different time intervals and aggregation was monitored using Thioflavin T fluorescence assay (excitation, 446 nm and emission, 482 nm). Values are expressed as average of triplicates.

2.3.3. Transmission Electron Microscopy

We examined the self-assembly (aggregates) of L and D enantiomers of Aβ(1-40) *in vitro* using transmission electron microscopy. Both L and D enantiomers of Aβ (1-40) formed insoluble fibrillar aggregates at more or less similar rates. Both have fibrillar morphology but Aβ(1-40) all-L gave rise to longer fibrils with branching (Figure 2.4A). D-form formed shorter fibrils and branching was not observed (Figure 2.4B). We also examined the fibrillation pattern of Aβ(1-40) all-L and found that it forms amorphous aggregates only (Figure 2.4C).

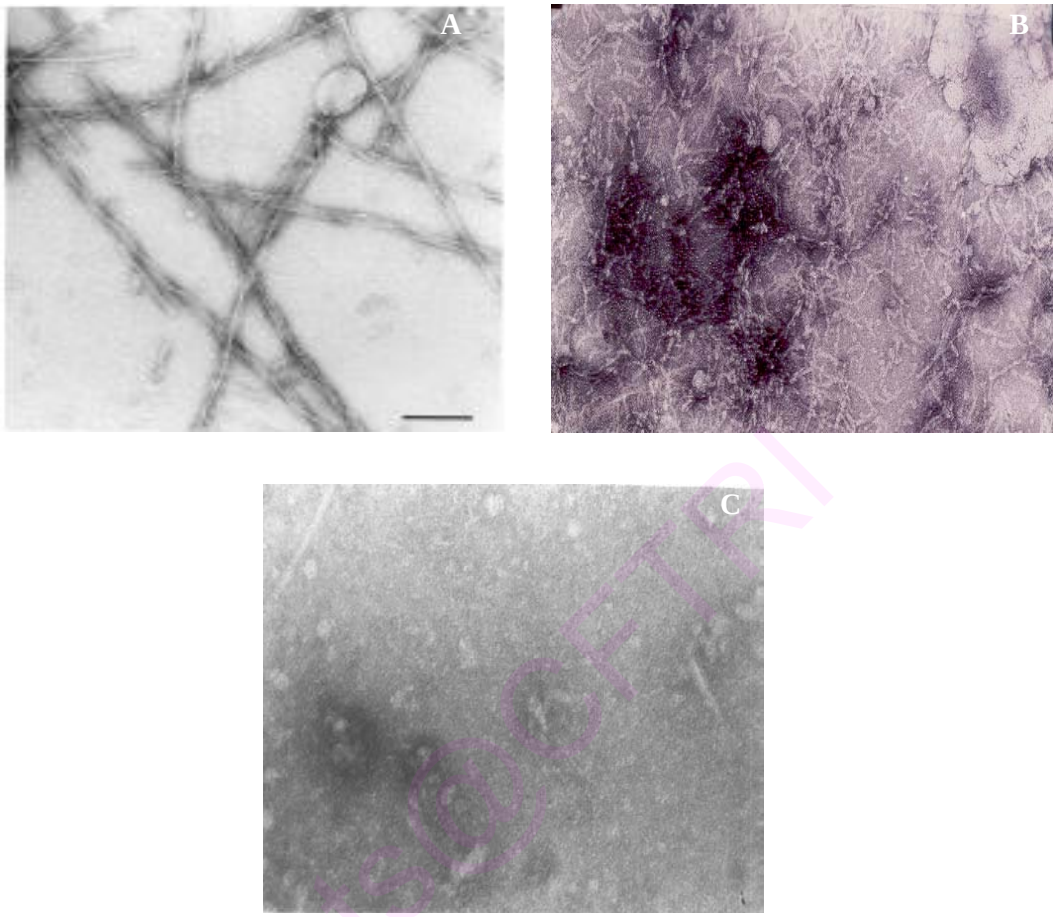


Figure 2.4: **Negatively stained transmission electron micrographs of fibrils formed from Aβ(1-40)enantiomers**: The protein samples were applied on carbon-coated Formver grids and allowed to dry in air. After staining for 2 min with 2% uranyl acetate, samples were observed under a JEOL 1010 transmission electron microscope. Magnification: scale bar, 100 nm. A-C represent fibrils formed by Aβ(1-40) all-L, Aβ(1-40) all-D and Aβ(40-1) all-L, respectively .

2.3.4. Intrinsic Tyrosine Fluorescence

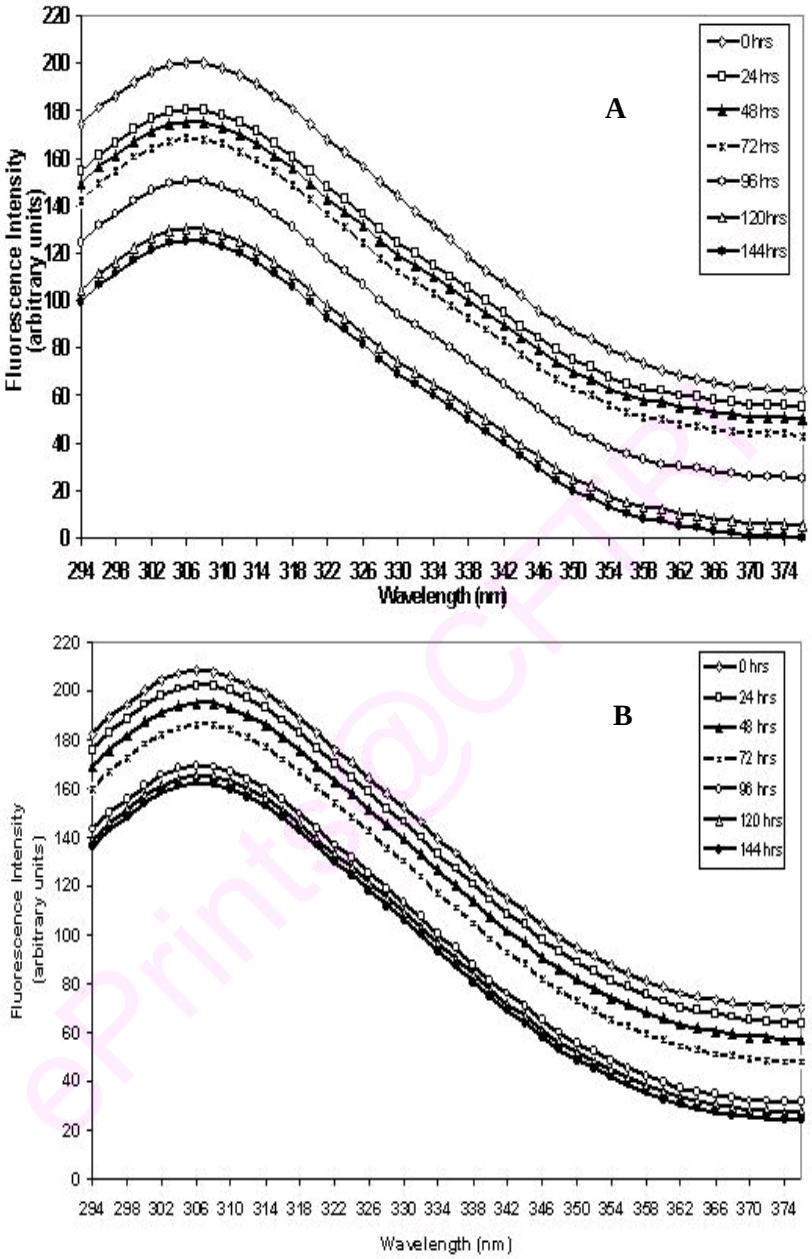


Figure 2.5: Effect of aging on A) Aβ(1-40) all-L and B) Aβ(1-40) all-D intrinsic tyrosine fluorescence: Excitation at 280nm, emission maximium at 306 nm (295-375 nm). Aβ40 concentrations were kept at 2.0 μM. Values are expressed as average of triplicates.

The pattern of self-assembly of Aβ is often correlated with its pathogenesis (Rodriguez et al., 1997). Intrinsic tyrosine fluorescence was used to investigate the differences in conformation of Aβ40 enantiomers assembly. In case of Aβ(1-40) all-L, a

significant decrease was observed after 24 h in tyrosine fluorescent intensity with the maturation of an oligomeric intermediate into amyloid fibrils (Figure 2.5A). This process of decrease was also observed in Aβ(1-40) all-D but the decrease in intensity was much less when compared to Aβ(1-40) all-L. After 144 h of incubation, the fluorescence intensity in case of Aβ(1-40) all-L decreased up to 100 U but in case of Aβ(1-40) all-D (Figure 2.5B), it decreased up to 140 U. This data suggests that the conformational changes take place in both Aβ(1-40) all-L and Aβ(1-40) all-D during the process of fibrillation are similar but relatively slower in Aβ(1-40) all-D.

2.3.4. Acrylamide Fluorescence Quenching

The assembly of natively unfolded peptide Aβ(1-40) to form relatively higher order structure such as fibrils is often associated with the protection of certain amino acid residues from the external aqueous environment. Other residues lie on the surface, where they are exposed to the polar solvent. In this study, we employed a strategy often used in studying the solution structure of proteins or peptides to map those residues that are exposed and those that are buried in the fibrillar Aβ(1-40). Stern-Volmer plots of tyrosine fluorescence quenching of Aβ(1-40) enantiomers at different incubation periods was shown in Figure 2.6A and Figure 2.6B with acrylamide as quencher. The lines in Figure 2.6 were obtained by fitting the data to the Stern-Volmer equation. The results showed that, for both fresh Aβ(1-40) all-L and Aβ(1-40) all-D, K_{sv} is smaller than the value for free L- and D- tyrosine respectively. This indicated that the tyrosine residues of natively unfolded Aβ enantiomers were partially protected from quencher molecules. In the course of time, from 0 to 144 h, the K_{sv} value further decreases, and in turn, the degree of protection increases. The K_{sv} for Aβ is less with aging because there is induction of folding, which protects tyrosine residues. The degree of quenching induced in Aβ(1-40) all-D by acrylamide is lesser as the peptide undergoes aging from 0 to 144 h, indicating stereospecificity as an important factor.

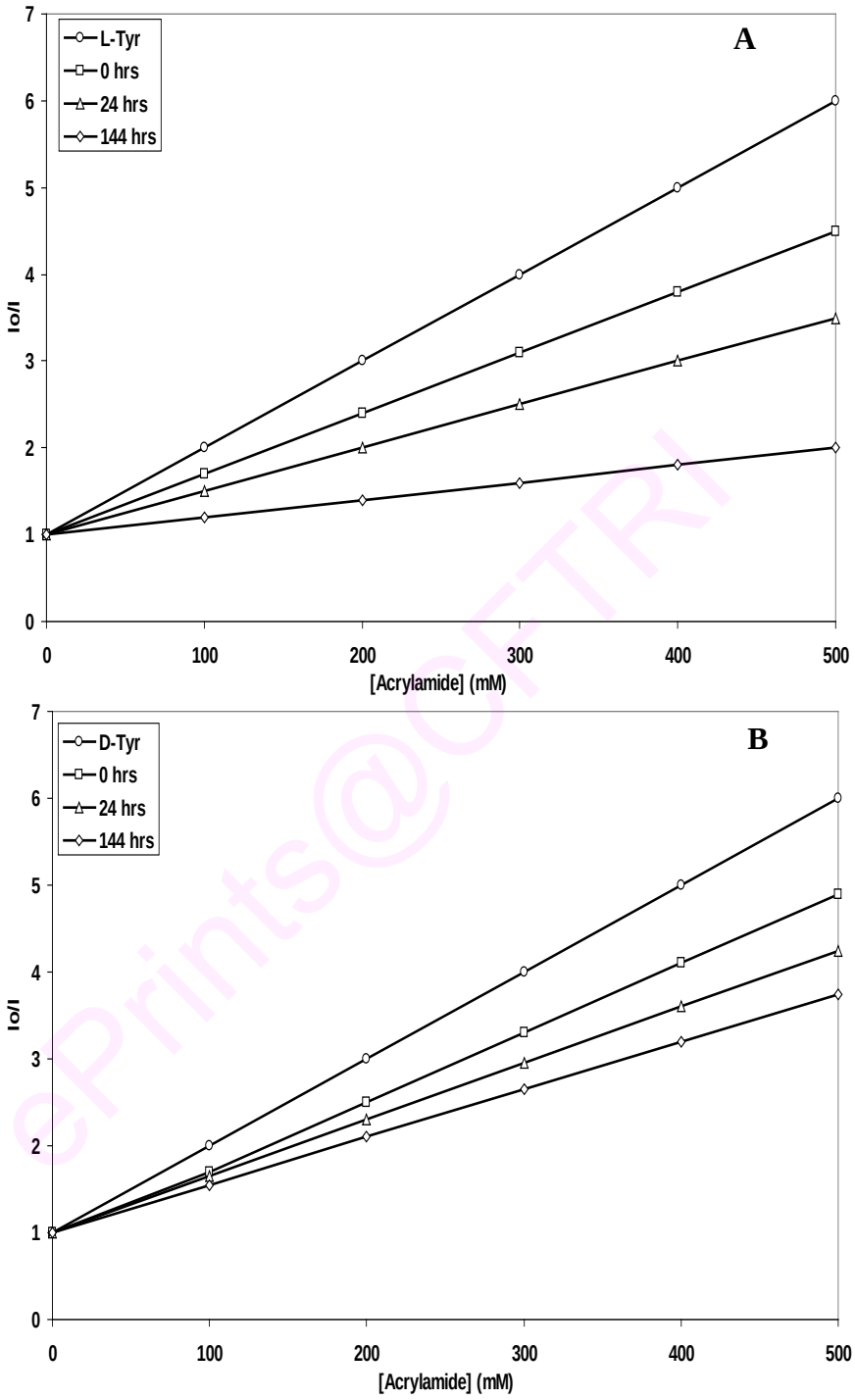


Figure 2.6: Stern-Volmer plots for acrylamide-mediated tyrosine fluorescence quenching of Aβ(1-40) enantiomers with aging: A) Aβ40 L B) Aβ40 D The conditions were as described under 'experimental procedures'. Values are expressed as average of triplicates.

2.3.4. 1-Anilinonaphthaleine-8-Sulphonic Acid Binding

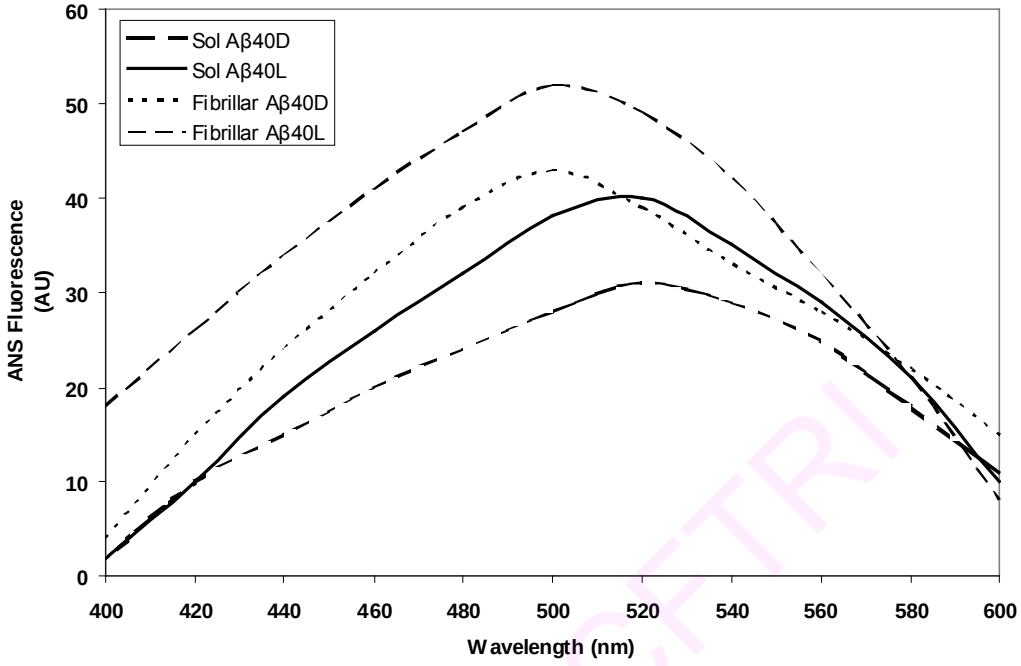


Figure 2.7: Effect of aging on ANS fluorescence of Aβ(1-40)all-L and Aβ(1-40) all-D: Emission spectra were recorded from 400 to 600nm with excitation at 350nm. Values are expressed as average of triplicates.

The hydrophobic dye ANS shows an intense fluorescence signal at ~480 nm when it binds to exposed hydrophobic clusters on the surface of partially folded intermediate states of proteins (Semisotnov et al., 1987). Changes in ANS fluorescence are characteristic of the interaction of this dye with solvent-exposed hydrophobic surfaces of partially folded proteins. Figure 2.7 represents the changes in ANS fluorescence of Aβ with aging. The results revealed that there was enhancement in ANS fluorescence of both Aβ enantiomers with aging. Interestingly, with aging there was induction of blue shift (~20 nm) of ANS λ_{max} in Aβ enantiomers but formation of partially folded conformation with solvent exposed hydrophobic patches was much quicker in Aβ(1-40) all-L when compared to Aβ(1-40) all-D. There was no change in ANS λ_{max} till 36 h in Aβ(1-40) all-D, indicating no conformational change in it till 36 h. In Aβ(1-40) all-L, the change in ANS λ_{max} was seen after 10 h of aging period.

2.4. Discussion

This study was designed to probe the role of stereospecificity on Aβ folding and aggregation pattern as Aβ aggregation in the brain is considered an important factor in the pathogenesis of AD. Aβ toxicity depends on both the soluble and aggregated forms of Aβ. Recently, Lesne et al., (2006) reported a significant observation on the existence of a specific Aβ protein assembly in the brain, termed as Aβ*56, which is involved in memory impairment. They showed that young Tg2576 mice, have normal memory and lack any neuropathology. However, middle-aged mice develop memory deficits along with extracellular accumulation of Aβ*56 without neuronal loss. The aged mice developed memory loss with abundant neuritic plaques containing Aβ. Further, they showed that Aβ*56 could induce memory loss when injected into young mice. They also demonstrated the presence of lesser quantities of nonamers along with the Aβ*56 which is a dodecamer of Aβ42. They proposed that Aβ*56 impaired memory independently of plaques. All these studies indicate that understanding Aβ assembly is essential to understand the biological significance of Aβ assembly in the brain.

Further, it is well known that proteins and peptides are prone to a variety of chemical modifications that can alter their structure and, in turn, biological activities. The isomerization of aspartic acid and glutamic acid are common events in proteins during aging (Kubo et al., 2003). Various biochemical studies revealed that Aβ peptides isolated from AD brains were post-translationally modified in various ways like isomerization, racemization, pyroglutamylation and truncation (Shimizu et al., 2000). A D- enantiomer of an amino acid or peptide would not be predicted to exhibit similar folding pattern or other physical properties comparable with the native L- form. We have analyzed the biophysical properties of all-L-enantiomer of Aβ1-40 and compared it with its corresponding all-D-enantiomer.

Our data suggests that the aggregation and folding parameters of Aβ are stereospecific. The differential aggregation pattern of both Aβ(1-40) all-L and all-D as revealed by Thio-T sigmoidal curves are in agreement with that of TEM results. The smaller fibrillar morphology of Aβ(1-40) all-D can be correlated with that of the lower saturation phase of the Thio-T curve showing aggregation pattern. To probe the

specificity of amyloid formation and growth, we examined the self-assembly of L- and D-enantiomers of Aβ *in vitro*. Both enantiomers formed insoluble aggregates at different rates with different fibrillar morphology, deposition of soluble Aβ peptide onto preexisting Aβ aggregates was stereospecific. The kinetics of aggregation of two enantiomers of Aβ (1-40) is dissimilar as they form morphologically varied fibrils. Further, our Thio-T and TEM results indicated that the aggregating property is specific for sequence of Aβ(1-40) with either L/D amino acids. The Thio-T pattern of Aβ (40-1) all-L indicates that the particular sequence of amino acids as present in Aβ L/D peptides is required for its aggregation/fibrillation property. The monitoring of intrinsic tyrosine fluorescence, its quenching by acrylamide and ANS binding studies demonstrated the slow folding of D- enantiomer when compared to that of L-enantiomer of Aβ(1-40). The intention of taking N-C reverse peptide was to set an aggregation control for the two enantiomers of Aβ (1-40). This study clearly shows that the aggregation property of Aβ is coded in its sequence and strongly depends upon the amino acid sequence and their stereospecificity. Our results indicate that deposition of Aβ in the brain may be stereospecific.

A number of studies have been done to elucidate the effect of racemization on the aggregation properties of Aβ, where they have shown that substitution of L-amino acid by D- amino acid at different positions in Aβ have different effect on Aβ aggregation kinetics. For example, racemization at amino acid position 23 accelerated the Aβ (1-35) peptide aggregation and fibril formation, whereas that at position 7 slowed down this reaction (Tomiyama et al., 1994). This study was important in suggesting that the site-specific racemization of Aβ may be involved in the amyloid fibril formation in AD. In another study, normal Aβ40 showed the gradual increase of aggregation, whereas [D-Asp7] Aβ40 and [D-Asp23] Aβ40 showed more enhanced aggregation at the final stage (Mori et al., 1994). However, Cribbs et al., (1997) reported that all L- and D-enantiomers of Aβ(1-42) and neurotoxic truncated form of Aβ(25-35) exhibit similar physical and biological properties. They also showed that both the enantiomers of Aβ peptides cause similar neurotoxicity. They attributed the neurotoxicity of peptides to both L-and D-enantiomers binding to neurons similarly and forming clusters of fibrils on the cell surface of neurons. This biological property was also attributed to similar fibrillar

assembly of both the enantiomers. Further, the comparison of computer-generated models of Aβ(25-35) enantiomers in antiparallel β-sheet conformation showed that the surface groups generated topochemically similar enantiomers (Cribbs et al., 1997). But in the case of Aβ(1-42), the length of the peptide was found to reduce the probability of formation of topochemically similar enantiomers. This was attributed to the more complex surface topography of Aβ(1-42) compared to Aβ(25-35). The difference in the results obtained between our studies and the study of Cribbs et al., (1997) could be attributed to the two amino acids (valine, alanine) at the C-terminal end of the Aβ(1-42). The literature showed quite interesting observations on the role of two amino acids at the C- terminal end. The biochemical properties are found to be significantly altered by two amino acids' difference, between Aβ(1-42) and Aβ(1-40). Aβ(1-42) forms fibrils more rapidly at pH 7.4 than Aβ(1-40), and the fibrils formed by Aβ(1-42) are more stable (Barrow and Zagorski, 1991). Furthermore, Aβ(1-42) forms higher molecular weight aggregates and these are resistant to sodium dodecyl sulfate (Burdick et al., 1992). Aβ(1-42) is more hydrophobic as a result of the presence of valine and alanine at the C-terminal end. The internalized Aβ(1-42) is resistant to degradation and accumulates in lysosomes, whereas Aβ(1-40) is sensitive for degradation (Knauer et al., 1992). Cribbs et al., (1997) by computer modeling studies, partially explained the role of the length of peptides in understanding the chemistry of enantiomers.

The data on the formation of racemized amino acids and their biological significance is limited. Deloncle and Guillard, (1990) have speculated over the reason for racemization of L-amino acids to D-amino acids that the chelation of amino acids by positively charged metal ions stabilizes the intermediate carbanion succinimide by loss of alpha hydrogen. The conversion also results when the carbonyl group of the side chain of the L- aspartyl residue is attacked by the nitrogen of the amino acid residue after the Asp residue. It is speculated that the rate of racemization depends upon the neighboring amino acid residue; the smaller the side chain of the neighboring residue, the easier it is for it to undergo racemization because of no steric hindrance (Geiger and Clarke, 1987). D-serine is considered to promote neuronal death in experimental models of Aβ induced-

neuroinflammation and of ischaemia by overactivating the N-methyl D-aspartate receptors (NMDA) (Martineau et al., 2006).

Aging of the proteins is considered a main cause for the racemization of the amino acid residues in Aβ peptide. Further, it is a known fact that AD is an age-related disorder; hence, there appears to be a correlation between aging of the peptide, racemization of amino acids and AD. This study probably helps us to correlate the morphology of aggregates formed by the racemized Aβ peptide with their neurotoxicity *in vivo*. Probably, having D- amino acids in Aβ40 prevents it to build β–sheet structure very efficiently on itself during aggregation. We explain our understanding of the self-assembly of L and D -enantiomers of Aβ (1-40) using TEM that deposition of soluble Aβ onto preexisting Aβ is stereospecific.

The fact that the all-D- analog of Aβ (1-40) has a different folding and aggregating pattern may present new avenues for therapeutic intervention by allowing us to actually locate the exact cause and triggering point of neurodegeneration process. A better understanding of the affect of racemization of amino acids in Aβ sequence probably helps us to better know the mechanism of neurodegeneration. Delayed folding, aggregation and varied morphology of fibrils in case of Aβ(1-40) all-D leads us toward two perceptions: First, racemization can prolong the disease symptoms to appear and slowly converts Aβ to a chemical form where it exhibits toxicity or in a form where it becomes chemically feasible to undergo proteolysis to release toxic fragments. Second, it is an age dependent process where this racemization is just a consequence of the disease process. But one thing is definite from our studies- that complete racemization causes varied folding and aggregation pattern, i.e., changes the physical properties; hence, their bioactivity is also expected to be different. Therefore, we suggest, based on our study and literature that presence of D- amino acids in Aβ can play a pivotal role to serve as stimuli or domains that are targets for different proteases, and hence, consequent production of toxic fragments and also their bioactivity.

CHAPTER 3

Differential DNA nicking activity of L- and D-enantiomers of Aβ(1-40)

3.1. Introduction

AD is a common neurodegenerative disease that affects cognitive function in the elderly. Large extracellular Aβ plaques and tau-containing intraneuronal NFTs characterize AD from a histopathologic perspective (Selkoe, 1989; Terry, 1994). However, the severity of dementia in AD is more closely related to the degree of the associated neuronal and synaptic loss. It is not known how neurons die and synapses are lost in AD. Most evidence indicates that APP processing has a central role in the AD process. The Aβ in the form of plaques is a metabolite of the APP that forms when an alternative (beta-secretase and then gamma-secretase) enzymatic pathway is utilized for processing (Zheng et al., 2006). A total of six mutations have been described in the APP gene, which lead to AD by influencing APP metabolism (Tanzi et al., 1996; Campion et al., 1996). One of the leading theories is that Aβ in plaques leads to AD because Aβ is directly toxic to the adjacent neurons (Hardy, 1992). Other theories advance the notion that neuronal death is triggered by intracellular events that occur during APP processing or by extraneuronal preplaque Aβ oligomers (Selkoe et al., 2000). Now, AD has been understood as a more general problem with protein processing, from accumulation of intraneuronal Aβ or extracellular, preplaque Aβ leading to neuronal cell death (Aigelsreiter et al., 2007). However, recently functional imaging studies implied that functional decline in humans can occur separately from both neuronal loss and neurofibrillary tangles (Moreno et al., 2007).

Aβ, a 39-43 aminoacid peptide derived from APP is the major component of SPs observed in AD (Armstrong et al., 2006). Evidences implicate a central role for Aβ in the pathophysiology of AD (Tamagno et al., 2006). Aβ(1-42) peptide and its fragments, 1-40, 1-28, 1-16 are reported to play a crucial role in neurodegeneration in AD (Chen et al., 1996). Aβ40 is the dominant species in human CSF accounting for approx. 90% of total Aβ under normal conditions. There is a disease-specific increase in Aβ40 brain tissue levels compared with the Aβ42 change observed both in the aged and in AD brain (Gregory et al., 2005). It is now speculated that Aβ pathological significance is also known to be associated with α-synuclein aggregation, a protein implicated in PD (Lippa et al., 2005). Aβ is a self-aggregating protein and the conformational transition from

unfolded state to a beta sheet rich conformation leads to deposition of protein aggregates (Deshpande et al., 2006). Though Aβ deposits are primarily extracellular, studies have demonstrated intraneuronal accumulation of Aβ in AD vulnerable regions. Masters et al., (1985) quoted that the "Amyloid is deposited first in the neuron and later in the extracellular space". There are various reports demonstrating the presence of Aβ in intraneuronal compartments (Wertkin et al., 1993; Takahashi et al., 2002). The oligomerization of Aβ protein has been shown to begin intracellularly in cells derived from human brain (Walsh et al., 2000). It is debated extensively that the soluble forms of intraneuronal Aβ (monomeric/ oligomeric) appear to be the predominant toxic species in AD and the extracellular Aβ aggregates/fibrils could be either a mere secondary consequence or a neuroprotective phenomenon (Walsh and Selkoe, 2004). The Aβ peptide is released either extracellularly (if the cleavage events occur while APP is at the plasma membrane) or within a membrane-bounded organelle (endoplasmic reticulum, Golgi, endosome, or secretory vesicle if APP is intracellular). Further there are reports on the intra-nuclear localization of Aβ in literature.

There are various reports indicating Aβ immunoreactivity in the nucleus, nucleolar membrane and in the vicinity of DNA:

i) Studies have demonstrated the neuronal localization of Aβ immunoreactivity in apoptotic nuclei in the vicinity of DNA/chromatin of the hippocampal neurons in human AD brain (Hegde et al., 2004). Further, i*n vitro* studies have shown that Aβ (1-42) and Aβ (1-16) fragments effectively bind to DNA and alter the conformation of DNA.

ii) Suram et al., (2007) evidenced a new toxic role of Aβ(1-42) in terms of its DNA nicking activity. This DNA nicking activity of Aβ was implicated to have biological significance in terms of causing DNA damage.

iii) Grant et al., (2000) reported immunoreactive signals in a number of subcellular organelles including nucleus, nuclear pores, nuclear envelope using the monoclonal antibody highly specific to an epitope in the human Aβ sequence.

iv) Kimberly et al., (2001) demonstrated that the cytoplasmic domain of APP is a highly labile fragment that gets stabilized by forming complexes with Fe65 and then enters nucleus in a manner similar to the function of Notch. These findings provide evidence that the cytoplasmic domain of APP molecules can participate in Fe65 containing complexes, which further induce transcription of heterologous reporter genes in the nucleus.

v) A model proposed by Cao and Sudhof, (2001) indicates that the cytoplasmic domain of APP after sequential α- and γ- secretase cleavages gets into the nucleus for signaling. They reported that the cytoplasmic tail of APP forms a complex with the nuclear adaptor protein Fe65 and the histone acetyltransferase, which inturn, induces transcription, indicating that C-terminal fragment regulates gene expression.

vi) There are number of *in vitro* systems that have established nuclear localization of neurotoxic APP fragments. Johnstone et al., (1996) reported nuclear and cytoplasmic localization of the Aβ(1–43) in transfected 293 cells. They further indicated that even though Aβ(1-43) species represent only a minor component in the amyloid plaques found in AD brain, this model peptide provides the critical C-terminal residues that are considered to be very important in nucleation or seeding of plaques. They reported that Aβ deposits both in the nucleus and cytoplasm. Based on these results, they hypothesized that Aβ first accumulates in the nucleus and later in the cytoplasm. But they could not conclude the reason and the mechanisms by which Aβ accumulates inside the nucleus.

vii) Buckig et al., (2002) clearly demonstrated that a fraction of Aβ42 targeted to the secretory pathway, is present in the cytosol in the form of aggregates. The aggregates are found to be localized near the nucleus of the cell, and they appear like aggresomes. But they are different from aggresomes in a way that only one aggresome is found in the pericentriolar region of the cell, where as, several Aβ42 aggregates (as aggresomes) are found in the cytosol and inside the nucleus.

viii) Aβ is observed in nuclear extracts from neural cells exposed to excess glutamate, comparable to the environment in post-striatal lesioned substantia nigra pars reticulata (Ohyagi et al., 1999). Later, DeGiorgio et al., (2000) evidenced using Aβ domain specific antibody that there is a significant accumulation of immunogold- silver reaction product in the nuclei of affected neurons. Hence, it is concluded from their results that intranuclear APP- CTF and Aβ may play a role in genomic events contributing to delayed neuronal degeneration.

ix) Ohyagi et al., (2005) proposed that there is a probability that Aβ42 might directly bind to the p53 promoter as the p53 promoter contains heat shock elements. They concluded from their results that Aβ42 may bind to p53 promoter in cooperation with other unknown nuclear proteins in vivo. They also indicated that an overload of oxidative stress results in oxidative DNA damage or overproduction of Aβ42.

Further, the possibility of Aβ binding directly to DNA cannot be denied because Aβ is theoretically known to form a β- hairpin shape followed by a helix-turn-helix motif (Durell et al., 1994), which is found to be an essential constituent of a DNA binding domain of heat-shock transcription factors (Wu, 1995). There are limited reports on the interaction of Aβ with nucleic acids. Ahn et al., (2000) reported a shift in the electrophoretic mobility of DNA on association with Aβ. The above studies provoked us to carry out the mechanistic studies on Aβ binding to DNA and to understand the DNA nicking property of Aβ40 and its biological significance. Further, in neurodegenerative brain, the amount of racemized forms of aminoacids, in particular D-Asp, D-Glu, D-Ser are relatively in large proportions in core amyloid plaques and NFTs (Fisher et al., 1991). However, the reasons for aminoacids racemization under *in vivo* conditions are not known. In the current study, we have utilized all- L- and all- D - aminoacid enantiomers of Aβ40 peptide as model peptides to elucidate the mechanism of DNA binding and nicking activity of Aβ40. Since numerous ligand-receptor interactions are stereospecific, one needs to both examine the nature of the Aβ-DNA interactions and to determine whether DNA- nicking

activity of Aβ exhibits stereospecificity. To the best of our knowledge, this is a new data, we are providing in literature.

Vast array of supercoiled pockets of DNA exist in viruses, animals and human cells, and are known to be involved in gene expression (Bauer et al., 1980). Such supercoiled pockets present in non-histone binding sites in human genome are comparable to the supercoiling of pUC-18 DNA used in the present study (Serban et al., 2002). Hence, in the present chapter, we characterized the stereospecific based binding and DNA nicking activity of soluble and aggregated forms of both Aβ40L and Aβ40D.

3.2. Materials and Methods

3.2.1. Chemicals

Supercoiled pUC 18 DNA (scDNA) (Cesium chloride purified, 90% supercoiled structure), Single stranded circular (ssc) DNA (M13 phage) (90% pure), Double stranded circular (dsc) DNA (M13 phage) (90% pure), DNA molecular weight markers, Low melting agarose, EcoRI restriction enzyme, Ethidium bromide (EtBr), Tris (hydroxymethyl) amino-methane and HEPES buffers were purchased from Bangalore Genei, India. Aurintricarboxylic acid (ATA), diethylpyrocarbonate (DEPC) were purchased from Sigma chemical Co., USA. Aβ(1-40)all-L, Aβ(1-40)all-D and Aβ(40-1) all-L peptides were also purchased from Sigma, USA and r-peptides (USA). *Escherichia coli* DNA polymerase I, terminal deoxynucleotidyl transferase, and [^{3}H] TMP (40 Ci/mmol) were purchased from Amersham (U.K). All other chemicals, sodium chloride (NaCl), acetic acid, EDTA were of analytical grade and were purchased from Sisco Research Labs, Mumbai, India.

3.2.2. Aβ-DNA interactions

ScDNA (1μg) was incubated with Aβ40 L and D enantiomers (15×10^{-6} M) overnight (12 h) in Tris-NaCl-EDTA buffer (pH 7.4) at 37^0C and the following analysis was done to characterize DNA nicking property using agarose gel electrophoresis.

i) The concentration dependent DNA nicking activity of Aβ40 enantiomers was studied at different concentrations of Aβ peptides viz. 5×10^{-6}, 10×10^{-6}, 15×10^{-6} M.

ii) The time kinetics of ScDNA nicking activity of Aβ40 enantiomers was studied at various time intervals of incubation of Aβ-DNA complex viz. 0,4,8,12,18,24 hrs.

iii) The effect of divalent metal ions (Mg^{2+}, Ca^{2+} and Zn^{2+}) was studied by adding metals at the concentration of 1mM to the ScDNA, followed by the addition of Aβ40 enantiomers.

iv) The role of aging stability of Aβ40 enantiomers on DNA nicking activity was studied by incubating Aβ at different time intervals of aging stability viz. 0, 6, 12, 24, 36, 48, 60, 72, 80 hrs with ScDNA.

v) The DNA nicking activity of aggregated form of Aβ40 enantiomers was also studied in the presence of metal ions (Mg^{2+}, Ca^{2+} and Zn^{2+}) at the concentration of 1mM.

vi) The effect of nuclease inhibitors like ATA and DEPC (2×10^{-4} M) in modulating DNA nicking property of Aβ40 enantiomers was also studied.

The DNA nicking activity of Aβ40 enantiomers was also studied on ssc and dsc DNA (1μg) by incubating DNA with Aβ40 L and D enantiomers (15×10^{-6} M) overnight (12 h) in Tris-NaCl-EDTA buffer (pH 7.4) at 37^0C using agarose gel electrophoresis.

3.2.3. Agarose Gel Electrophoresis studies

DNA mobility and damage studies in presence of Aβ peptides were carried out by running agarose gel electrophoresis. Samples contained 1μg pUC18 plasmid DNA and Aβ40 L/D peptides (15×10^{-6} M). To assess the damage induced by Aβ to DNA by gel analysis, samples were prepared, incubated overnight (12 hrs) in 0.01mM Tris-NaCl-EDTA buffer (pH 7.4) at 37^0C, and loaded on a 1% agarose gel buffered with 1x Tris-acetate-EDTA (TAE) pH 8.4 conductive media at 4v/cm at room temperature. The gels were stained with EtBr (0.1μg/ml) for UV photography and imaged on a gel documentation system. This was repeated six times from six different batches of Aβ procured from Sigma and r-peptide Company.

3.2.4. Linearisation of pUC 18 DNA by EcoRI treatment

Linear form of Sc DNA was prepared using EcoRI restriction enzyme. The reaction was carried out in 50mM Tris-Cl buffer (pH 8.0), 100mM NaCl, 10mM $MgCl_2$ and 5mM β-mercaptoethanol. 1μg of DNA was treated with one unit of enzyme in a 50μL reaction volume. EcoRI recognizes the G*AATTC sequence. After incubating the

reaction mixture for 60 min at 37^0 C, the reaction was stopped by heating the reaction mixture at 60^0C for 20 min, which inactivates the EcoRI.

3.2.5. Circular Dichroism studies

The CD spectra (190-330nm) were recorded for Sc DNA in the presence/ absence of increasing concentrations of Aβ40L and Aβ40D (1×10^{-6}, 5×10^{-6}, 10×10^{-6}, 15×10^{-6} M) in 0.01mM Tris-NaCl-EDTA buffer (pH 7.4) at 25^0C on a JASCO-J 700 spectropolarimeter with 2 mm pathlength quartz cuvette. 20μg of ScDNA was used for each interaction study and each resulting point ($[\theta]_{obs}$, deg) was converted to mean residue ellipticity ([θ], deg cm^2 $dmol^{-1}$). Each spectrum was the average of four repetitions and all spectra were corrected by subtracting the buffer baseline. The CD contributions from Aβ alone were subtracted in the DNA-Aβ complex spectra. The DNA conformations were characterized from the CD spectra using the reference of Gray et al., (1978); Hanlon et al., (1975); and Shin and Eichhorn, (1984).

3.2.6. Ethidium Bromide (EtBr) binding studies and Scatchard plots

The quantification of EtBr bound in moles per base pair of Sc DNA in absence and presence of 15×10^{-6} M Aβ40L and Aβ40D was studied in 0.01M HEPES buffer, pH 7.4 by taking DNA/EtBr in a ratio 1:1 (w/w) (Chatterjee and Rao, 1994). The fluorescence was measured using a constant amount of DNA with increasing EtBr concentration against the blank containing no DNA. DNA/EtBr and DNA- Aβ/EtBr solutions were excited at 535nm, and emission intensity was monitored at 600 nm using HITACHI F-2000 Fluorescence Spectrophotometer. The path-length was 10 mm.

The maximum amount of EtBr bound per bp DNA was calculated using Scatchard plots of 'r' versus 'r/Cf ', in the DNA-EtBr reaction mixture at various titration intervals when increasing amount of EtBr was titrated to constant amount of DNA by employing the independent binding equation of Scatchard (Scatchard, 1949). The concentration of bound EtBr in 1.0 ml dye-DNA –Aβ peptide (C_b) was calculated using the equation:

$C_b = C_t (I-I_0)/(V-1) I_0$,

where, C_t is the known total EtBr concentration (picomoles) in the dye-DNA- Aβ peptide mixture, I is the observed fluorescence intensity at any point of dye-DNA- Aβ peptide mixture, I_0 is the observed intensity of the identical concentration of EtBr in the absence of DNA and Aβ, and V is the experimentally determined ratio of the fluorescence

intensity of totally bound EtBr to that of free EtBr. Free EtBr concentrations (C_f) were obtained from the relationship $C_t = C_b + C_{f.}$ Ct, C_b and C_f were expressed in picomoles. The amount of bound EtBr/ base pair (r) was calculated by

$r = C_b$ (pmoles) /[DNA bp] $_{total}$ (pmoles of base pair).

A plot was made for r vs r/C_f and the point where the straight line intersects the axis r was defined as the maximum amount of dye bound per base pair (n), where $C_{f=} C_f' \times 10^{15}$ M.

3.2.7. Melting temperature and hyperchromicity calculation

In order to determine the stability of the DNA in the presence of Aβ40L and Aβ40D, the melting profiles for ScDNA alone and DNA-Aβ complexes were carried out in 0.01 M HEPES buffer, pH. 7.4 by recording UV absorbance at 260nm at different temperatures (25-95^{0}C, 1^{0}C/min) using Amersham spectrophotometer equipped with thermostat control. The hyperchromicity changes of the ScDNA alone (10μg/ml) and ScDNA in presence of 15×10^{-6}M concentration each of Aβ40L and Aβ40D were recorded from 25^{0}C- 95^{0}C with 1^{0}C increment/minute. Tm values were determined graphically from the hyperchromicity versus temperature plots. The temperature point at which there was a 50% hyperchromic shift was taken as melting temperature (Tm) of the DNA sample. The precision in Tm values was estimated from variance in three repeated experiments was ± 0.05^{0}C.

3.2.8. Nick Translation assay for estimation of single and double strand breaks in Sc DNA

Single strand breaks (SSBs)- SSBs induced by Aβ peptides in ScDNA were calculated through incorporation of ^{3}[H]-TMP in to Aβ treated ScDNA samples when incubated with *E. Coli* DNA polymerase I (Klenow Fragment) in a Nick Translation assay. DNA polymerase I adds nucleotides at the 3′-OH end of a SSBs generated by various means, using the other strand as template (Mosbaugh and Linn, 1982).

When one of the deoxynucleotide triphosphates is labeled, then the incorporation of radioactivity into substrate DNA would be proportional to the number of SSBs present in the DNA sample. 1μg of Sc DNA was incubated with 15×10^{-6}M of each Aβ(1-40)all-L and Aβ(1-40)all-D with and without 7.5mM $MgCl_2$ in 40mM Tris-HCl, pH 8.0 for overnight (12 hrs). During the standardization of the assay, using the Cos T fragment of λ phage

DNA having known number of SSBs, it was found that ~375 TMP residues were added at each of the 3′-OH group. As the incubation mixture contains all the four deoxynucleotides and only one of them is radiolabelled (^{3}[H]-TMP), the total number of nucleotides added to the 3′-OH groups is taken as four times the number of TMP residues, i.e. 1,500 (375×4). The number of TMP residues added at each 3' OH are calculated from the mole percentage of ^{3}[H]-TMP incorporated into DNA and Avagadro's number. Thus each picomole of TMP incorporated would be equivalent to 6×10^{11} molecules. This number is divided by 375 to arrive at the number of 3'-OH groups (SSBs) present in the DNA sample taken for assay, which would be equivalent to 1.6×10^{9}. From this, it was inferred that each picomole of TMP incorporated was equivalent to 1.6×10^{9} 3´-OH groups or SSBs (Mandavilli and Rao, 1996).

In a total reaction volume of 50 µl, the assay mixture consisted of: 40mM Tris-HCl, pH 8.0, 1mM β-mercaptoethanol, 7.5mM $MgCl_2$, 4mM ATP, 100µM each of dATP, dCTP and dGTP and 25µM of dTTP, 1µCi of ^{3}[H] TTP and 1µg of ScDNA (incubated overnight with 1, 5, 10, 15 µM of Aβ peptide in $MgCl_2$) and 1 unit of *E. coli* DNA polymerase. (1 unit is the amount of enzyme activity that leads to an incorporation of 10nmol of total nucleotide into the acid-precipitable fraction in 30 min under the given assay conditions).

After incubation of ScDNA (incubated overnight with Aβ peptides) with *E.coli* polymerase I for 30 mins, the DNA was precipitated by adding 10% TCA containing 10 mM Na pyrophosphate in the presence of 100µg of Calf Thymus DNA and bovine serum albumin (BSA), which act as carrier molecules. Pellet was separated after centrifugation at 12000 rpm for 15 sec and dissolved in 400µl NaOH (0.2 N) by vortexing. DNA was reprecipitated in 10% Trichloro acetic acid (TCA) containing 10 mM Na pyrophosphate, which was separated by centrifugation at 6000 rpm for 5 min. The DNA precipitate was washed twice with 5% TCA, and 95% ethanol on Glass Fiber Filter (GF 6). The filters were dried at room temperature for ~12 h and counted for radioproperty in BRAY'S mixture (containing 4gms/litre of PPO, 200 mg/ liter POPOP in Dioxan) in Beckman LS1800 Liquid scintillation counter. The values were expressed as DPM (disintegrations per minute) incorporated into 1µg of DNA.

Double strand breaks (DSBs)- Terminal deoxynucleotidyl transferase catalyzes the addition of deoxynucleotides to the 3′ termini of DNA and does not need direction from template strand. ScDNA (treated overnight with similar concentrations of Aβ peptides as for SSBs estimation) was incubated with Terminal transferase at 37^0C for 30 min. The incorporation of the ^{3}[H]-dTTP into DNA would be proportional to the number of DSBs in the DNA. From the conditions of incubation (Mandavilli and Rao, 1996; Deng and Wu, 1983), it was assumed that about 50 TMP residues were added at each of the 3´- ends of the duplex DNA. From this it was calculated that each femtomole of TMP incorporation would be equivalent to 1.2×10^7 3´ ends or half that number minus one DSB. The assay mixture for terminal transferase assay consisted of a total volume of 50μl: 100mM sodium cacodylate buffer (pH7.0), 1mM $CoCl_2$, 0.2mM DTT, 1μCi of ^{3}[H]-dTTP, 1μg DNA and 1 unit of terminal transferase (1 unit is the amount of enzyme activity that leads to an incorporation of 1nmol of a given nucleotide into acid-insoluble products in 30min at 37^0C).

3.2.9. Sequence homology of Aβ40

The Aβ40 sequence was obtained from SWISSPROT (entry: P05067; 40 residues) database. A FASTA search for a suitable template within the PDB was performed. KALIGN was used to find the homology of Aβ40 sequence with other DNA binding proteins.

3.3. Results

3.3.1. Aβ40 enantiomers bind to DNA and alter DNA topology

Effect of Aβ40 enantiomers in modulating ScDNA conformation was examined by CD spectroscopy. ScDNA alone showed typical B-DNA conformation with a characteristic positive peak at 275nm and a negative peak at 245nm. Substantial changes in the CD spectra of ScDNA were observed upon interaction with both Aβ40 L and Aβ40 D peptides. Figure 3.1A indicates the spectra of ScDNA complexed with Aβ40 L. The spectra indicated strong binding of Aβ40 L with ScDNA, causing shift in the conformation of Sc DNA from normal B to ψ-DNA with increasing concentration of Aβ40L. The spectral changes involved shift of the negative CD signals towards the far UV region with a significant increase in the magnitude of peak at around 210 nm

indicating asymmetric compaction of Sc DNA to ψ (+) like conformation. The spectral modifications of the negative peak were continuous with the increasing concentration of Aβ. On the addition of higher concentration (15×10^{-6} M) of Aβ40 L, the negative CD band extended in the non-absorbing region in the form of CD tails, with a large CD magnitude compared to the intrinsic CD of scDNA. This was a characteristic feature of ψ-DNA conformation.

But in the presence of Aβ40D, the CD spectral pattern of DNA was different. With increasing concentration of Aβ40D, a small decrease in the magnitude of negative peak at 245 nm accompanied with a small decrease in the magnitude of positive peak at 275 nm is observed (Figure 3.1B). At higher concentrations (15×10^{-6} M) of Aβ40 D, a significant shortening of 210 nm negative peak was also observed. The presence of isodichroic point at 258 nm was also a characteristic feature of all the spectra. The observed changes indicated the transition of B-DNA conformation to altered B-DNA conformation. Hence, we found that both Aβ40L and Aβ40D peptides bind to DNA and bring about changes in the conformation of DNA. The CD signals contributed by Aβ40 in each interaction study were subtracted from each complex spectra.

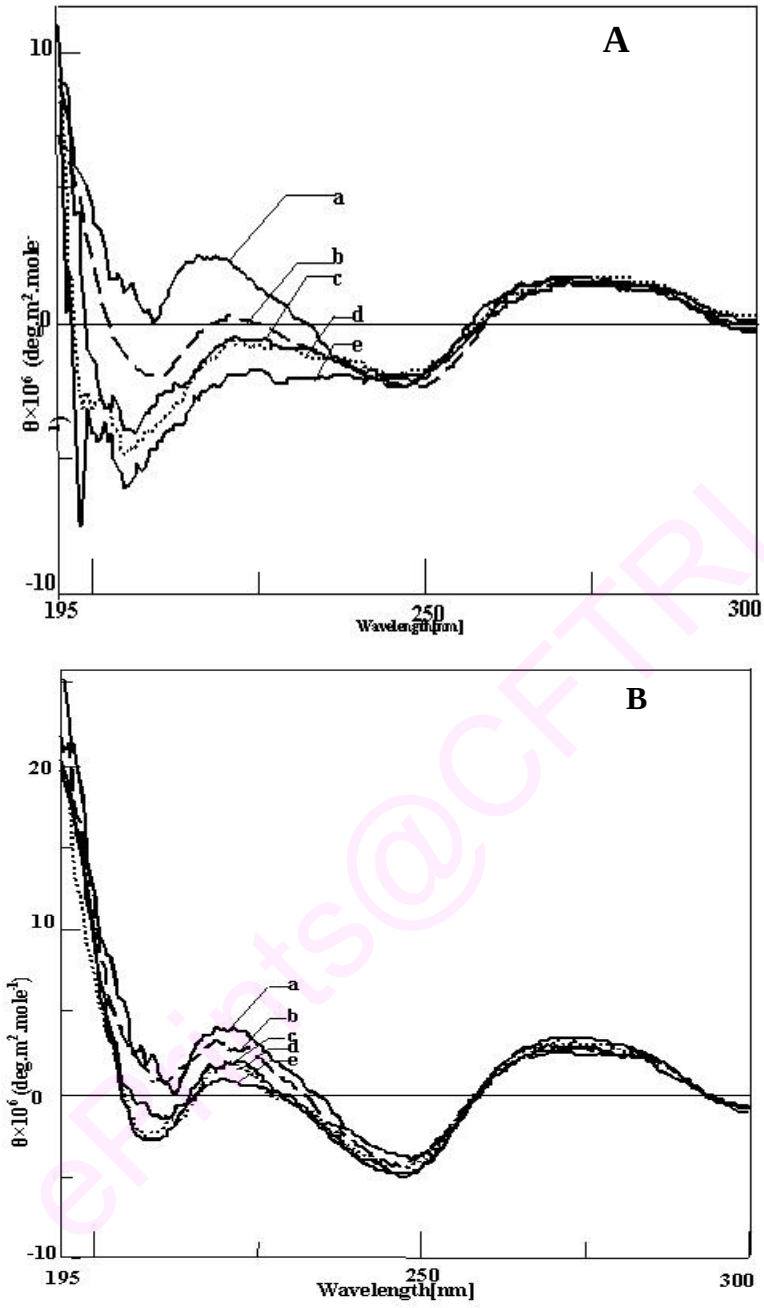


Figure 3.1: Circular Dichroism Spectra of pUC18 supercoiled DNA-Aβ40 complex: A) Aβ40L binds to ScDNA and alters the conformation of ScDNA from B-DNA to ψ-A mixed conformation; B) Aβ40D binds to ScDNA and alters the conformation of ScDNA from B-DNA to altered B-DNA conformation (a) 25µg scDNA; (b) scDNA+ 1µM Aβ40; (c) scDNA+ 5µM Aβ40; d) scDNA+10 µM Aβ40; e) scDNA+15 µM Aβ40

3.3.2. Aβ40 enantiomers have DNA nicking activity like nucleases

Figure 3.2 represents neutral agarose gel where M1 represents 1Kb marker; M2 represents EcoR1 digested linearized supercoiled DNA. It indicates the concentration dependency of Aβ40L induced DNA nicking activity. The increasing concentration of Aβ40L (5×10^{-6}, 10×10^{-6}, 15×10^{-6} M) resulted in a gradual decrease in the supercoiled band intensity with simultaneous increase in the open circular form. The nicking effect increased linearly with increasing Aβ40L concentrations with the appearance of bands of DNA dimers and trimers (Figure 3.2, b-d lanes). But in the case of Aβ40D, (Figure 3.2, e-g lanes) there was a decrease in the intensity of supercoiled form of DNA, with increased open circular and linear form with increasing concentration of Aβ (5×10^{-6}, 10×10^{-6}, 15×10^{-6} M). Linear DNA was identified using the comparison of migration of the linearized DNA resulted due to the treatment of Sc DNA with EcoR1 restriction enzyme.

The time kinetics of DNA nicking activity of Aβ40L enantiomers was studied using agarose gel electrophoresis. Aβ40 enantiomers at the concentration of 15×10^{-6} M were incubated with 1μg scDNA for various time intervals (0, 4, 8, 12, 18, 24 hrs) in separate reaction mixtures. With increase in time of incubation of ScDNA with Aβ40L, there is an increase in the open circular form and at 16 hrs of incubation, we could also observe the presence of linear form of DNA (Figure 3.3A). Similarly, upon incubation of ScDNA with 15×10^{-6} M Aβ40 D, there was an increase in the open circular form with increased time of incubation along with the appearance of linear form of DNA from 12 hrs onwards (Figure 3.3B).

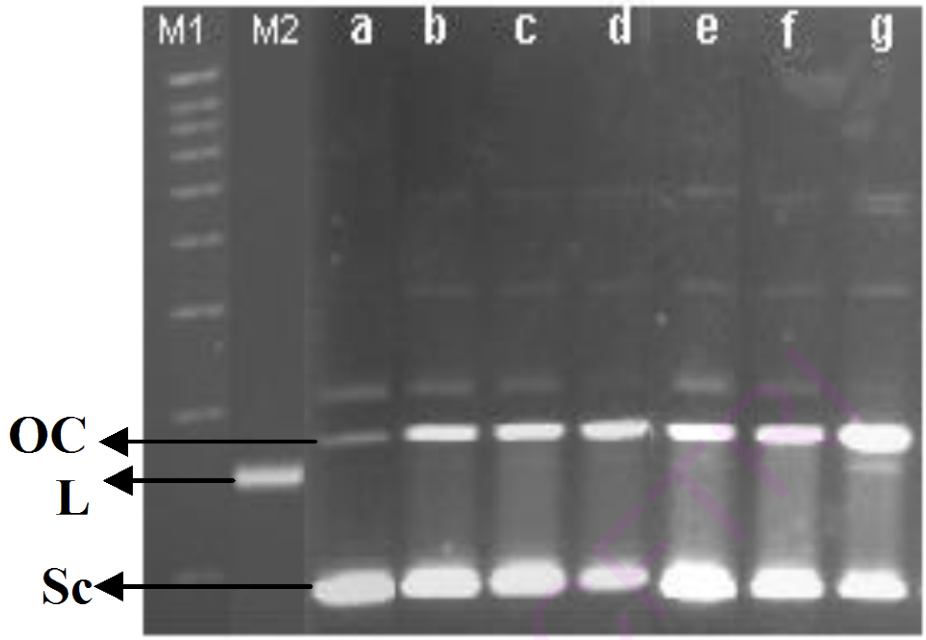


Figure 3.2: Neutral Agarose Gel Electrophoresis showing concentration dependency in Aβ40 L/D induced scDNA nicking and damage: M1) 1Kb marker M2) Linearized supercoiled DNA -*EcoR*1 digested a) 0.5 µg Sc DNA alone b) Sc DNA+5uM Aβ40 L c) Sc DNA+10uM Aβ40 L d) Sc DNA+15uM Aβ40 L e) Sc DNA+5uM Aβ40 D f) Sc DNA+10uM Aβ40 D g) Sc DNA+15uM Aβ40 D. OC: Open circular DNA; L: Linear DNA; Sc: Supercoiled DNA).

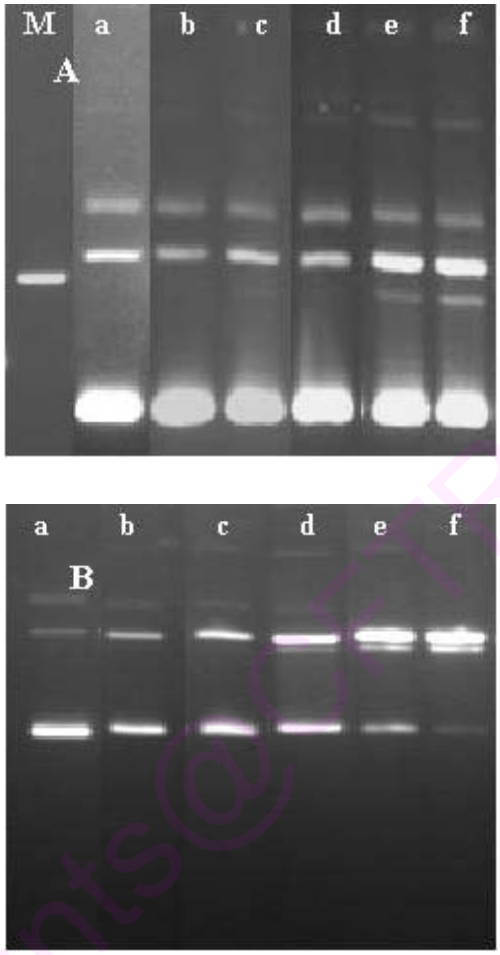


Figure 3.3: Time kinetics of DNA nicking activity of Aβ40 L (A) and Aβ40 D (B): Agarose gel electrophoresis (1% agarose) run at 4V/cm for 6 hrs. a, scDNA alone; a to f, scDNA incubated with 15μM Aβ at 37^0C for 0, 4, 8, 12, 16 and 24 hrs. 0.5 μg of scDNA was loaded in each well.

DNA nicking activity of Aβ40 enantiomers was further confirmed by quantitating the number of SSBs and DSBs in ScDNA using Nick Translation assay. It was observed that accumulation of SSBs and DSBs in ScDNA increased significantly with increasing concentrations of Aβ40L (1×10^{-6}, 5×10^{-6}, 10×10^{-6}, 15×10^{-6} M) on incubation at 37^0C for ~ 12 hrs (Figure 3.4A). Minimal number of SSBs were already present in the untreated control ScDNA used in the present study. Further, a ScDNA when incubated with increasing concentrations of Aβ40D (1×10^{-6}, 5×10^{-6}, 10×10^{-6}, 15×10^{-6} M) at 37^0C for ~12

hrs also showed significant increase in the number of SSBs and DSBs (Figure 3.4B). The results revealed that number of SSBs and DSBs accumulated in the ScDNA treated with Aβ40D were ~1fold more compared to the ScDNA treated with Aβ40L (Table 3.1).

Sample	Aβ40L					Aβ40D				
Concentration (10^{-6}M)	0	1	5	10	15	0	1	5	10	15
SSBs ($\times10^{9}$)/ µg DNA	0.3	0.9	1.3	1.9	3.6	0.3	1.2	1.6	2.1	4.0
DSBs ($\times10^{9}$)/ µg DNA	0	0.5	0.9	1.3	3.4	0	0.8	1.1	1.6	3.6

Table 3.1: Nick Translation assay to study concentration dependent DNA nicking activity of Aβ: SSBs and DSBs accumulated in the ScDNA treated with Aβ40L and Aβ40D peptides at their increasing concentrations viz; 0, 1, 5, 10, 15 µM. Values are expressed as average of triplicates.

Nick Translation method was also used to study the time kinetics of DNA nicking activity of Aβ40 enantiomers. The Nick Translation studies revealed that the kinetics of formation of SSBs in presence of both L and D Aβ40 enantiomers (15×10^{-6} M) was a linear pattern whereas the kinetics of formation of DSBs showed sigmoidal pattern. There was an initial lag phase of ~6 hrs in case of DSBs and then increased exponentially reaching a plateau at 20 hrs and 24 hrs for Aβ40 L (Figure 3.5A) and Aβ40 D (Figure 3.5B) respectively forming a sigmoidal curve pattern. Interestingly, only SSBs were formed initially till 6 hrs and the DSBs appeared after 6 hrs and SSBs accumulated were always more in number than DSBs. It was observed that the accumulation of strand breaks in the form of both SSBs and DSBs was almost ~ 1 fold higher in presence of Aβ40 D than Aβ40 L.

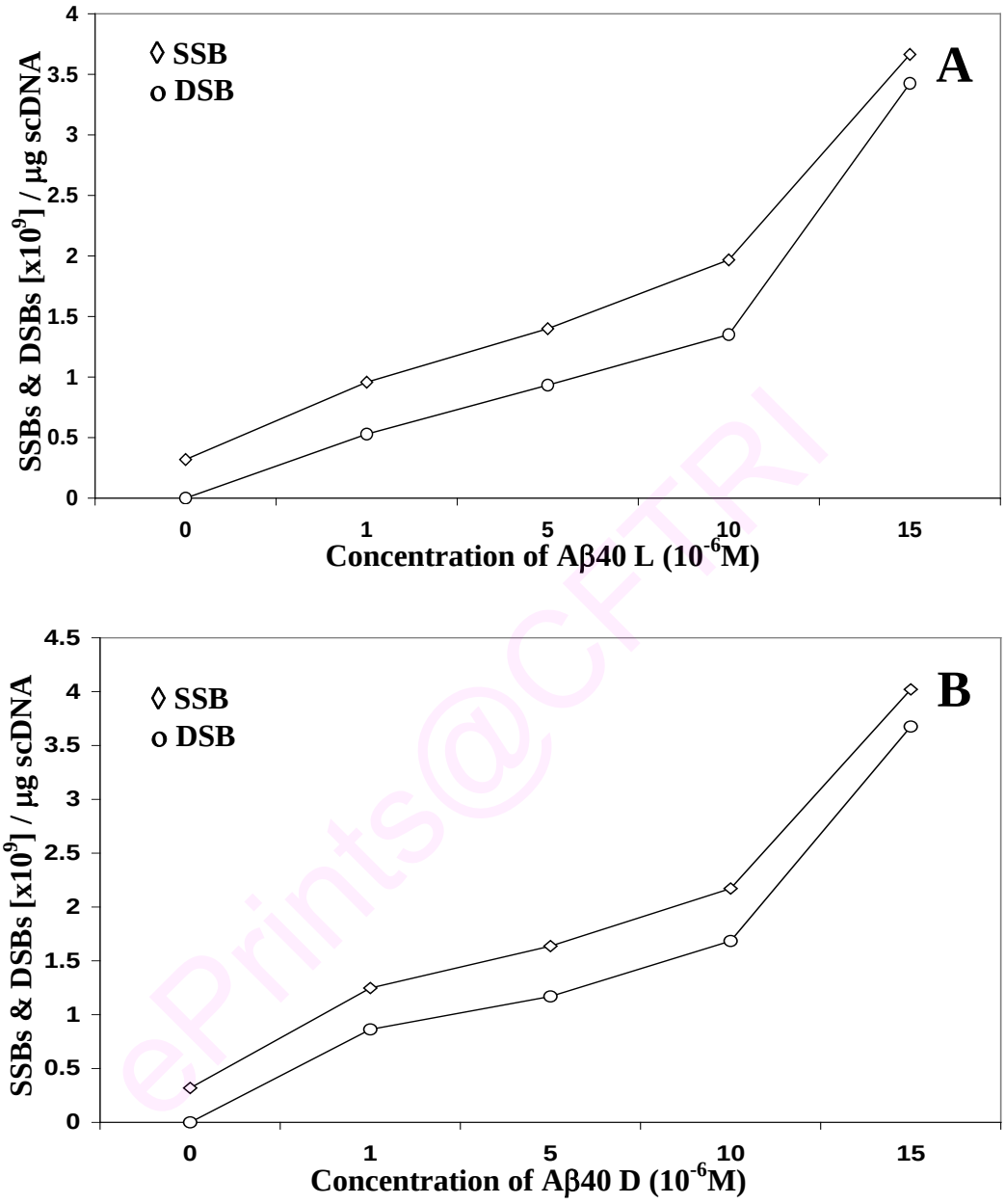


Figure 3.4: Concentration dependency in the accumulation of SSBs and DSBs in ScDNA treated with A) Aβ40 L and B) Aβ40 D: The strand break assay was carried out at different concentrations of Aβ stereoisomers in the assay mixture i.e. 0µM, 1µM, 5µM, 10µM and 15µM. Values are expressed as average of triplicates.

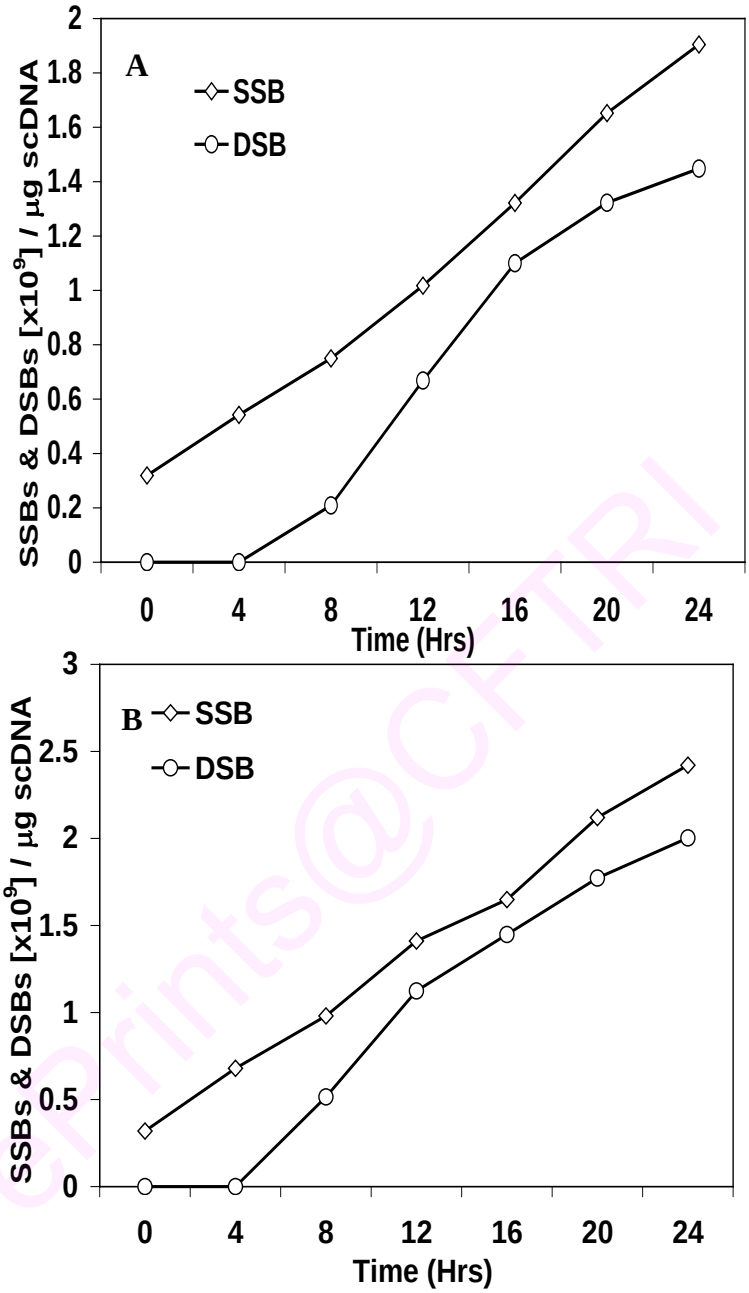


Figure 3.5: Time kinetics of formation of SSBs and DSBs in scDNA by Aβ40 L (A) and Aβ40 D (B): 1 µg of scDNA was treated with 15 µM Aβ for various time intervals in separate reactions at 37^0C and subjected for Nick translation assay. Values are expressed as average of triplicates.

3.3.3. Effect of divalent metals on Aβ40 enantiomers induced DNA nicking

Cell death was known to precede in many instances by fragmentation of DNA by Ca^{2+}-Mg^{2+} -dependent DNases (Arends et al., 1990) and Zn was also known to be important for various protein-DNA interactions *in vivo* (Duan and Nilsson, 2006). Moreover, Mg^{2+} functions as a cofactor for proteins involved in interaction with DNA and also required for the activity of certain nucleases (Sirover et al., 1977; Barzilay et al., 1995). Therefore, we expanded our results on Aβ influence on DNA damage in presence of Mg^{2+}, Ca^{2+} and Zn^{2+}. The effect of divalent metals like Mg^{2+}, Ca^{2+} and Zn^{2+} was studied at the concentration of 1mM by agarose gel electrophoresis.

These divalent metals did not affect the integrity of ScDNA when incubated without Aβ at the same concentration (Figure 3.6A). Both Mg^{2+} and Ca^{2+} enhanced the DNA nicking activity of Aβ40L and Aβ40D. The formation of open circular and linear form of DNA was more in presence of Mg^{2+} when compared to Ca^{2+}. On the other hand, Zn inhibited DNA nicking activity of Aβ40L/D (Figure 3.6B).

The effect of both Aβ40L (Figure 3.7A) and Aβ40D (Figure 3.7B) on ScDNA was also studied using Nick translation assay in presence of Mg^{2+} and Zn^{2+}. Mg^{2+} enhanced the accumulation of both SSBs and DSBs induced by Aβ40 L/D, But Zn^{2+} inhibited the formation of SSBs and DSBs in the presence of Aβ40 L/D.

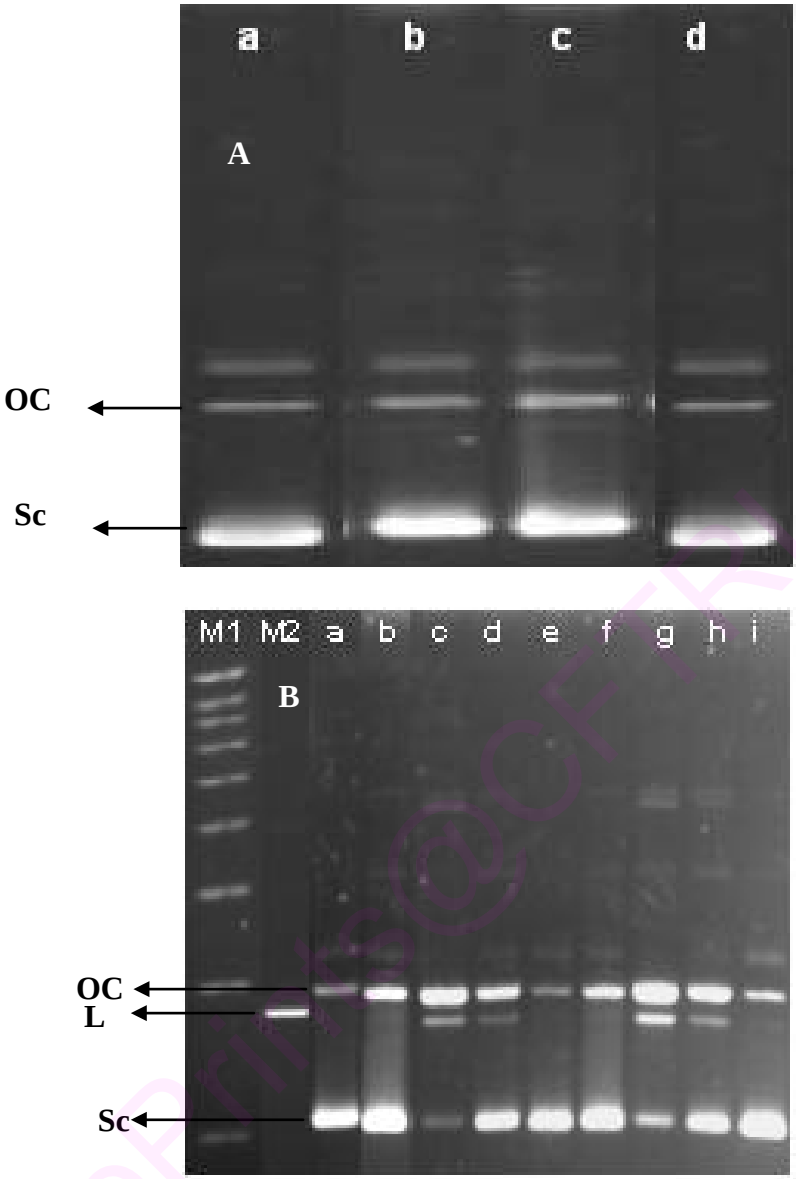


Figure 3.6: A) Effect of Ca^{2+}, Mg^{2+}, and Zn^{2+} on Sc DNA: Agarose gel electrophoresis (1%) (a) sc DNA alone (1.0 μg); (b) sc DNA +1mM $CaCl_2$; (c) sc DNA +1mM $MgCl_2$ and (d) sc DNA +1mM $ZnCl_2$

B) Agarose Gel Electrophoresis showing modulation of DNA nicking activity of Aβ40 L/D in presence of Metals like Ca, Mg and Zn: M1) 1Kb marker M2) Linearized supercoiled DNA -*EcoR*1 digested a) 0.5 μg Sc DNA alone b) Sc DNA+ 15μM Aβ40 L c) Sc DNA+ 15μM Aβ40 L +1mM $MgCl_2$ d) Sc DNA+ 15μM Aβ40 L +1mM $CaCl_2$ e) Sc DNA+ 15μM Aβ40 L +1mM Zn Cl_2 f) DNA+ 15μM Aβ40 D g) Sc DNA+ 15μM Aβ40 D +1mM $MgCl_2$ h) Sc DNA+ 15μM Aβ40 D +1mM $CaCl_2$ i) Sc DNA+ 15μM Aβ40 D +1mM $ZnCl_2$.

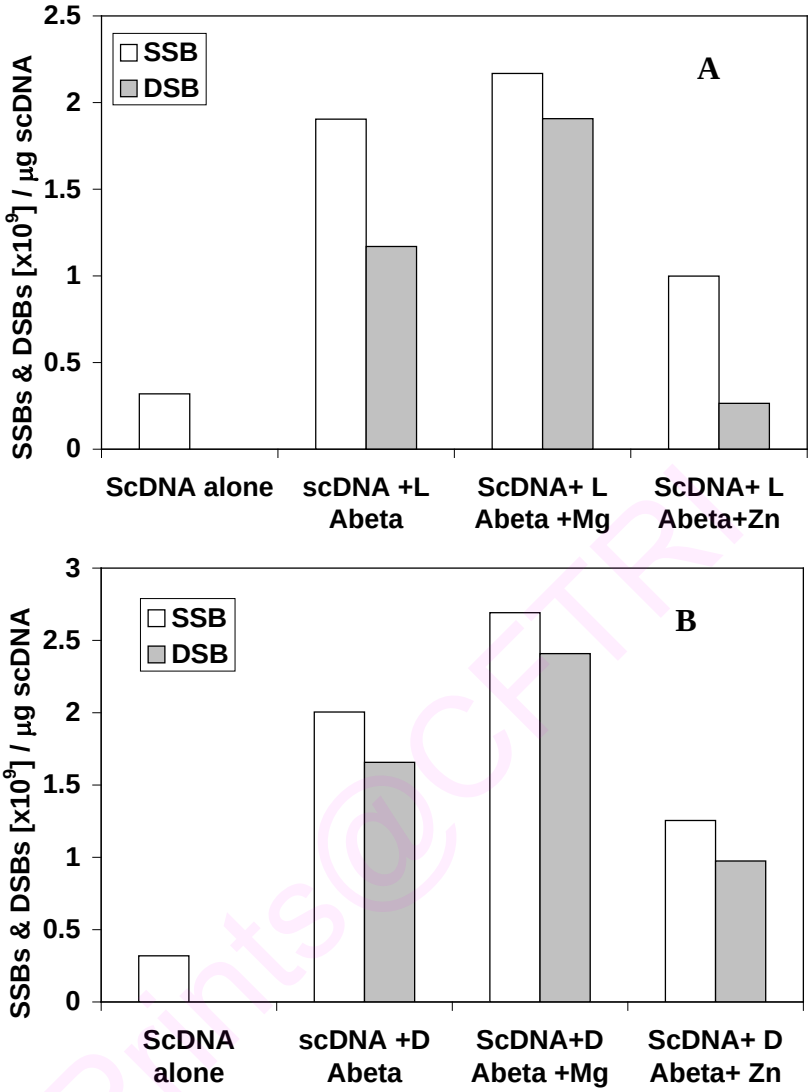


Figure 3.7: Assessment of Single stranded breaks (SSB) and Double stranded breaks (DSB) in scDNA induced by A) Aβ40 L and B) Aβ40 D through nick translation type incubation with E.coli DNA Polymerase I: Action of both the enantiomers on ScDNA was also studied using nick translation studies in presence of Mg^{2+} and Zn^{2+}. Values are expressed as SSBs and DSBs induced per µg of ScDNA per hour of incubation. Values are expressed as average of triplicates.

3.3.4. Role of Amyloid beta aging stability on its DNA nicking activity

The conformational changes of Aβ from random coil to β-sheet and subsequent fibril formation takes from hours to days depending on the particular peptide fragment and the conditions of fibril formation (Harper et al., 1997). During this process, Aβ exists as monomers, oligomers and aggregates. We studied the DNA nicking activity of Aβ40L and

Aβ40D peptides at different time intervals of peptide aging stability in relation to its soluble/ aggregation state. At different time intervals, an aliquot of the peptide stock solution (100 μM) was collected. Agarose gel electrophoresis (Figure 3.8) showed that maximum DNA nicking activity was observed at 12 hrs of peptide aging where maximum linear DNA was observed. It was clear that at 12 hrs of incubation, Aβ40D exhibited more DNA nicking activity than Aβ40L. The nicking activity of both Aβ40L and Aβ40D decreased after 12 hrs of aging. It was clear that both Aβ40L and Aβ40D in aggregated state had comparatively less nicking activity when compared to freshly dissolved one.

Further, the number of SSBs and DSBs were estimated by nick translation assay in ScDNA treated with aliquots of Aβ40 enantiomers withdrawn at different time intervals of incubation. Both the SSBs and DSBs increased from 1 to ~20 hrs and started decreasing after ~20 hrs. The Nick Translation studies revealed that the kinetics of formation of SSBs in presence of both the enantiomers of Aβ40 was a linear pattern whereas the kinetics of formation of DSBs showed sigmoidal pattern. There was an initial lag phase of ~6 hrs followed by an exponential increase and then reached a plateau at 20 hrs and 24 hrs in case of Aβ40L (Figure 3.9A) and Aβ40D (Figure 3.9B) respectively. Initially till 6 hrs, only SSBs were formed and the DSBs appeared later. SSBs accumulated were always more in number than DSBs (Table 3.2).

Sample	Aβ40L(10^{-6})M							Aβ40D(10^{-6})M						
Hours	0	5	10	20	30	44	60	0	5	10	20	30	44	60
SSBs($\times 10^9$)/μg DNA	0.3	1.01	1.07	2.1	1.3	1.08	0.95	0.3	1.3	1.6	2.8	1.6	1.5	0.38
DSBs($\times 10^9$)/μg DNA	0	0	0.6	1.6	1.2	0.9	0.7	0	0	0.8	2.1	1.6	1.2	0.9

Table 3.2: Nick Translation Assay to study the effect of Aβ aging stability on its DNA nicking activity: SSBs and DSBs accumulated in the ScDNA treated with Aβ40L and Aβ40D peptides at different time intervals of their aging stability in relation to its soluble/ aggregation state. Values are expressed as average of triplicates.

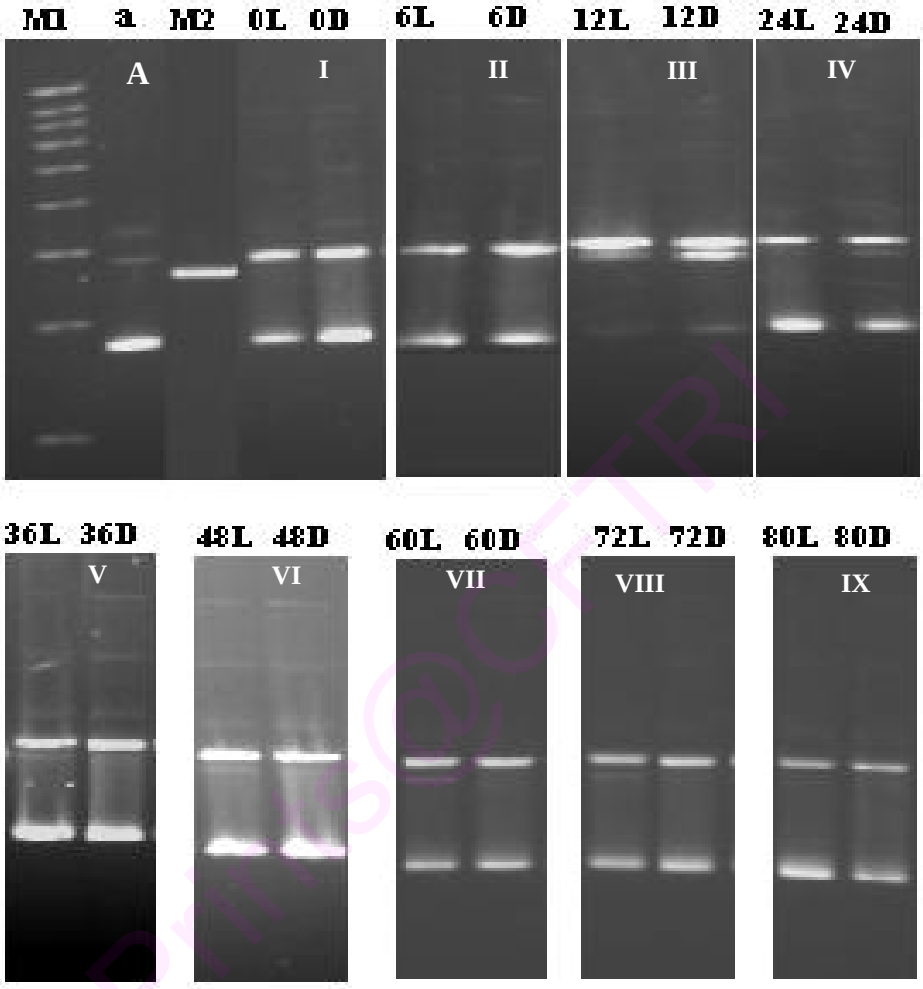


Figure 3.8: Agarose Gel Electrophoresis: Effect of Aβ40 enantiomers aging on its inherent DNA nicking activity: I, II, III, IV, V, VI, VII, VIII, IX show the effect of 0, 6, 12, 24, 36, 48, 60, 72, 84 hrs old peptides on Sc DNA**.** (L represents Aβ 1-40 with all L-aminoacids**,** D represents Aβ 1-40 with all D-aminoacids, M1- 1Kb marker; M2- Linearized Supercoiled DNA (EcoR1 digested); a-ScDNA alone).

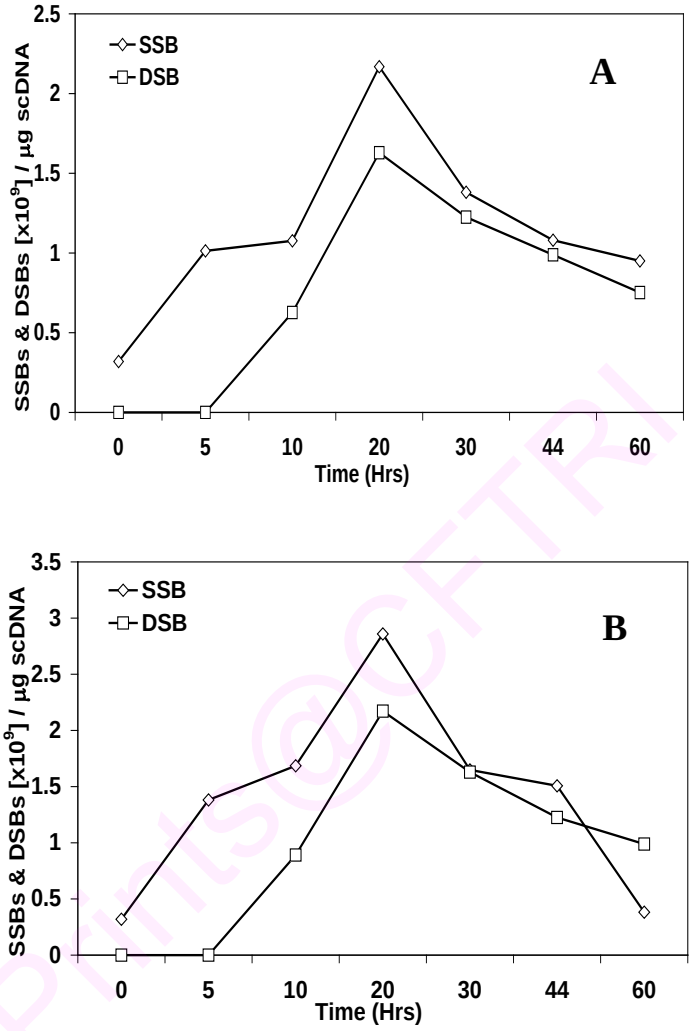


Figure 3.9: **DNA nicking activity of A) Aβ40 L and B) Aβ40 D aliquots withdrawn from the incubated sample at various time intervals:** The SSBs and DSBs were measured by Nick Translation method using DNA polymerase I assay for SSBs and Terminal transferase assay for DSBs. L indicates Aβ40 with all L- aminoacids and D indicates Aβ40 with all D- aminoacids. Values are expressed as average of triplicates.

Further, the interaction of aggregated form of Aβ40L and Aβ40D with Sc DNA in presence of Mg^{2+}, Ca^{2+} and Zn^{2+} was studied. These metals enhanced the DNA nicking activity of aggregated form of Aβ40L and Aβ40D in the order of $Mg^{2+} > Ca^{2+} > Zn^{2+}$ (Figure 3.10).

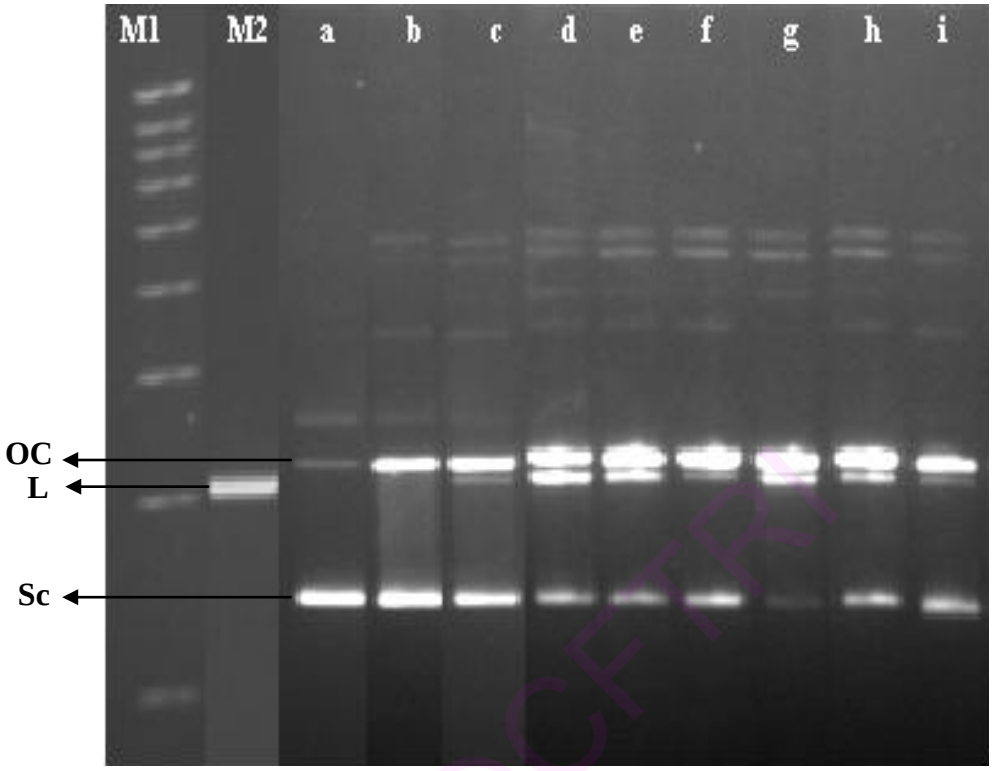


Figure 3.10: Agarose Gel Electrophoresis: Effect of metal ions Ca^{2+}, Mg^{2+}, Zn^{2+} on DNA nicking activity of aggregated Aβ40 enantiomers: M1) 1Kb marker M2) Linearized supercoiled DNA -*EcoR*1 digested
a) 0.5 µg Sc DNA alone b) Sc DNA + 15 µM Aβ40 L; c) Sc DNA + 15 µM Aβ40; D d) Sc DNA + 15 µM L Aβ +1 mM $MgCl_2$ e) Sc DNA + 15 µM L Aβ +1mM $CaCl_2$ f) Sc DNA + 15 µM L Aβ +1mM $ZnCl_2$ g) Sc DNA + 15 µM D Aβ +1mM $MgCl_2$ h) Sc DNA + 15 µM D Aβ +1mM $CaCl_2$ i) Sc DNA + 15 µM D Aβ +1mM $ZnCl_2$

3.3.5. Effect of nuclease inhibitors (ATA and DEPC) on Aβ induced nicking

To better understand the mechanism of Aβ DNA nicking activity, the effect of classical nuclease inhibitors like ATA (2 x 10^{-4} M) and DEPC (2 x 10^{-4} M) on Aβ induced DNA nicking was studied. Both ATA and DEPC prevented Aβ40L and Aβ40D induced DNA nicking (Figure 3.11).

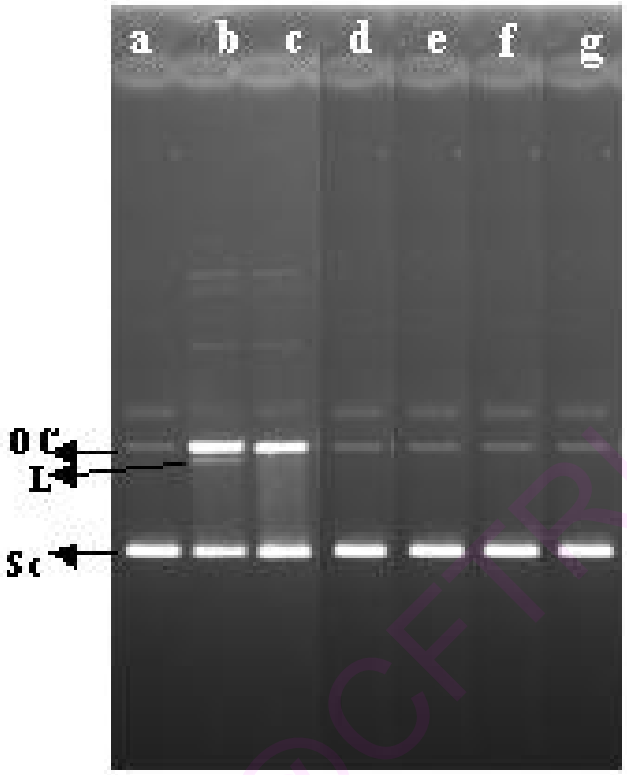


Figure 3.11: Agarose Gel Electrophoresis: Effect of ATA and DEPC on Aβ40 L, Aβ40 D induced nicking of Sc DNA: a) 0.5 µg Sc DNA alone b) Sc DNA +15µM D-Aβ c) Sc DNA +15µM L-Aβ d) Sc DNA +75µM D-Aβ + 200µM ATA e) Sc DNA +15µM L-Aβ + 200µM ATA f) Sc DNA +75µM D-Aβ + 200µM DEPC g) Sc DNA +15µM L-Aβ + 200µM DEPC

3.3.6. Amyloid beta enantiomers interaction with ssc and dsc DNA

Further, the interaction of Aβ40L and Aβ40D was also carried out with ssc and dsc DNA in order to understand the specificity of Aβ nicking for single and double stranded circular DNA. The enantiomers of Aβ40 acted differently on both ssc and dsc DNAs. Both Aβ40L and Aβ40D only nicked ssc DNA as evident in the gel by its reduced density (Figure 3.12) and did not have an effect on dscDNA. The effect of Aβ40D on sscDNA was more than Aβ40L. Hence, our results evidenced that Aβ40 enantiomers nicking is single stranded specific.

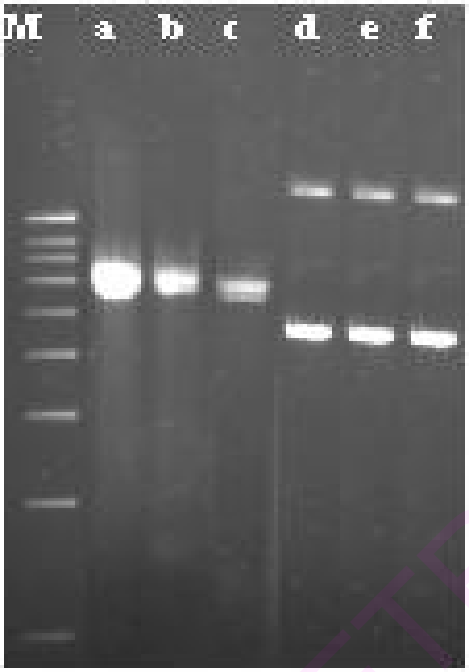


Figure 3.12: Nicking activity of Aβ40 enantiomers on ssc and dsc DNA: M) 1 Kb marker a) 0.5 µg sscDNA alone b) sscDNA+5uM Aβ40 L c) sscDNA+10uM Aβ40 D d) dscDNA alone e) dscDNA+5uM Aβ40 L f) dscDNA+10uM Aβ40 D

3.3.7. Sequence homology of human Aβ40

Our results indicated that Aβ40 acts like an endonuclease. Hence, we investigated for the similarity of Aβ with DNA binding proteins and nucleases. Protein Data- Bank (PDB) was used to search for the sequence neighbor of Aβ40 as the query, it revealed that it has weak to moderate homologies with many DNA binding proteins and DNAse. The top scoring sequences for similarity with Aβ40 were, Nuclear matrix protein p84 (NMP); (Uniprot KB/ Swiss Prot entry: Q96FV9), Purine rich single stranded DNA-binding protein alpha (ssDBP) (Uniprot KB/ Swiss Prot entry: Q00577), Zinc finger protein PLAG1 (ZFP) (Uniprot KB/ Swiss Prot entry: Q9Y4L2), Apoptosis inducing factor (AIF) (Uniprot KB/ Swiss Prot entry: Q5RZ99), Poly [ADP-ribose] polymerase 1 (PARP) (Uniprot KB/ Swiss Prot entry: P09874), DNA fragmentation factor subunit beta (DFFB) (Uniprot KB/ Swiss Prot entry: O76075), Deoxyribonuclease I splicing isomer 2 (DNAse) (Uniprot KB/ Swiss Prot entry: Q14UU9). Aβ40 showed homology with NMP

sharing 30% identical residues (residues 14-46), ssDBP sharing 30% identical residues (residues 144-196), ZFP sharing 27% identical residues (residues 127-184), AIF sharing 22% identical residues (residues 79-122), PARP sharing 22% identical residues (residues 248-287), DFFB sharing 18% identical residues (residues 146-194) and DNAse sharing 18% identical residues (residues 188-247). The sequence alignment of Aβ40 with other DNA binding proteins is represented in Figure 3.13.

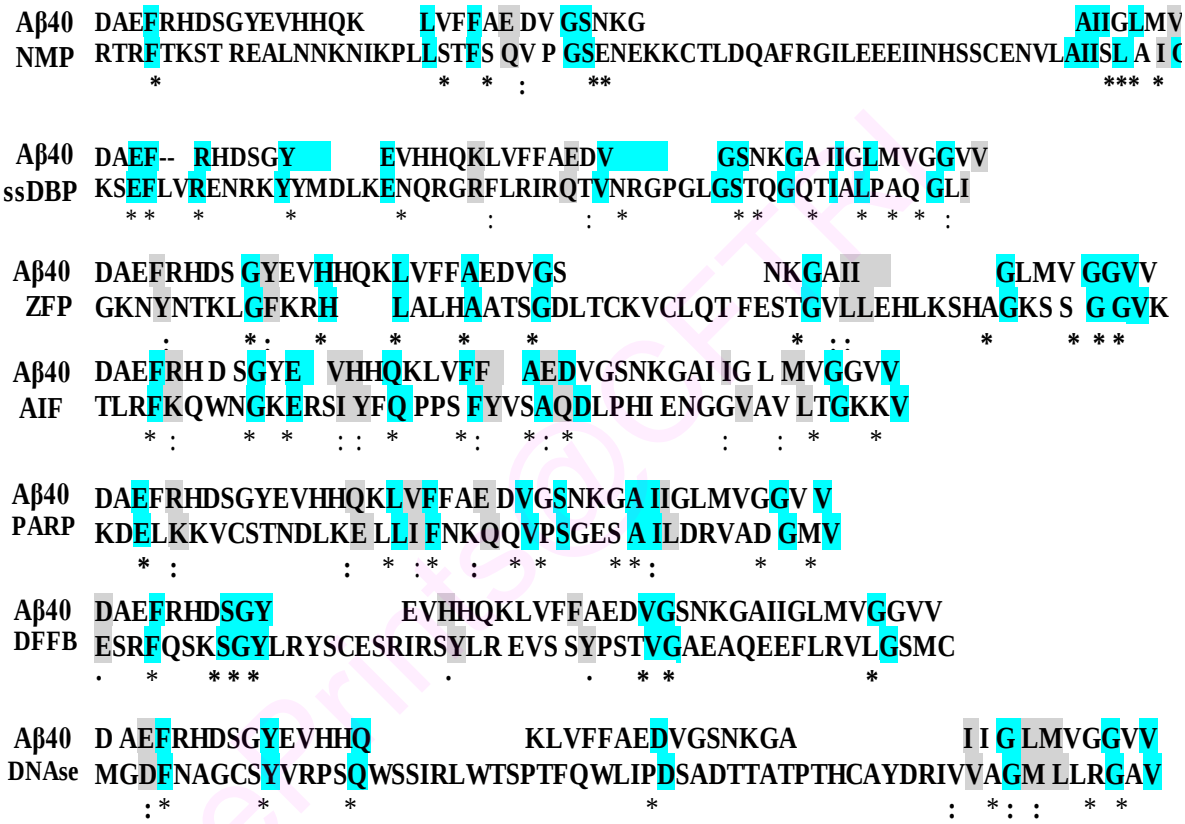


Figure 3.13: Sequence homology of Aβ40 with other DNA binding proteins: Sequence alignment of human Aβ40 with Nuclear matrix protein p84 (NMP), Purine rich single stranded DNA-binding protein alpha (ssDBP), Zinc finger protein PLAG1 (ZFP), Apoptosis inducing factor (AIF), Poly [ADP-ribose] polymerase 1 (PARP), DNA fragmentation factor subunit beta (DFFB), Deoxyribonuclease I splicing isomer 2 (DNAse). Identical residues are shown by '*' and similar residues by ':'.

3.3.8. Amyloid beta enantiomers alter the stability of DNA

Both the enantiomers of Aβ40 (15×10^{-6} M) resulted in differential pattern of EtBr binding to ScDNA. The amount of EtBr molecules bound per base pair (bp) of DNA was

represented in Table 3.3. Scatchard plots of 'r vs r/Cf' for EtBr complexed with ScDNA alone and with ScDNA- Aβ40L and Aβ40D were plotted in Figure 3.14. The results showed that EtBr bound per bp to scDNA was 22.2% less in presence of Aβ40L compared to scDNA alone and this may be due to ψ DNA conformation. But EtBr bound per bp to scDNA was 33.3% less in presence of Aβ40D, which may be due to modified B-form of DNA.

Sample	EtBr (r/bp)	% change
ScDNA alone	0.018	
ScDNA +Aβ(1-40) with all-L aminoacids	0.012	33.3
ScDNA +Aβ(1-40) with all-D aminoacids	0.014	22.2

Table 3.3: EtBr Binding assay of DNA and DNA-Aβ complexes: Bound EtBr per base pair (bp) of ScDNA were calculated using Scatchard plots (Scatchard, 1949; Chatterjee and Rao, 1994). Values are as mean of number of EtBr molecules bound per bp DNA ± SD.

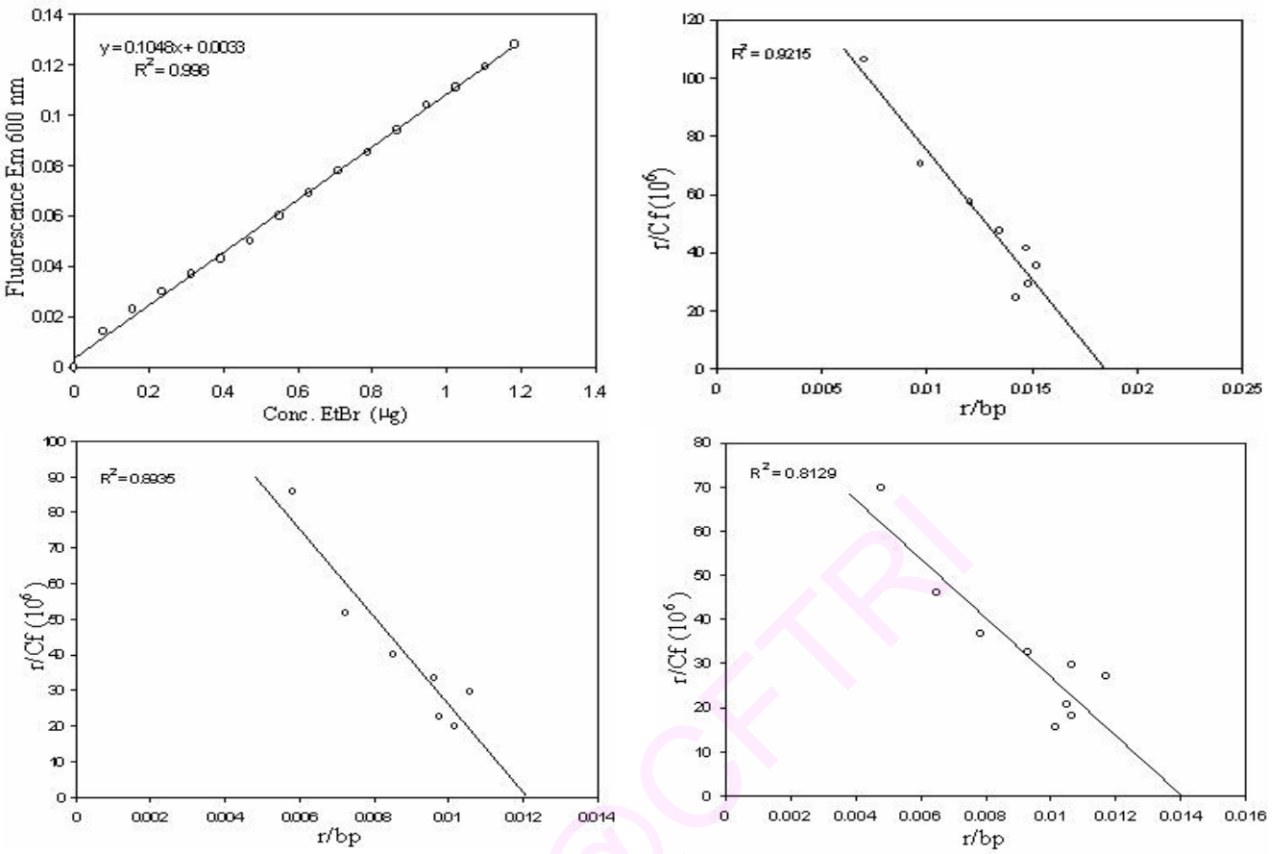


Figure 3.14: **Scatchard plots of EtBr binding to ScDNA/ ScDNA-Aβ complexes:** A) Fluorescence Emission for a range of EtBr concentrations (0-1.2 µg) B) ScDNA alone (1µg) C) ScDNA+ 15µM Aβ40 L D) ScDNA+ 15µM Aβ40 D.

Thermal denaturation studies were used to understand stability of DNA in the presence of Aβ. Figure 3.15A and 3.15B shows the Tm and hyperchromicity graphs plotted for the ScDNA alone and ScDNA complex with Aβ40L and Aβ40D. The data showed that the Tm of the Sc DNA significantly decreased upon interaction with Aβ40L and Aβ40D. The Tm graph of ScDNA showed biphasic pattern indicating transition at 54^0C and 88^0C. But in presence of Aβ40L and Aβ40D, it became monophasic with Tm of 64^0C and 62^0C respectively (Table 3.4).

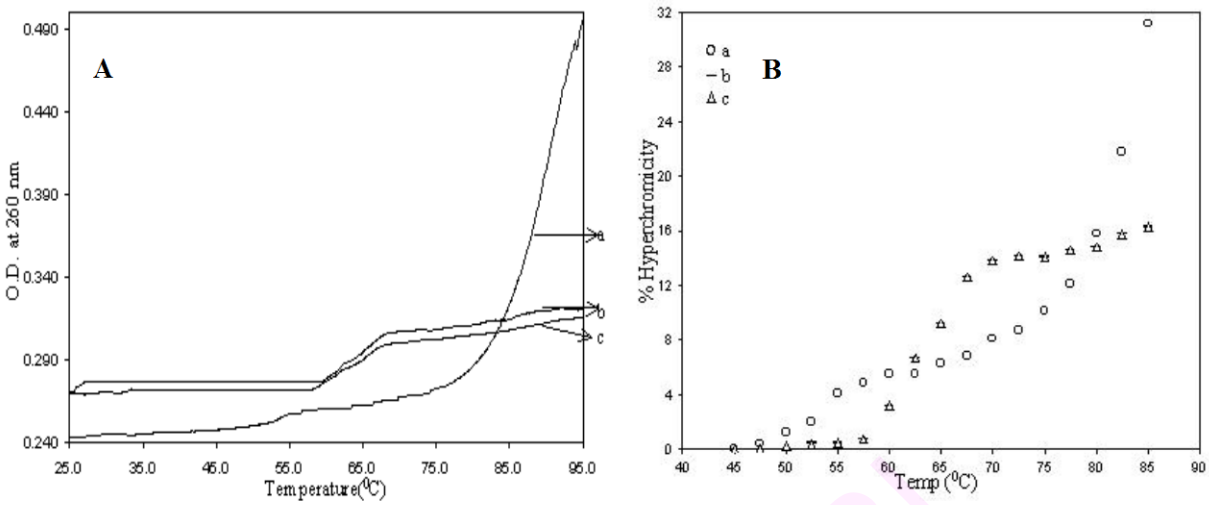


Figure 3.15: A) DNA melting temperature profiles of ScDNA -Aβ complexes in 0.01M HEPES (pH 7.4). B) Percent hyperchromicity vs Temperature plots; All the values are mean ± SD. a) Sc DNA alone b) Sc DNA+ Aβ40L c) ScDNA + Aβ40D

Sample	Tm (% hyperchromicity)
ScDNA alone	Biphasic Tm; 54^0C, 88^0C
ScDNA +Aβ(1-40) with all-L aminoacids	64^0C
ScDNA +Aβ(1-40) with all-D aminoacids	62^0C

Table 3.4: Melting temperature (Tm) of ScDNA alone and ScDNA–Aβ complexes: The Tm was calculated as the point of 50% hyperchromic shift. Values are expressed as average of triplicates.

3.4. Discussion

Accumulating evidence describes the deposition of amyloid beta peptides (Aβ) as the major feature in the neuropathology of Alzheimer's disease (AD) (Govaerts et al., 2007). Although the precise mechanism of Aβ pathogenesis in AD is not clear, conformational transition in its native state leading to its fibrillation has been suggested to be a critical event in neuronal damage (Simmons et al., 1994). Studies have shown that conformationally altered Aβ is neurotoxic (Giovannelli et al., 1998). Aβ under physiological conditions can undergo fibrillation to form fibrils consisting of antiparallel

β- pleated sheets (Simmons et al., 1994). The conformational modulation of Aβ peptides under the pathological conditions of AD results in the formation of various partial/ mis-folded species, which in turn leads to Aβ oligomers and aggregates (Finder and Glockshuber, 2007). The exact pathological mechanisms of these different forms of Aβ are not clear to date. However, studies have shown that soluble oligomers of Aβ are active species of the peptide that ultimately cause the synaptic loss and dementia associated with AD (Hardy, 1992). The role of plaque associated Aβ became unclear with the postmortem studies in human brain where it was shown that soluble Aβ correlates better than insoluble Aβ or extracellular Aβ plaques with cognitive dysfunction in AD patients (Lue et al., 1999).

Further, studies have shown that intracellular Aβ activates biochemical cascades like caspase activation, mitochondrial membrane depolarization, dysregulation of the cytosolic, mitochondrial and ER calcium homeostasis (Ginsberg et al., 1999; Atwood et al., 1998; Atwood et al., 2000; Huang et al., 1999). Apoptosis may be one of the mechanisms by which neurons die in neurodegenerative disorders (Anderson et al., 1995). There is no clear understanding regarding the inducers of apoptotic events in neurodegenerative disorders (Gupta et al., 2006). Studies have shown that Aβ peptides could exert apoptotic effects on neurons (Anderson et al., 1995; Forloni et al., 1996). However, the results presented here could raise a possibility of Aβ involvement in AD pathogenesis through a non-apoptotic pathway. DNA damage has been considered as one of the major events in the pathology of AD (Lyras et al., 1997). There are few reports on DNA topology getting altered in AD (Crapper et al., 1979; Anitha et al., 2002). Previously, Crapper et al., (1979) had reported changes in the integrity of chromatin structure in AD. Both the DNA integrity and topology have been proposed to have a crucial role in DNA function and any alteration makes it susceptible to damage. Further, the studies have shown the presence of Z-DNA in hippocampal region of AD post-mortem brain (Anitha et al., 2002) while normal brain has B-DNA. There are number of biological implications of Z-DNA conformation in AD brain cells. Since Z-DNA is a condensed DNA with left handed conformation and has only a minor groove, it has altered ligand-binding ability (Herbert and Rich, 1999). Further, Z-DNA formation excludes nucleosome assembly thus affecting the function of chromatin (Anitha et al.,

2002). Hence, the presence of altered DNA topology in AD could hypothesize to have implication in AD in terms of distorted nucleosome formation, terminal differentiation, altered ligand binding ability and DNA damage (Anitha et al., 2002). Anderson et al., (1995) also reported neuronal damage in terms of DNA fragmentation/ damage in neurodegenerative disorders. It is beyond any doubt now that cell death in AD is not caused by a single entity but by combination of many complex biochemical events (Gupta et al., 2006). It is also shown that DNA in AD undergoes greater damage than proteins and lipids (Lyras et al., 1997). The relationship between altered DNA topology and neurodegeneration in AD is not yet established but the DNA fragmentation observed in AD might be preceded by crucial step of transition from normal B-DNA to altered DNA topology (Anitha et al., 2002). There appears to be a definite link between DNA damage and DNA topology and Aβ in AD because Aβ is a strong candidate for modulating DNA topology. Further, it is known that AD specific proteins like Aβ are rich in lysine and Takeuchi et al., (1994) has shown lysine rich proteins favor the B→Z transition. It helped in further speculating the role of Aβ in inducing altered DNA topology. Hegde et al., (2004) evidenced the nuclear localization of Aβ immunoreactivity in the apoptotic nuclei in the vicinity of DNA/chromatin of the hippocampal neurons in human AD brain but not in age matched control brain. Grant et al., (2000) studied the immunoreactive signals in a number of subcellular organelles including nucleus using the monoclonal antibody highly specific to an epitope in the human Aβ sequence. The Aβ immunoreactivity is observed in trans-Golgi network (TGN) and Golgi-related endosomal vesicles but no Aβ immunoreactivity was observed in lysosomes. Aβ immunoreactive deposits are frequently found to be associated with the outer mitochondrial membrane and microtubules. Aβ immunoreactivity is also observed in the nucleus of neuroectodermally differentiated pAD 28 cells. The multiple Aβ immunoreactive deposits are found over the nuclear envelope. The deposits are also reported to be visualized in amongst the nuclear pores (Grant et al., 2000). Hence, it is evocative to understand the significance of nuclear localization of Aβ immunoreactive material. Moreover, the nuclear localization of Aβ might play a role in bringing about changes in DNA topology.

Further, i*n vitro* studies have shown that Aβ(1-42) and Aβ(1-16) fragments effectively bind to DNA and alter the conformation of DNA. Aβ(1-42) induced a B→Ψ DNA conformation, while Aβ(1-16) caused an altered B-DNA conformation (Hegde et al., 2004). It is interesting to correlate the Aβ induced conformational change in DNA from B→Ψ to B→Z observed in severe AD hippocampus (Anitha et al., 2002). The Ψ– DNA conformation induced by Aβ(1-42) is observed to be similar in conformation to Z-DNA. Thomas and Thomas, (1989) showed that Ψ– DNA is an ordered, twisted, tight packing arrangement of the double helix and also immunologically related to Z- DNA family. Ψ-DNA is also left-handed in conformation like Z-DNA. It provides an insight confirmed that Aβ induces condensation of DNA and hence has biological significance. Recently, Suram et al., (2007) provided intriguing information on the Aβ(1-42) induced DNA nicking property. Aβ(1-42) DNA nicking property was shown to be differentially modulated by metal ions Mg^{2+} and Ca^{2+}. Both Mg^{2+} and Ca^{2+} were reported to be required for the DNA nicking property whereas, Zn^{2+} exhibited no effect on Aβ(1-42) induced DNA nicking activity.

In the present study, we have explored the mechanism of Aβ-DNA binding by employing all-L–and all-D-aminoacid enantiomers of Aβ (1-40). The reason behind using complete enantiomers as model was to understand the effect of aminoacid stereospecificity on Aβ-DNA nicking. We used Aβ40 in the present study as there is a disease specific increase in Aβ40 brain tissue levels in AD. Aβ42 accumulation increases with age but Aβ40 levels remain consistently low. However, in AD, there is a large increase in both Aβ40 and Aβ42 levels in the brain. Hence, in contrast to Aβ42, Aβ40 deposits are AD specific.

Our results demonstrated that the two enantiomers of Aβ40 alter conformation of ScDNA differently. The ψ -DNA conformation induced by Aβ40L was observed to be similar in conformation to Z -DNA. The altered B-DNA resulted by the action of Aβ40D may also have its basic characteristics altered when compared to B-DNA. Our results also evidenced that D-enantiomer of Aβ40 was more potential in nicking ScDNA when compared to L-enantiomer of Aβ40. The differential DNA binding and nicking activity of DNA- Aβ complexes may be due to the stereoisomerism and chirality of the aminoacids

constituting Aβ. Further, since SSBs formed were more compared to DSBs, we assume that the accumulation of SSBs by Aβ was through the single strand nicks and the appearance of DSBs were formed as a result of cumulative single strand breaks in ScDNA. Moreover, fibrillar forms of Aβ exerted less DNA nicking activity when compared to soluble Aβ. Hence, there appeared to be a relationship between nicking activity and protein conformation, which was critical to spot out the mechanism of Aβ neurotoxicity.

Further, Aβ- DNA interactions *in vivo* can be influenced by Mg^{2+}, Ca^{2+} and Zn^{2+} as they are essential in various protein-DNA interactions. Mg^{2+} ions are known to occur inside the cell at a high concentration of upto 30 mM (Alberts et al., 1983). It is the most abundant divalent metal ion in mammalian cells, plays structural and catalytic roles in many cellular processes that affect genome stability (Hartwig, 2001). Mg^{2+} functions as a cofactor of proteins involved in DNA replication and repair pathways. It is required for activity and fidelity of DNA polymerases (Sirover and Loeb, 1977), nucleases (Barzilay et al., 1995; O'Donovan and Wood, 1993; Welsh et al., 1987). Ca^{2+} overload intracellularly is known to be a key factor underlying ischaemic neuronal necrosis, whereas, intracellular reduction of Ca^{2+} favors apoptosis (Choi, 1988). Zn-ribbons are present in a variety of nucleic-acid-binding proteins, including several ribosomal proteins, translation factors, aminoacyl-tRNA synthetases, RNA polymerase cofactors in archaea and eukaryotes, and several transcription factors (Nelson and Cox, 2000). Moreover, Zn^{2+} concentration released during neurotransmission is reported to be ~300 μM (Frederickson, 1989). Hence, the brain must have efficient homeostatic mechanisms to prevent the abnormal discompartmentalization of metal ions. Both Aβ40L and D showed enhanced DNA nicking activity in presence of Mg^{2+} and Ca^{2+} whereas Zn inhibited Aβ40L/D from nicking DNA. Kozin et al., (2001) reported that Ca^{2+} and Mg^{2+} did not cause any conformational changes in Aβ whereas Zn^{2+} was reported to induce conformational change from random coil to some regular secondary structure. These studies suggested that Zn^{2+} might be a key factor in AD plaque formation and/ or stabilization. Keeping this in view, we speculated that Zn^{2+} probably caused aggregation of soluble Aβ and hence the DNA nicking activity of Aβ is abolished. Cherny et al., (1999) showed that Mg^{2+} and Ca^{2+} might participate in the resolubilization of Aβ. Kuroda and

Kawahara, (1994) also reported that Ca^{2+} and Mg^{2+} did not promote aggregation of Aβ. Hence, metal ions (Ca^{2+} and Mg^{2+}) that solubilize the Aβ have enhanced the DNA nicking activity whereas the metal ion (Zn^{2+}) which promotes aggregation prevented the DNA nicking activity of Aβ.

The ability of known nuclease inhibitors like ATA and DEPC to abolish DNA nicking activity of Aβ peptides revealed mechanism of Aβ action in behaving like nucleases. ATA is a well-known compound capable of interacting with a broad variety of nucleic acid binding proteins. Gonzalez et al., (1980) have shown that the active components present in ATA preparations are phenol-formaldehyde type. ATA is a powerful inhibitor of proteins whose biological function depends on the formation of complex with nucleic acid (Gonzalez et al., 1980). The mechanism of action of ATA in the inhibition of protein-nucleic acid complex formation is by competition between nucleic acid and the polymeric ATA for binding in the active site. Further, ATA is also capable of interfering with electrostatic interactions, which are a contributory driving force in protein- nucleic acid complex formation (Gonzalez et al., 1980). DEPC derivitizes histidine residues to carboethoxy histidine and is therefore an effective method to inactivate nucleases including RNAse (Safarian et al., 2003). These inhibitors are known to derivatize Histidine residues in the active site of the proteins/enzymes resulting in abolishing the protein- nucleic acid interactions. Histidine is considered as one of the most common aminoacids *in vivo* and one of the strongest low molecular weight ligands for metal ions. Hence, treatment of Aβ-DNA complex with ATA and DEPC was a key experiment to understand the mechanism underlying the nicking of DNA by Aβ. The protection of Aβ induced DNA nicking in presence of both ATA and DEPC revealed the possibility of Histidine involvement in the nicking activity of Aβ peptides.

The DNA binding activity of Aβ implicated in the pathogenesis of AD cannot be ignored by just considering it as a non-specific phenomenon. DNA binding activity has been shown to be a prerequisite for apoptosis inducing factor (AIF) to exert its apoptogenic action which has been identified as a major player in caspase-independent cell death (Ye et al., 2002). Apart from the above similarity that brought Aβ in parallel to AIF, it has also been reported that in cells treated with an apoptotic stimulus, endogenous AIF becomes colocalized with DNA at an early stage of nuclear morphological changes.

This observation of colocalization of AIF with DNA is very much similar to the nuclear localization of Aβ in the apoptotic nuclei in the vicinity of DNA in the hippocampal neurons in human AD brain (Hegde et al., 2004). Hence, it appears that to execute some common function, these proteins are localized in the vicinity of DNA. Further, an earlier report by Mathura et al., (2005) showed the homology of Aβ42 with an RNA binding protein, AF-Sm1 from *Archaeoglobus fulgidus*, giving an insight about its interaction with RNA. Similarly, in our study, we looked for the similarity of Aβ with nucleases and DNA binding proteins to further validate our findings that Aβ behaves like a nuclease. When Protein Data- Bank (PDB) was used to search for the sequence neighbor of Aβ40 as the query, it revealed that it has homology with many DNA binding proteins such as Zinc finger binding protein, nuclear matrix protein, Apoptosis inducing factor etc. and nucleases such as DNAse.

The DNA binding could be a unifying phenomena associated with amyloid proteins implicated in various neurodegenerative disorders as recent studies revealed that α-synuclein and prion proteins implicated in PD and Prion disease, respectively, have also been shown to have DNA binding property (Nandi et al., 2002; Hegde et al., 2003; Cherny et al., 2004, Hegde et al., 2007). Hegde et al., (2003) had earlier shown that α-synuclein binds DNA and induces B to A-DNA transition. Further, Hegde et al., (2007) characterized the effect of DNA binding on the conformation and fibrillation kinetics of α-synuclein. ScDNA was shown to induce partially folded conformation in α-synuclein and promoted the fibrillation while ssc DNA induced α-helix and delayed the fibrillation, indicating that the partially folded intermediate conformation is critical in the aggregation process. Prion proteins are also known to interact with nucleic acids and this interaction has been speculated to play a role in the prion diseases (Nandi et al., 1997). They demonstrated nucleic acid induced polymerization of the normal and scrapie prion isoforms accompanying a change in the nucleic acid conformation, resulting in condensed globular nucleic acid structures (Nandi et al., 1998; Nandi and Sizaret, 2001). They also reported the unfolding of prion protein followed by a nucleation-dependent polymerisation of prion protein to amyloid. These spherical amyloid structures can later on act as probable constituents of the coat of the spherical particles found in vivo (Nandi

et al., 2002; Nandi and Nicole, 2004). Recently, Bera et al., (2007) evidenced DNA binding and unwinding by prion protein, therefore establishing the crucial role of prion protein and nucleic acids interaction in prion diseases.

Hence, based on our present results and several reports establishing nuclear localization of Aβ immunoreactivity as well as unifying feature of amyloid proteins binding to DNA, we hypothesize that Aβ may induce toxicity through an independent non-apoptotic mechanism in addition to other well known mechanisms. Hence, it can be hypothesized that ScDNA component of chromatin may have a significant effect on nuclear– translocated Aβ functioning. Another probability is that Aβ may interact with histone-free, transcriptionally active DNA segments and thereby lead to a decreased transcriptional activity of some genes responding to environmental stimuli.

CHAPTER 4

Anti-amyloidogenic activity of Garlic, SAC &, Curcumin derivative on Aβ(1-40)

4.1. Introduction

AD is one of the most common forms of senile dementia and Aβ deposition is a hallmark feature of its pathogenesis (Masters et al., 1985). Aβ, a self-aggregating peptide, is derived by proteolytic cleavage of Amyloid Precursor Protein (APP). Differential activity between three different secretase, α, β and γ at their specific cleavage sites yields a number of different products, including Aβ(1–40) and Aβ(1–42) (Sisodia, 1992). While Aβ (1–40) is the predominant product of this proteolytic pathway, Aβ(1–42) is more fibrillogenic in vitro and is the major Aβ species present in the core of SPs (both AD and non-AD related) (Burdick et al., 1992; Jarrett and Lansbury, 1993). Amyloid beta (Aβ) - protein toxicity is considered to be one of the underlying causes of Alzheimer's disease (AD) pathogenesis (Martin, 1999). The aggregation of Aβ is thought to be a critical step in the pathogenesis of AD. The disease modifying treatment of AD involves reduction in Aβ aggregation and toxicity. Under i*n vitro* conditions, Aβ readily assembles into fibrils, with morphologies and staining characteristics similar to those of fibrils extracted from AD affected brain tissue. Aβ fibrillization involves formation of dimers, small oligomers, protofibrils and fibrils via a complex multistep-nucleated polymerization. The critical primary stage is the formation of a *partially folded intermediate and* it has been suggested that these oligomers, precursors of fibrils of Aβ might be more toxic than the fibril itself.

Therefore, many therapeutic efforts are targeted at reducing Aβ production, increasing Aβ clearance and inhibiting Aβ aggregation. The self-association of synthetic Aβ monomer to fibrils *in vitro* has provided an assay to identify compounds that inhibit fibril formation. Compounds that hinder the aggregation of Aβ into multimeric species (oligomers, protofibrils, and fibrils) are most effective as they stop the protein polymerization prior to the formation of the primary cytotoxic moieties. For example, melatonin has been reported to interact with Aβ, inhibiting fibrillogenesis and neurotoxicity (Pappolla et al., 1998). Monoclonal antibodies raised against the N-terminal region of Aβ have been shown to cause fibril disaggregation and inhibition of neurotoxicity (Solomon et al., 1996). Beta-sheet breaker peptides inhibit amyloid aggregation and prevent neurotoxicity (Soto et al., 1996). A number of antioxidants have

been reported to inhibit the formation and extension of Aβ fibrils, as well as to destabilize preformed fibrillar Aβ in vitro (Ono et al., 2006). Guided with this precept, a search for the natural inhibitors of fibrillogenesis such as, bioavailable components of garlic and curcumin derivatives could be realistic to clinical practice.

Garlic, *Allium sativum*, has been consumed as a spice and also as a medicine for thousands of years all over the world. Garlic (Allium sativum), a flavor-enhancing vegetable, has long been used as a health supplement and herbal medicine since ancient times (Block, 1985; Rivlin, 2001). It was Cavallito et al., (1944), who first isolated and characterized *allicin*, the antibacterial principle of *Allium sativum*. Many other beneficial effects of Garlic have come into picture such as antineoplastic, anti-cardiovascular diseases, immuno-stimulatory, hypoglycemic, anti- ageing properties.

Garlic extract helps to prevent cognitive decline by protecting neurons from Aβ induced oxidative stress and apoptosis, thereby preventing ischemia- or reperfusion related neuronal death (Borek, 2006). Garlic extract and its components inhibit the oxidative damage that is implicated in neurodegenerative disorders and aging. Garlic compounds are shown to reduce Aβ induced neuronal apoptosis, possibly by enhancing the endogenous antioxidant defenses (Peng et al., 2002). Jackson et al., (2002) reported the dose dependent inhibition of caspase-3 activity by aged GE. GE has been shown to have multiple biological activities, including an antiaging effect (Nishiyama et al., 1997) improvement of learning and memory impairment (Moriguchi et al., 1996), neurotrophic effects (Moriguchi et al., 1997) and antioxidant activity (Yamasaki et al., 1997) and these activities increase with the age of the extract. Scientists from around the world have identified a number of bioactive substances in garlic that are water soluble e.g. S-allyl-L-cysteine, S-allyl methylcysteine (SAC, SAMC), and fat soluble e.g. diallyldisulfide (DADS) (Imai et al., 1994). The primary sulfur containing constituents in whole intact garlic are the γ-glutamyl-S-alkyl-L-cysteines and S-alkyl-L-cysteine sulfoxides, including alliin. S-(2-Carboxypropyl) glutathione, γ-glutamyl-S-allyl-L-cysteine, γ-glutamyl-S-(trans-1-propenyl)-L-cysteine and γ-glutamyl-S-allyl-mercapto-L-cysteine are also present in garlic cloves (Fenwick and Hanley, 1985). In particular, SAC, an organosulfur compound purified from aged GE, protected neurons against Tunicamycin and Aβ induced neurotoxicity region specifically (Kosuge et al., 2006). SAC is an organosulfur

compound that is a natural constituent of garlic. It is a derivative of the amino acid cysteine in which an allyl group has been added to the sulfur atom. It is a water-soluble compound yet is presumably sufficiently hydrophobic to cross the blood brain barrier and gain access to the CNS. Further, ischemic neuronal damage has also been shown to be inhibited by SAC in rat brain (Numagami and Ohnishi, 2001). SAC also has been shown to have antioxidant and radical scavenging effects (Imai et al., 1994), anti-cancer activity (Thomson and Ali, 2003) and cholesterol-lowering activity (Yeh and Liu, 2001). The selection of SAC as a test compound was based on the reports showing its protective effect against Aβ induced neurotoxicity (Chauhan, 2006; Chauhan et al., 2007 and Imai et al., 2007; Ishige et al., 2007; Ito et al., 2003; Kosuge et al, 2003; Peng et al., 2002). Chauhan et al., (2006) showed aged GE and its constituents reduce cerebral plaques and also reduce both soluble and fibrillar Aβ species with increased α-cleaved sAPPα, reduce inflammation and reduce conformational change in tau. However, there are no studies carried out to show the effect of Garlic and its constituents on modulation of Aβ aggregation.

In the present study, we used an *in vitro* model of Aβ fibrillization to show that SAC could bind Aβ and inhibit its aggregation as well as disaggregation of preformed fibrils. We also studied the protective effect of aqueous fresh Garlic extract (FGE) and boiled garlic extract (BGE) against Aβ aggregation. We have chosen aqueous extracts of garlic to study its interaction with Aβ as organic solvents affect the conformational state of Aβ (Wei et al., 2006). The results presented here indicated that garlic and its constituents can be used to design inhibitors of Aβ toxicity and deposition in AD.

Further, micro-environmental changes in the brain, such as pH, metal ion (Cu, Fe and Zn) availability and oxidants, likely impact upon Aβ structural conformation and its deposition in amyloid plaques (Atwood et al., 1998; Smith et al., 1997). There is a severe dysregulation of metal ion homeostasis in AD (Lovell et al., 1998; Gonzales et al., 1999). Evidence suggests that metals such as copper, zinc, or iron play a role in AD. Injury and impairment of energy metabolism in AD may also release metals from intracellular stores, contributing to an increase in free metal concentration in AD (Atwood et al., 1998). Copper was also found to be elevated in serum of AD patients with an Apo E4 allele (Gonzales et al., 1999). Increased concentrations of these metals are detected in the

neuropil of the AD- affected brain and in vitro at physiological concentrations they can bind to Aβ and induce its aggregation or its creation of reactive oxygen species (Atwood et al., 1998; Atwood et al., 2000; Dyrks et al., 1992; Hartter et al., 1988; Huang et al., 2000; Lovell et al., 1998; Samudralwar et al., 1995). In particular, Cu is present at a concentration of ~1mM in amyloid plaque deposits in AD (Lovell et al., 1998). Bush et al., (1994) evidenced direct interaction between Aβ(1-40) and Cu^{2+} by the stabilization of an apparent Aβ(1-40) dimer by Cu^{2+}on gel chromatography. Further, Cherny et al., (1999) demonstrated that Cu^{2+} and Zn^{2+} specific chelators enhance the solubilization of Aβ collections in postmortem brain specimens from AD subjects.

Substantial efforts have been made to discover chelators that selectively bind these metals and inhibit Aβ aggregate and fibril formation which could be used to treat or prevent amyloidoses (Cherny et al., 2001). Clioquinol is a known chelator which can prevent Aβ aggregation by binding the copper and zinc on Aβ (Cherny et al., 2001). Desferrioxamine is another chelator, binding copper, zinc, iron and aluminium ions, and it has slowed AD progression in a human clinical trial (Crapper et al., 1991). On the contrary, search for a dietary metal chelator could be realistic to clinical practice. The natural compound curcumin acts through a range of activities and represents a hopeful approach for preventing or delaying the progression of AD (Cole et al., 2004). The curcumin is yellow in color and used as spice and as a coloring agent in Indian food. Curcumin has a history in India for its use in Indian traditional medicine; ayurveda (Aggarwal et al., 2007). Curcumin is a polyphenolic diketone from turmeric, known to exhibit anti-protozoal, anti-bacterial, anti-inflammatory, and anti-oxidant activity. In rural India, where turmeric is commonly used in food, such as curry, AD prevalence is only 1% of those over age 65 (Chandra et al., 1998). Curcumin is a yellow pigment extracted from the rhizome of the plant *Curcuma longa* (Bala et al. 2006) and in vitro studies have shown that curcumin attenuates inflammatory response of brain microglial cells (Kim et al. 2003; Jung et al. 2006). In Alzheimer's transgenic model Tg2576, diet-containing curcumin significantly reduced levels of brain amyloid, plaques (Frautschy et al., 2001; Lim et al., 2001). Curcumin was tested in these animal models primarily because of its known anti-inflammatory effect because inflammation and oxidation are known to contribute to AD (Cummings and Cole, 2002). Curcumin also inhibits the formation of

Aβ oligomers and fibrils in vitro (Ono et al. 2004; Yang et al. 2005). Other studies have shown that curcumin prevents neuronal damage (Shukla et al. 2003), and reduces both oxidative damage (Lim et al. 2001) and amyloid accumulation (Yang et al. 2005) in a transgenic mouse model of AD. Clinical trials with curcumin have shown that the compound is not only safe but may be a chemoprotective (Cheng et al. 2001) and anti-inflammatory (Holt et al. 2005) drug. Garcia-Alloza et al., (2007) showed that curcumin crosses the blood brain barrier (BBB) using APPSwe/PS1dE9 transgenic mice. Further, curcumin was also shown to bind senile plaques (SPs) as observed through multiphoton microscopy. Treatment of transgenic mice with curcumin for 7 days dissolved the SPs and reduced the existing plaques which was monitored through longitudinal imaging. Garcia –Alloza *et al* (2007) concluded that curcumin reverses the amyloid pathology and associated neurotoxicity in AD. But the mechanism of binding of curcumin to Aβ is still not known. Curcumin ,CG and Congo red share common structures, that is they contain substituted aromatic groups separated by a rigid ;planar backbone called linker. There are reports linking the curcumin like ligands and inhibition of Aβ aggregation (Necula et al., 2007; Riviere et al., 2007; Lee et al., 2005; Porat, 2006).

However, one alternative possible mechanism by which curcumin may exert its action is metal chelation, which may reduce metal-induced Aβ aggregation (Atwood et al., 1998; Atwood et al., 2000; Cherny et al., 2001; Cherny et al., 1999; Crapper et al., 1991; Yoshiike et al., 2001). Studies suggest that bioavailable, curcumin, a natural compound can bind metal ions such as copper and iron (Araujo and Leon, 2001; Began et al., 1998; Began et al., 1999; Patro et al., 2002; Sreejayan and Rao, 1994). The only disadvantage with curcumin is that it is soluble in organic solvents. Barrow et al., (1992) reported that hexafluoroisopropanol, trifluoroethanol and ethanol are known to affect the conformational change in Aβ. The mechanism of organic solvent induced folding differs for different solvents. This led us to use the modified form of curcumin in our *in vitro* studies, viz., CG, wholly soluble in water. CG dissociates by enzymatic degradation into curcumin and mono, di-glucosides, thereby curcumin readily available for experimental purpose.

In a quest to understand whether CG can inhibit Cu induced Aβ40 aggregation, we examined the effects of CG to inhibit the oligomer formation, as well as to destabilize fibrillar Aβ40.

We used Aβ40 in the present study as there is a disease specific increase in Aβ40 brain tissue levels in AD. Aβ42 accumulation increases with age but Aβ40 levels remain consistently low. However, in AD, there is a large increase in both Aβ40 and Aβ42 levels in the brain. Hence, in contrast to Aβ42, Aβ40 deposits are AD specific. It is an important though overlooked pathological change compared with the well-documented Aβ42 change observed both in the aged and in AD (Gregory and Halliday, 2005). Further, it is know that Aβ40 is normally rapidly cleared from the brain in non-AD cases. This suggests that a substantial increase in Aβ40 in brain tissue in AD may itself be detrimental to normal neuronal function (Gregory and Halliday, 2005). Moreover, there is a preferential deposition of Aβ40 over Aβ42 in senile plaques in presence of ApoE4 allele, a risk factor associated with AD (Mann et al., 1997).

4.2. Materials and Methods

4.2.1. Materials

Aβ (1-40) was purchased from rPeptides, USA and purity of peptide is supported by mass spectral data. Tris, Glycine and HEPES buffers were purchased from Bangalore Genei, India. $CuCl_2$, Copper grids (300 mesh size) for TEM, Low molecular weight protein markers and Thio-T were purchased from Sigma chemical company, USA. Uranyl acetate was purchased from B.D.H. laboratory chemicals division. SAC was procured from Fluka/ Sigma-Aldrich, Bangalore, India. Healthy dry bulbs of Garlic (Allium sativum L.) were purchased from local market. Curcumin glucoside was generously provided by P. Sreenivas (CFTRI, India).All other chemicals NaCl, EDTA, KCl, $MgCl_2$, polyvinylpyrrolidone, ascorbic acid were of analytical grade and were purchased from Sisco Research Labs, Mumbai, India.

4.2.2. Preparation of aqueous FGE and BGE

The aqueous FGE was prepared by homogenizing freshly peeled garlic in liquid nitrogen. Ten-millimolar phosphate buffer (pH 7.8) containing 1% polyvinylpyrrolidone,

1% ascorbic acid, 1mM potassium chloride, 10 mM $MgCl_2$ and 50 mM EDTA was added in the ratio of 1/3 (gram fresh weight/ ml of extraction buffer). After centrifugation at 12,000 g for 20 min at 4^0C, the supernatant liquid was collected (aqueous extract) and immediately used for the following studies. BGE was obtained by subjecting FGE to boiling at 100^0 C for 5 minutes. The final concentration of GE was made upto 100µg/ml.

4.2.3. Assay of Protease activities in FGE and BGE

Proteinase assay using casein as substrate was performed according to the method of Bergmeyer (1984). FGE and BGE were diluted with double- distilled water to about 0.25 mg protein per ml as enzyme sources. Substrate solution was prepared by mixing 2.5g casein in 10 ml H_2O, then add 15 ml 0.1N NaOH and dissolve completely. Add 50 ml of 0.05 M Tris-Cl buffer and adjust pH to 7.4. The volume is made upto 100 ml. Substrate solution must be used on the day it is prepared. The enzyme reaction was started by adding 500µl of aqueous GE to a mixture of buffer and substrate solution (1ml each), incubated for 10 min, followed by the addition of 5% TCA as precipitating agent and was then incubated at 37^0C for 30 min. Absorbance was taken at 280 nm after centrifugation. Absorbance at zero time was used as the blank value for each corresponding assay.

4.2.4. Inhibition of aggregation

Lyophilized Aβ40 peptide at 200µM concentration was dissolved in triple distilled water in a 4^0C room and then stored at -80^0C. SAC stock was prepared at a concentration of 1mM in 0.05 M Tris-Cl, pH 7.4. Aβ40 was allowed to fibrillize in the presence and absence of both FGE and BGE (1:1, 1:5 w/w ratios) in Tris –Cl, pH 7.4 for 6 days without stirring at 37^0C. Aβ40 at a final concentration of 50µM was also mixed with 0.1, 1, 10 and 50 µM concentrations of SAC and incubated under the same conditions as above.

Aβ (1-40) in H_2O at 30µM concentration was mixed with 30µM Cu^{2+}. The reaction mixture was incubated at a 1:1 volume ratio with CG in Eppendorf tubes in Tris –Cl, for 6 days without stirring at pH 7.4, 37^0C. Final concentrations of curcumin glucoside in the reaction mixtures were 0, 3, 5, 10, 20, 30 µM.

4.2.5. Disaggregation of preformed fibrils

Aβ40 (50μm) was incubated without stirring for 3 days at 37^0C to generate fibrils. Preformed fibrils were mixed with the FGE and BGE in the same ratios as above for additional 3 days at 37^0C. Preformed fibrils were also mixed with the same concentrations of SAC as above for additional 3 days at 37^0C.

Aβ40 at 30μM with 30μM Cu^{2+} was incubated without stirring for 3 days at 37^0C to generate fibrils. Preformed fibrils were mixed with the CG in the same ratios as above for additional 3 days at 37^0C.

4.2.6. Thioflavin-T assay for monitoring Aβ40 aggregation

The degree of Aβ aggregation was determined using the fluorescent dye Thio-T (LeVine, 1993). A 100 μM aqueous solution of Thio-T were prepared and filtered through 0.2-μm polyether sulfone filter. Aliquots of 25 μl were drawn from each incubated sample at interval of every 12 hrs for 6 days and treated with 100 μM Thio-T and adjusted to a final volume of 1000 μl with 50 mM Glycine NaOH buffer, pH 8.5. Each test sample was shaken for 10 sec prior to each measurement. Measurements were carried out at an excitation wavelength of 446 nm and an emission of 482 nm, wavelengths that result in the optimum detection of bound Thio-T. The relative degree of Aβ aggregation was assessed in terms of fluorescence intensity, which was measured at 37^0C using a model F4500 Hitachi fluorescence spectrophotometer. To account for background fluorescence, the fluorescence intensity measured from each control solution without Aβ was subtracted from that of each solution containing Aβ.

Along with the experimental measurement of Aβ aggregation, we also employed nucleation based polymerization model to analyze the aggregation pattern in presence of Cu^{2+} and CG with a sigmoidal time response curve. The resultant sigmoidal response curve could be well represented by a simple stretched exponential decay function as follows:

$F(t)= F(\infty)+ \Delta F \exp\{-[kt]^n\}$ ……Eq (1)

where t is the duration time of aggregation, F(t) the observed fluorescence intensity at time t, F(∞) the final Thio-T fluorescence intensity, ΔF the Thio-T fluorescence difference between time t=0 and ∞, and k is the rate constant of fibril formation.

4.2.7. Fluorescence quenching studies

For fluorescence quenching measurements, Aβ was dissolved at a concentration of 100μg/ml. Fluorescence quenching of Aβ by SAC was followed at 25^0C. All the samples were centrifuged at 26,000 g for 30 min to remove any aggregates. 0.5mM stock solution of SAC was added in aliquots of 0, 5, 10, 15, 25, 35, 45, 60, 75, 95, 115, 140, 165 μl to 23 μM Aβ40 in 0.05 μM Tris-Cl, pH 7.4. The excitation wavelength was set at 280 nm and emission was scanned in the range of 300 nm to 500 nm. The % of fluorescence quenching was plotted against [SAC] to determine maximum quenching.

The value of n, stoichiometry of the SAC-Aβ complex, has been estimated by plotting $[SAC]_b/([SAC]_f.[A\beta]$ vs. $[SAC]_b/[A\beta]$, where $[SAC]_b$ and $[SAC]_f$ is the molar concentration of bound and free SAC respectively. $[SAC]_b$, is given by the equation $(\Delta F/\Delta F_{max}).[A\beta]$, where ΔF is the corrected percentage quenching, ΔF_{max}, the maximal quenching, and [Aβ] is the concentration of Aβ used in fluorescence quenching measurements. K_α, binding constant is obtained by mass action plot of β/(1-β) vs. $[SAC]_f$ and is given by the equation, $K_\alpha=\beta/(1-\beta).1/[SAC]_f$, where $\beta=\Delta F/\Delta F_{max}$.

4.2.8. Transmission Electron Microscopy analysis of fibril formation

This study was implemented to determine whether the test compounds were capable of causing inhibition Aβ aggregation and disassembly/disruption of preformed Aβ fibrils. The carbon- coated copper grids (300 mesh size) were glow discharged for 1-2 min (~150 millitorr: discharge current, 2-3mA), dipped in absolute alcohol for 1 sec and were air -dried. A drop (5μl) of each incubated sample was placed on the grid and was allowed to dry in air for 30 min. A second drop was applied after blotting the first drop with filter paper. The sample was then wicked off with lens paper, washed with distilled water and then negative stained by transferring the grid face down to a droplet of 2% (w/v) uranyl acetate for 5-10 min before wicking off the solution and air drying. Uranyl acetate solutions were filtered through a 0.2 μM sterile syringe filter before use. Four individual experiments were carried out for each sample. The grids were completely dried so as to avoid moisture and then observed under JOEL 1010 EM examination.

4.2.9. Circular Dichroism

Far-UV (190-260 nm) CD spectra of incubated samples were recorded in 0.01M Tris-Cl buffer (pH 7.4) at 25°C on a JASCO-J 700 spectropolarimeter. Final Aβ

concentration was 30 μM. The quartz cuvette was of 1 mm slit width and 1 mm path length. Each spectrum was the average of four repetitions. The CD contributions from SAC and CG were subtracted in the SAC-Aβ and Aβ-Cu^{2+}-CG complex spectra respectively. The deconvolution of the CD spectra at various time intervals is carried out using j-fit software which indicates the % of the secondary structural content.

4.2.10. SDS-Polyacrylamide Gel Electrophoresis

To gain an idea of the mass of the species that exist when Aβ (50μM) is incubated along with GE (1:5 w/w) and SAC (50μM) and Aβ (30μM) with 30μM Cu^{2+} is incubated along with CG 30μM. We carried out SDS-PAGE of the incubated samples (Laemmli, 1970). 15 μl of pre-incubated samples were mixed with 15μl of SDS-PAGE sample buffer and loaded on 12% SDS- polyacrylamide gel without boiling. Low molecular weight markers were used as molecular weight standards. The samples were electrophoresced at 100V at room temperature for 4 hrs. The gels were stained for protein with 0.1% Coomassie Brilliant Blue.

4.2.11. Size exclusion chromatography

The pre-incubated samples were analyzed for their aggregation state with size exclusion (SE) chromatography using Biosep-SEC-S-2000 (300×7.8mm) column, using a Water Associate HPLC system equipped with a 1525 binary pump and water 2996 photo diode array detector. The mobile phase used was 0.1M sodium phosphate buffer, pH 7.0; flow rate was set 0.7ml min^{-1} and elution peaks were detected by measuring UV absorption at 275 nm. The column was calibrated using the standard markers. The data were collected and analyzed by Millenium software.

4.2.12. Molecular modeling of SAC-Aβ and Aβ-Cu^{2+}-CG interaction

In order to understand the possible binding mode of Aβ with SAC and CG with Aβ-Cu^{2+} complex, we used the solution NMR structure of Aβ40 (PDB ID: 1BA4). To create initial coordinates for the docking studies, all water molecules of the involved solution NMR structure were removed and excluded from the calculations. Hydrogen atoms were added in their idealized positions. The ligand SAC was constructed and submitted to the PRODRG site (Van Aalten et al., 1996), and the initial geometry and topology was retrieved. Similarly, firstly, the binding of Cu^{2+} ion was studied and then CG was constructed and submitted to the PRODRG site (Van Aalten et al., 1996), and the

initial geometry and topology was retrieved. The SAC and CG thus obtained was used for all ligand-docking purposes. The hamiltanion energy for SAC binding to Aβ and CG binding to Aβ-Cu^{2+} complex was calculated by employing the Hartee-Fock operator as computed by Austin Model 1 (AM1) parameterization of the MNDO (modified neglect of diatomic differential overlap) method (Dewar et al., 1985). All the energy calculations were performed at a net molecular charge of zero. Flexible docking was carried out using the genetic algorithm implemented in the program Argus lab (A Score scoring method). This dock engine was set to perform an exhaustive search for automated docking with complete ligand flexibility. The run was optimized for 150 different poses. A high grid resolution of 0.4 A^0 was used with average grid dimensions of 81× 52 × 115 A^0. The dimensions of binding site box were excluded to 31.6 × 20.2 × 45.3 A^0. The optimal model was chosen based on energy minimization; and bond angle stereochemistry using PROCHECK (Laskowski et al., 1993) and WHATIF (Vriend, 1990).

4A.1. Results

4A.1.1. Inhibition of Aβ40 aggregation and disaggregation of preformed fibrils by GE and SAC

Thio-T is known to bind amyloid fibrils accompanied by a dramatic increase of fluorescence at around 482nm, when excited at 446nm. As shown in Figure 4A.1a, when freshly dissolved Aβ40 was incubated at 37^0C as a function of different time intervals, the fluorescence of Thio-T followed a characteristic sigmoidal curve. There was a dose-dependent inhibition of Aβ40 aggregation with increasing w/w ratio of FGE viz. 1:1<1:5. A larger lag time without the fibril formation was observed on incubation of GE with Aβ when compared to Aβ alone. For Aβ alone, a lag time of 1day was observed before the aggregation started as indicated by the increase in the Thio-T fluorescence emission, which reached equilibrium after 5 days and increased negligibly till 6 days. The kinetics of fibrillation was significantly lower, reflected in the increase in lag time to 3 and 6 days at 1:1 (Figure 4A.1b) and 1:5 w/w (Figure 4A.1c) ratios respectively.

Further, the GE is considered to be a rich source of proteases. The protease activity assay revealed that the FGE had 382 units of activity/gm and FGE obtained by boiling for 5 minutes at 100^0C had 0 units of activity/gm. it was important to study the effect of BGE on Aβ aggregation to eliminate the possibility of Aβ degradation by proteases present in FGE. BGE could also inhibit Aβ aggregation at 1:5 w/w ratio (Figure 4A.1d). Hence, degradation of Aβ due to proteases present in GE is not significant and Garlic could inhibit Aβ aggregation through non-enzymatic pathway in the order FGE> BGE.

In order to determine whether GE could also be inhibitory when added after initial aggregation events, its effect on kinetics of disaggregation of preformed fibrils of Aβ40 was evaluated. Aβ pre-formed fibrils, were mixed with FGE at 1:1 and 1:5 w/w ratios in two separate reaction mixtures and incubation was continued for another 3 days at 37^0C. There was a dose- dependent disaggregation of preformed fibrils i.e. 1:5 w/w ratio (Figure 4A.1e) resulted in a more substantial decrease in the kinetics of already set in aggregation when compared to 1:1 w/w ratio (data not shown). The Thio-T fluorescence intensity significantly decreased after addition of the FGE. However, the fibril degrading

ability of BGE was significantly lost as reflected in the Thio- T studies (Figure 4A.1f). This may be due to the loss of stability of certain heat labile components of garlic.

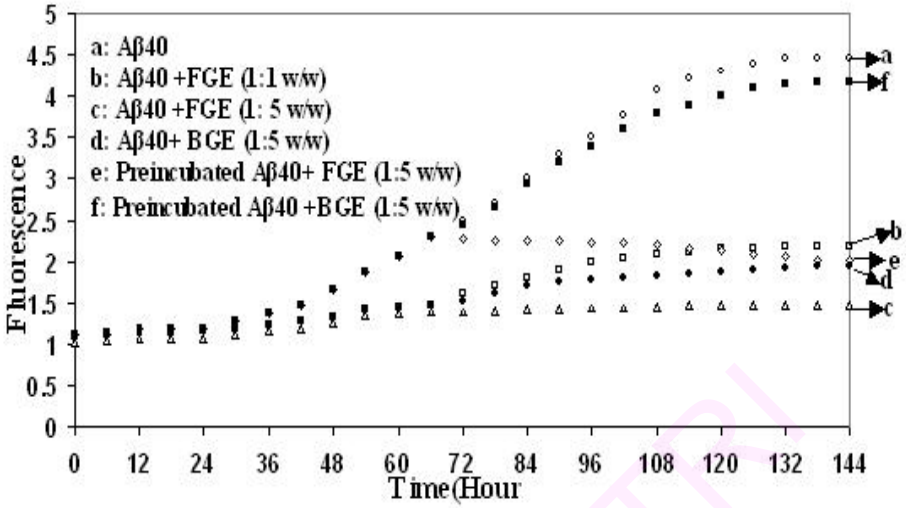


Figure 4A.1: Effect of GE on the kinetics of aggregation and disaggregation of Aβ(1-40) studied using Thio-T assay: The reaction mixtures containing (a)100µg/ml Aβ40, (b) Aβ40 and FGE (1:1w/w ratio) for 6 days at 37^0C, (c) Aβ40 and FGE (1:5 w/w ratio) for 6 days at 37^0C, (d) Aβ40 and BGE (1:5 w/w ratio) for 6 days at 37^0C, (e) preincubated 100µg/ml Aβ40 for 3 days+ FGE (1:5 w/w ratio), (f) preincubated 100µg/ml Aβ40 for 3 days+ BGE (1:5 w/w ratio). Points represent means of three independent experiments with S.D.± 0.05.

Further, there was a dose-dependent inhibition of Aβ40 (50µM) aggregation with increasing concentration of SAC viz. 10 µM and 50 µM (Figure 4A.2a). A larger lag time without the fibril formation was observed on incubation of SAC with Aβ when compared to Aβ alone. For Aβ alone a lag time of 1day was observed before the aggregation started as indicated by the increase in the Thio-T fluorescence emission, which reached equilibrium after 5 days and increased negligibly till 6 days. The kinetics of fibrillation was significantly lower, reflected in the increase in lag time to 3 and 6 days at 10 and 50 µM SAC respectively. This indicates that SAC delays Aβ fibril formation to a considerable extent. The observations were confirmed repeating the experiment three times, where similar trend was observed.

In order to determine whether SAC could also be inhibitory when added after initial aggregation events, its effect on kinetics of disaggregation of preformed fibrils of Aβ40 was evaluated. Aβ pre-formed fibrils, were mixed with 10 and 50 µM SAC in two

separate reaction mixtures and incubation was continued for another 3 days at 37^0C. The Thio-T fluorescence intensity significantly decreased after addition of SAC (Figure 4A.2b). There was a dose- dependent disaggregation of preformed fibrils i.e. 50 μM SAC resulted in a more substantial decrease in the kinetics of already set in aggregation when compared to 10 μM SAC.

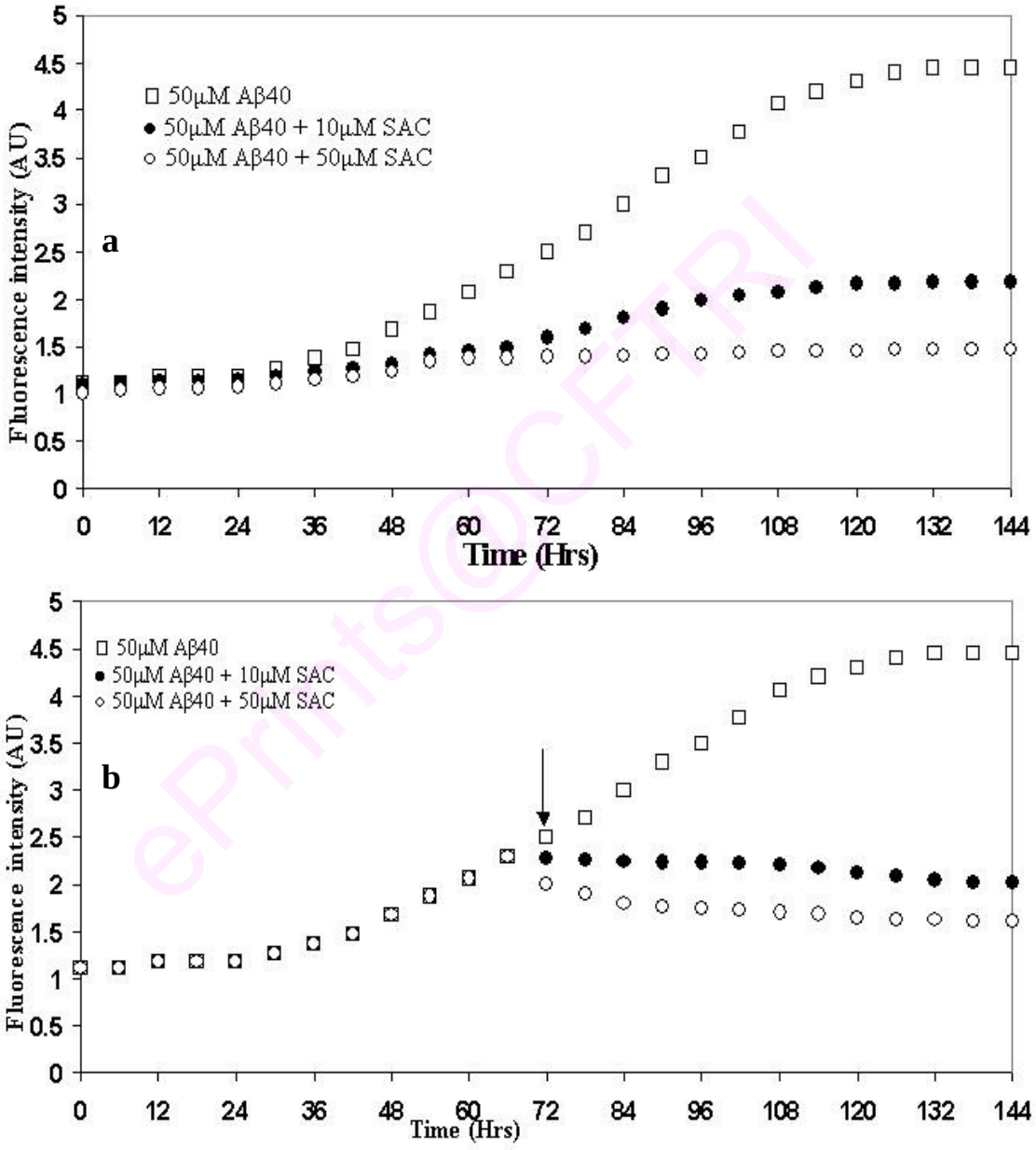


Figure 4A.2: Thio-T assay: Effects of SAC (a,b) on the kinetics of aggregation (a) and disaggregation (b) of Aβ(1-40): The reaction mixtures containing 50μM Aβ40 (a) and 50μM Aβ40 preincubated for 3 days at 37^0C (b), 0.05 M Tris-Cl, pH 7.4, and 0(□), 10(●), 50(O) μM of SAC (a,b) were incubated at 37^0C for the indicated times. ↓: An

arrow shown in the 2b indicates the time at which SAC was introduced in the reaction mixture (i.e. after 3 days of Aβ incubation at 37^0C). Values are expressed as average of triplicates.

4A.1.2. Transmission Electron Microscopy

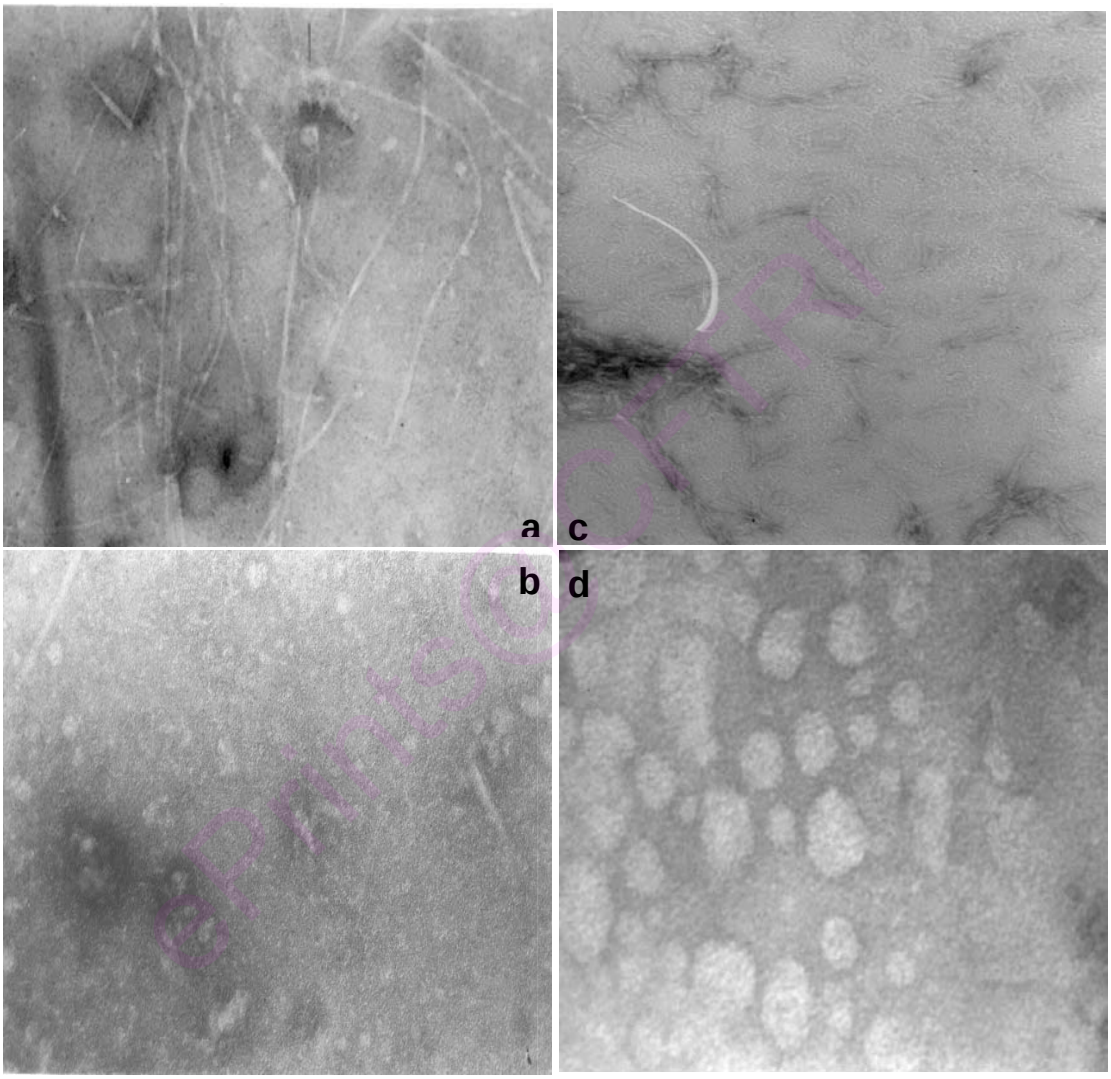


Figure 4A.3: Electron micrographs of Aβ40 in the presence and absence of GE: a) 100μg/ml Aβ40 incubated for 6 days at 37^0C; b) Aβ40 incubated for 6 days at 37^0C in presence of FGE at 1:5 w/w ratio; c) Aβ40 incubated for 6 days at 37^0C in presence of BGE at 1:5 w/w ratio, d) Aβ40 (pre-incubated for 3 days) incubated for subsequent 3 days at 37^0C in presence of FGE at 1:5 w/w ratio.

TEM was used to confirm the inhibitory effects of GE on Aβ fibril formation and defibrillation effect on pre-formed Aβ fibrils (Figure 4A.3). Aβ40 incubated for 6 days at 37^0C formed extensive fibrils. The aggregates observed were seen as long, predominantly fibrillar forming large networks. The fibril formation was reduced at 1:1 w/w ratio (data not shown). There was more potent inhibition of fibril formation on incubation of Aβ with both FGE and BGE at 1:5 w/w ratio. Aβ pre-formed fibrils obtained by incubation at 37^0C for 3 days were incubated successively for another 3 days after addition of FGE at 1:5 w/w ratio. The number of fibrils were reduced markedly. Hence, FGE could even defibrillate preformed Aβ fibrils.

TEM was also used to confirm the inhibitory effects of SAC on Aβ fibril formation and defibrillation effect on pre-formed Aβ fibrils (Figure 4A.4). The fibril formation reduced at 10 μM SAC (data not shown), and more potent inhibition of fibril formation was seen at higher concentration of 50 μM SAC. The fibrils formed after incubation at 37°C for 3 days had no branching and had less mature fibrillar morphology. Aβ pre-formed fibrils obtained were incubated successively for another 3 days after addition of SAC (50 μM). The number of fibrils were reduced markedly and small amorphous aggregates were occasionally observed. Hence, SAC limits fibril formation even when added halfway through the incubation time.

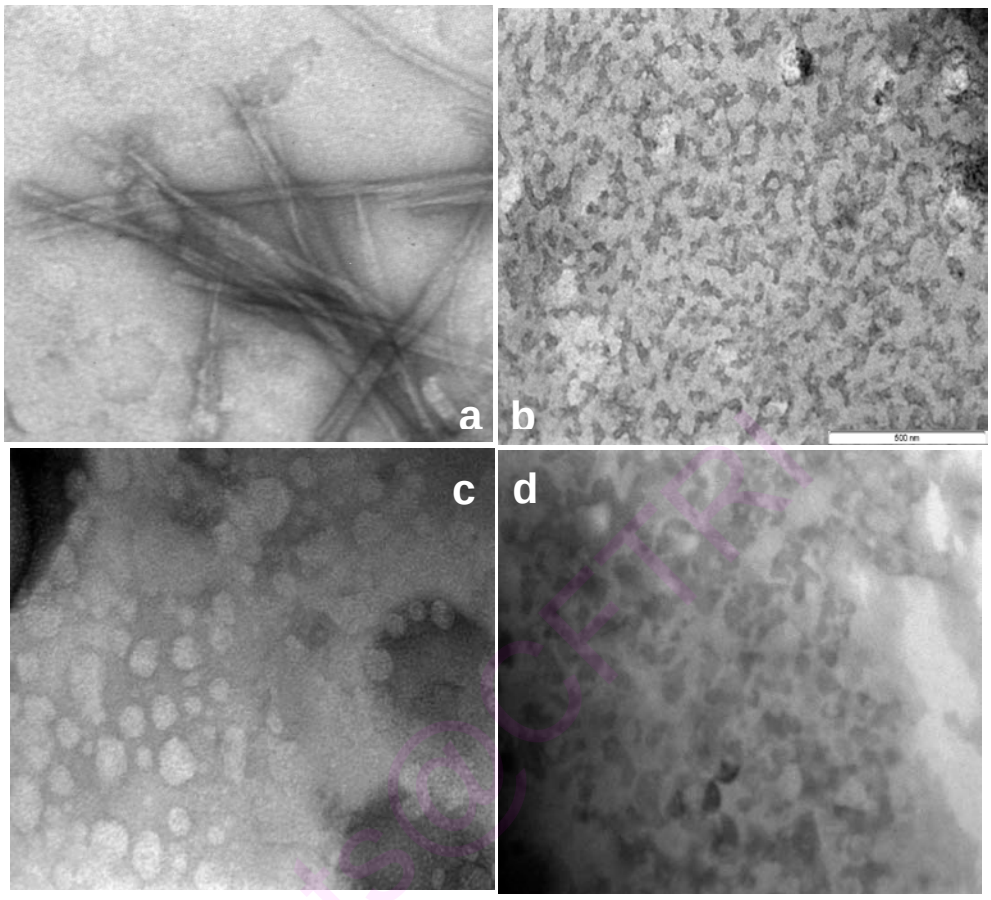


Figure 4A.4: Electron micrographs of Aβ40 in the presence and absence of SAC: a) Aβ40 incubated for 6 days at 37^0C; b) Aβ40 incubated for 3 days at 37^0C in the absence of SAC; c) Aβ40 incubated for 6 days at 37^0C in presence of 50μM SAC; d) Aβ40 (pre-incubated for 3 days) incubated for subsequent 3 days at 37^0C in presence of 50μM SAC.

4A.1.3. SDS- Polyacrylamide Gel Electrophoresis

To gain an idea of the mass of the species that exist when Aβ (100μg/ml) is incubated along with GE, we carried out SDS-PAGE of the incubated samples (Figure 4A.5). Aβ incubated without the addition of GE showed oligomerisation as viewed by the presence of higher molecular weight assemblies ranging from 29 to 45 kD. The freshly dissolved Aβ appeared as a single 4kD band in the gel. We found that both FGE and BGE inhibited oligomerization of Aβ as evidenced by a single band equivalent to that of 4kD. Thus it inhibits the formation of higher molecular weight assemblies and stabilizes the 4 kD soluble Aβ. SDS-PAGE was also carried out to study the disaggregation effect

of FGE on preformed fibrils. The majority of Aβ preformed fibrils got solubilized to soluble Aβ, as evident by a major band corresponding to 4kD and a few bands ranging from 20-45 kD.

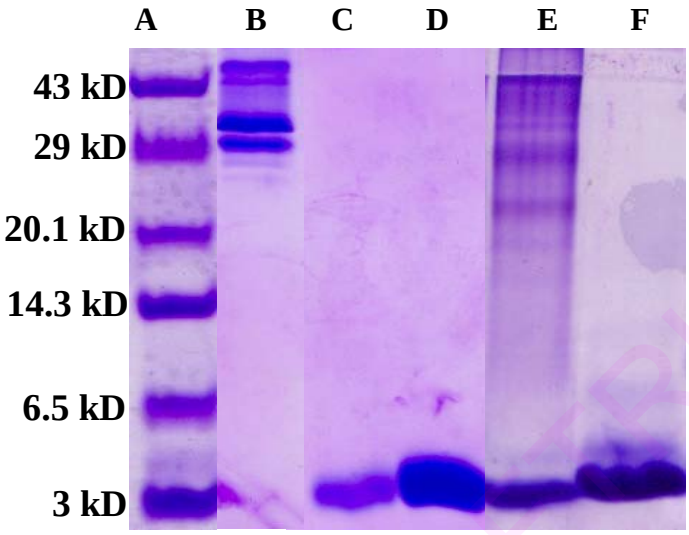


Figure 4A.5: SDS-PAGE of Aβ40 in presence and absence of GE: A) Molecular weight Marker (3-43kD), B) Aβ40 incubated for 6 days at 37^0C in absence of GE, C) Fresh Aβ40 (100μg/ml), D) Aβ40 incubated for 6 days at 37^0C in presence of FGE at 1:5 w/w ratio E) preincubated 100μg/ml Aβ40 for 3 days incubated subsequently for another 3 days with FGE (1:5 w/w) at 37^0C, F) Aβ40 and BGE incubated at1: 5 w/w ratio for 6 days at 37^0C.

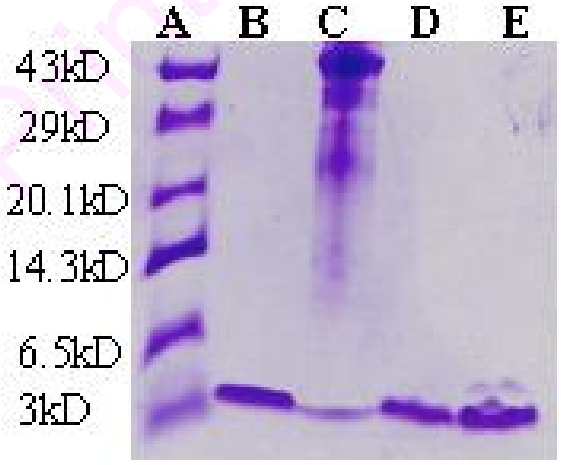


Figure 4A.6: SDS-PAGE of Aβ40 in presence and absence of SAC: A) Molecular weight Marker (3-43kD) B) Fresh Aβ40; C) Aβ40 incubated for 6 days at 37^0C in absence of SAC; D) Aβ40 incubated for 6 days at 37^0C in presence of 50μM SAC; E) Aβ40 (pre-incubated for 3 days) incubated for subsequent 3 days at 37^0C in presence of 50μM SAC.

Further, SDS-PAGE studies also showed inhibition of Aβ oligomerization by SAC as evidenced by a single band equivalent to that of 4kD (Figure 4A.6). Aβ incubated without the addition of SAC showed oligomerisation as viewed by the presence of higher molecular weight assemblies ranging from 10 to 45 kD. Thus it inhibits the formation of higher molecular weight assemblies and stabilizes the 4 kD soluble Aβ. The preformed fibrils on incubation with SAC (50μM), got disaggregated to monomeric Aβ, as evident by a major band corresponding to 4kD and a faint band slightly above it.

4A.1.4. Size exclusion-HPLC

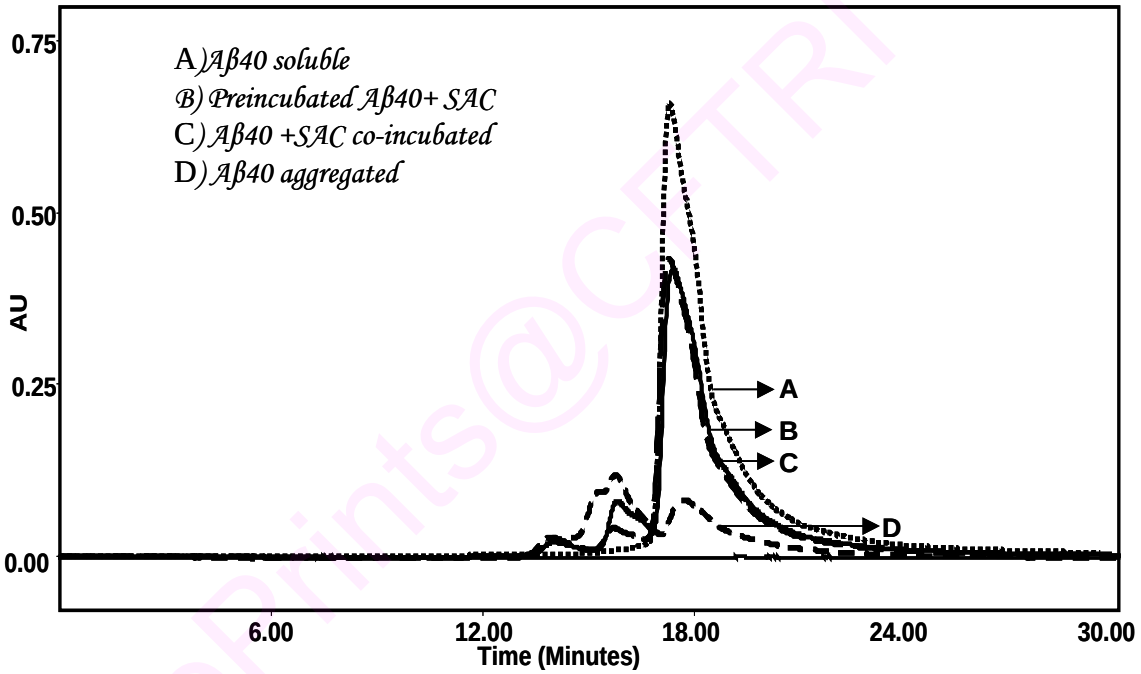


Figure 4A.7: Size exclusion-HPLC to study association state of Aβ40 on incubation with SAC: A) Freshly dissolved Aβ40; B) Aβ40 (pre-incubated for 3 days) incubated for subsequent 3 days at 37^0C in presence of 50μM SAC; C) Aβ40 incubated for 6 days at 37^0C in presence of 50μM SAC; D) Aβ40 incubated for 6 days at 37^0C in absence of SAC.

SE- HPLC was used to determine the association state of Aβ40 on incubation with SAC. The incubated samples were centrifuged to remove precipitated aggregates, and the supernatant was run on SE-HPLC. Chromatograms of Aβ samples incubated with SAC can be seen in Figure 4A.7. Aβ (50μM) incubated without SAC appeared as multiple peaks, indicating the presence of Aβ in multimer state, whereas in presence of

SAC (50μM), the major peak obtained corresponded to that of soluble Aβ. The addition of SAC (50μM) in the middle of the fibrillation reaction (after 3 days of incubation), as monitored by Thio-T, caused the peaks corresponding to the multimeric forms of Aβ to disappear. Hence, the predominant end product after defibrillation of preformed fibrils corresponded to that of soluble Aβ.

4A.1.5. Binding affinity of SAC to Aβ40

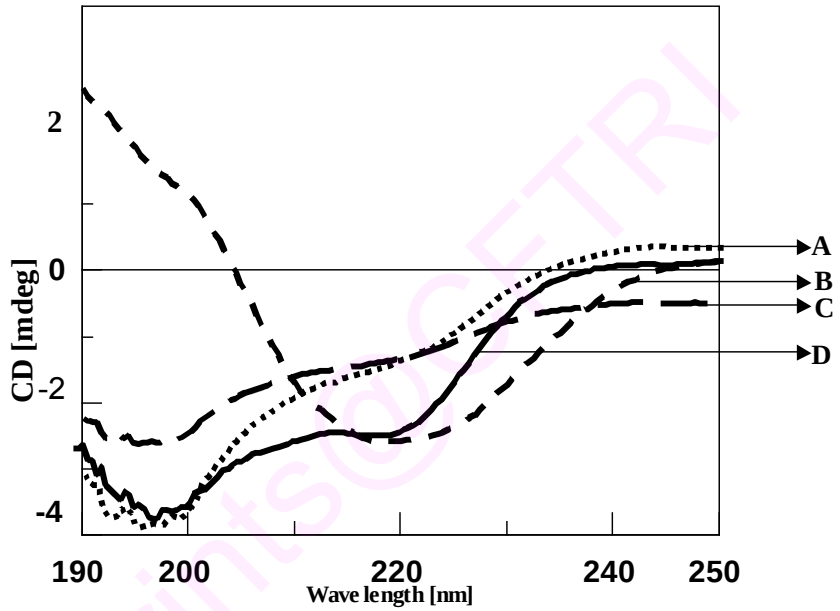


Figure 4A.8: CD spectra indicating the effect of SAC on the secondary structure of Aβ40: A) Freshly dissolved Aβ40 without SAC; B) Aβ40 incubated for 6 days at 37^0C in absence of SAC; C) Aβ40 incubated for 6 days at 37^0C in presence of 50μM SAC; D) Aβ40 (pre-incubated for 3 days) incubated for subsequent 3 days at 37^0C in presence of 50μM SAC.

CD studies aimed to determine the effect of SAC binding on the secondary structure of Aβ40 (Figure 4A.8). The CD spectrum of native Aβ40 was characterized by a strong negative CD band in the 195 to 200-nm region, indicative of a random coil conformation (A; 94.40% coil, 4.1% α-helix, 1.5% β-sheet). On incubation of Aβ for 6 days at 37°C, the CD spectrum was characterized by a broad negative CD band with a characteristic minima at 220 nm, indicative of β-sheet conformation (B; 91.2% β-sheet,

7.4% α-helix, 1.4% coil). On the other hand, when Aβ was incubated in presence of SAC under same conditions, the CD spectrum obtained had a broad negative band ranging from 198nm to 200nm, along with another shorter negative band in the 220 to 225 nm region (C; 88.1% coil, 17% α-helix, 11.2% β-sheet). This spectrum clearly indicated that SAC was capable of holding majority of Aβ in random coil. Further, Aβ pre-formed fibrils on incubation with SAC for 3 days gave a CD spectrum characteristic of partially folded conformation (D; 79.3% coil, 3.7% β-sheet, 17% α-helix). There was a significant decrease in the magnitude of negative band at 220 nm and appearance of a negative band at 198 nm, thus revealing a shift in the secondary structure of preformed Aβ fibrils towards partially folded conformation on binding to SAC.

Binding of SAC to Aβ40 has also been monitored following the quenching of relative fluorescence intensity of Aβ. Quenching of fluorescence by SAC does not lead to detectable changes in wavelength of maximum emission (E_m:330 nm). The fluorescence intensity of Aβ in the absence of SAC did not change during the course of the experiment. A maximum quench of 57.4% has been observed as deduced from the plot of % Q vs. [SAC] (Figure 4A.9a). The value of n, stoichiometry of the Aβ-SAC complex, has been estimated to be 1:1.2 (Figure 4A.9b). The binding constant, K_α was estimated to be 2.1 x 10^{-5} M from mass action plot (Figure 4A.9c).

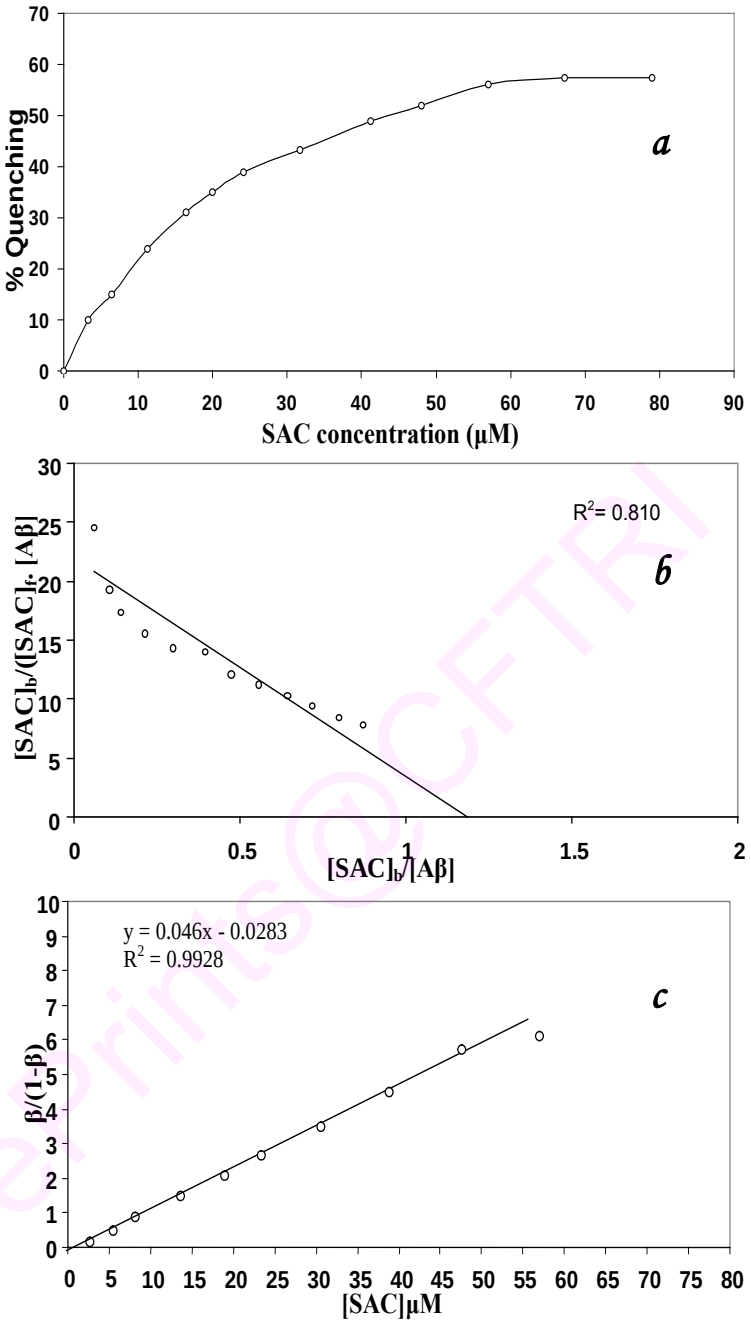


Figure 4A.9: Fluorescence quenching experiments to quantitate the interaction of Aβ40 with SAC: a) % quench of fluorescence intensity, as a function of constituent SAC concentration. b) A plot of $[SAC]_b/([SAC]_f.[A\beta]$ vs. $[SAC]_b/[A\beta]$, where $[SAC]_b$ and $[SAC]_f$ is the molar concentration of bound and free SAC resp. c) A plot of β/(1-β) vs. $[SAC]_f$ and K_α, association constant is obtained by, $K\alpha=\beta/(1-\beta).1/[SAC]_f$. Values are expressed as average of triplicates.

4A.1.6. Molecular modelling of Aβ-SAC interaction

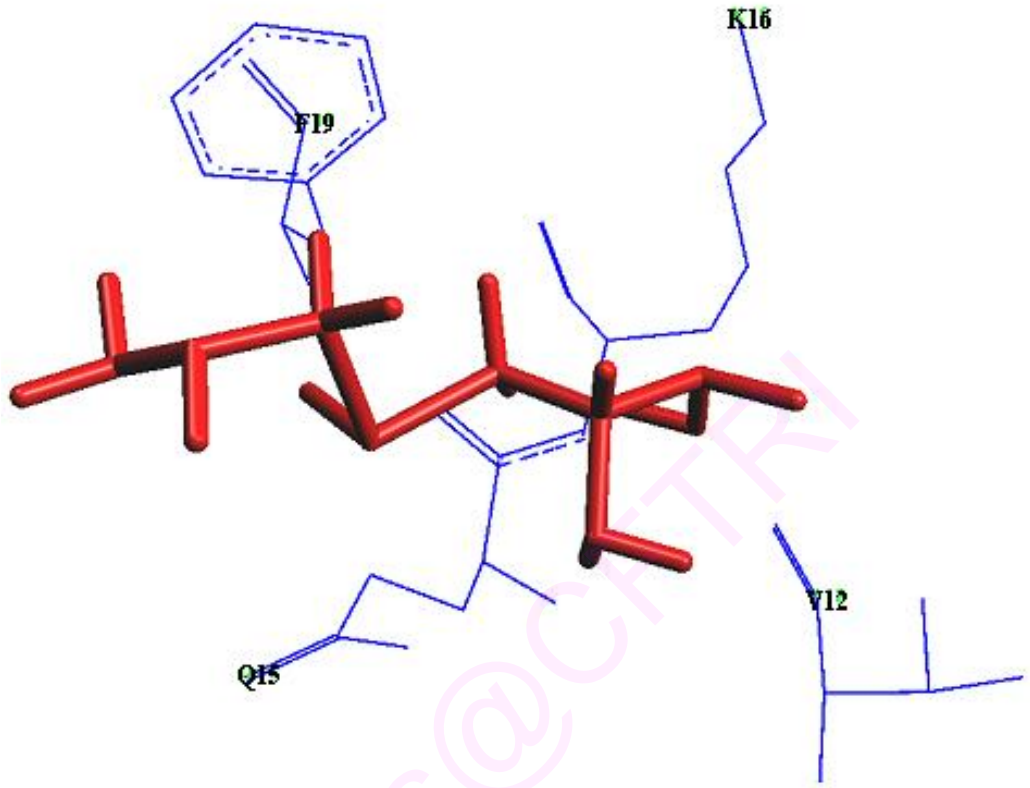


Figure 4A.10: Molecular modelling of Aβ-SAC interaction: The wire frame model showing molecular visualization of binding of SAC with Aβ40. The residues of Aβ40 (Gln^{15}-Lys^{16}) involved in their interaction have been highlighted. Phe 19 and Val 12 are also shown in the proximity.

The main purpose of the computational analysis of 3D structure and modelling is to ensure that the space and suitable residues for interaction are available at the binding site of Aβ. The docking search in and around the Aβ40 structure readily resulted in the identification of a site suitable for accommodating SAC. SAC has the possibility of interaction with the positively charged Gln^{15}-Lys^{16} segment. Phe 19 and Val 12 are also present in the proximity (Figure 4A.10). These residues are likely to interact depending upon the precise orientation and positioning of the SAC within a broader predicted binding site. The best ligand pose energy for docking was calculated to be -6.308 kcal/mol for SAC-Aβ interface. As expected for well-minimized models no bad contacts or clashes were observed. It is extremely difficult to predict the accurate 3D structure of

SAC-Aβ complex with precise details of interactions between protein residues and SAC. However the current analysis clearly shows that space and optimal residues congenial for interaction with SAC exist in Aβ structure. Thus the modeling results are consistent with the experimental findings.

4A.2. Discussion

The formation of insoluble Aβ deposits in the brain is a pathological hallmark of AD. If the hypothesis that the neurotoxicity of Aβ is mediated by amyloid fibril formation is correct (Hashimoto et al., 2003; Lorenzo and Yankner, 1996; Pike et al., 1993; Blanchard, 2004), inhibition of Aβ fibril formation might slow progression or prevent the disease. However, more recent studies have shown that fibrils are not the only neurotoxic structures and that Aβ also assembles into soluble forms like small oligomers and protofibrils, which could be responsible for neurotoxicity (Barghorn et al., 2005; Kokubo et al., 2005; Lesne et al., 2006). Consequently, screening the compounds that reduce fibrillation and disaggregate the fibrils of Aβ may lead to therapies to prevent or to control AD as well as to a better understanding of the process. One of the current therapeutic approaches in AD is to prevent Aβ fibrillation and to destabilize preformed fibrils using small biomolecules. Various biomolecules along with their ability to attenuate the oxidative stress have also been reported to possess anti-amyloidogenic effects in AD (Ono et al., 2006). On the basis of various studies, such as antiaging effect (Nishiyama et al., 1997), improvement of learning and memory impairment (Moriguchi et al., 1997a; Nishiyama et al., 1997), neurotrophic activities (Moriguchi et al., 1997b), it can be proposed that GE ameliorates symptoms associated with aging. Hence, Garlic has acquired a reputation as the most promising herb with which to treat or prevent diseases, including neurodegeneration. The present study was designed to investigate the effects of aqueous GE and SAC on Aβ fibrillogenesis *in vitro* and strongly implicates their inhibitory role in Aβ aggregate formation in AD brain. Our results demonstrate that both FGE and BGE as well as SAC inhibit amyloid fibril formation and FGE and SAC even cause disaggregation of preformed fibrils in solution. These findings suggest the possible

use of garlic and its constituents for inhibition of aggregation and deposition of Aβ in the human brain.

Aβ fibril formation is believed to be the main determinant in the pathogenesis of AD, although it is not clear how Aβ fibrils exert their cytotoxic effects. Aβ fibrils are reported to permeabilize lipid membranes (Arispe et al., 1993) and induce calcium inflow (Sanderson et al., 1997) in neurons. Hence, inhibition of cerebral Aβ aggregation is an important goal in AD therapy. Several compounds have been reported to have such an effect such as nicotine (Ono et al., 2002), melatonin (Pappolla et al., 1998), laminin (Castillo et al., 2000). Further, because of the known relationship between oxidative stress and AD and due to the established antioxidative properties of garlic, it has been proposed that GE exerts its neuroprotective effects mostly as an intracellular antioxidant (Peng et al., 2002). However, results presented herein suggest that the anti-amyloidogenic property of GE could be a consequence of its direct interaction with Aβ. Aqueous GE is a relatively complex mixture of several compounds. Its components like SAC have been reported to have neurotrophic activity (Moriguchi et al., 1997), prevent Aβ toxicity in cell cultures (Kosuge et al., 2006) and also have antioxidant and radical scavenging effects (Borek, 2006). In summary, according to the reports available in literature and results reported in the present study, GE exerts a combination of anti-oxidative, anti-amyloidogenic and anti-apoptotic effects. The results provided here confirm the inhibitory action of GE on Aβ aggregation and fibrillogenesis, they provide no information on the molecular basis of this phenomenon. Hence, we also studied the anti-amyloidogenic action of SAC on AB aggregate formation.

In addition to odoriferous oil-soluble compounds, less odoriferous, nonvolatile, water-soluble organosulfur compounds such as, SAC, SAMC are also present in garlic preparations, although the content varies considerably (Imai et al., 1994; Lawson, 1993). During aging process, the odorous, harsh and irritating compounds in garlic are converted naturally into stable and safe sulfur compounds. SAC, having a thioallyl group, the most abundant water-soluble organosulfur compound in AGE, is present at a concentration of 1.47 g / L. SAC is formed from γ-glutamyl cysteine catabolism and has been reported to contribute to the health benefits of some garlic preparations. The concentration of SAC increases during extraction /aging. The pharmacokinetics of SAC are also well

established (Nagae et al, 1994). SAC may account for the reduced toxicity of the hydroalcoholic extracts of garlic compared with raw preparations (Kanezawa et al., 1984). SAC can be detected in the plasma, liver, and kidney after oral intake (Nagae et al, 1994). The bioavailability of SAC is 103 % in mice, 98.2 % in rats and 87.2 % in dogs (Nagae et al, 1994). N-acetyl-SAC has been identified as a metabolite of SAC in the urine of dogs and humans. SAC increases quantitatively in the blood after oral intake of garlic (Steiner and Li, 2001). This suggests that SAC could be transformed by N-acetyltransferase. SAC and its metabolites are possible compliance markers for clinical studies involving garlic (Steiner and Li, 2001). Because SAC is found in many preparations, it might be used for standardization and /or used to compare various sources. The United States National Cancer Institute tested the toxicity of SAC vs. other typical garlic compounds and found that it has 30-fold less toxicity than allicin and DADS (Imada, 1990).

SAC has a compact structure, which appears to be quite suitable for specifically binding to Aβ and subsequent destabilization of the β-sheet rich conformation in fibrils. SAC extends both the length of the lag phase and the time to proceed to equilibrium in a dose-dependent manner in Thio-T assay. Aβ has experienced a halt in conformational transition upon SAC treatment, which is necessary for the prevention of Aβ aggregation. SAC prevented the self-oligomerization of Aβ by inducing a partially folded conformation, which does not undergo further oligomerization. Based on our disaggregation studies, SAC may act as a breaker of the preformed Aβ fibrils. Further, the fluorescence quenching studies indicated a gradual decrease in the fluorescence intensity on addition of SAC indicating burying of the tyrosine in the core of the newly formed structure. Aβ may have certain local structures to accommodate the structural characteristics of SAC although the protein has been known to exist in natively unfolded state. When the protein recognizes its specific ligands, it exhibits structural plasticity to accommodate the ligand. Binding could also be induced by hydrophobic interactions between allyl chain of SAC and the hydrophobic region of Aβ, thus blocking association between Aβ molecules. Similarly, Tomiyama et al. (1996), suggested hydrophobic interactions between rifampicin lipophilic chain and the hydrophobic region of Aβ. These interactions could also be reinforced by the H- bond between the –OH group of the

carboxylic group of SAC and donor/acceptor groups of Aβ. Hence, SAC might have 3 kinds of interactions: i) hydrophobic interactions ii) conformational mutual adaptation owing to the flexibility of Aβ and SAC and allowing them to adopt a steric complementarity and to create additional vanderwaals bonds; and iii) additional H- bonds between –OH of SAC. Thus SAC with potent antioxidant motifs (Kosuge et al., 2003), could bind specifically to Aβ and inhibit fibrillar Aβ formation and destabilize preformed fibrillar Aβ through mechanisms yet unknown. However, SAC is specifically known to protect neurons against the caspase-12 dependent neurotoxicity induced by Aβ (Kosuge et al., 2003). It is also known to attenuate the Aβ induced increase of intracellular reactive oxygen species in hippocampal neurons (Kosuge et al., 2003). Hence, SAC may prevent the progression of AD by multiple mechanisms *in vivo*. Chauhan, (2006) have recently demonstrated anti-amyloidogenic, anti-inflammatory and anti-tangle effects of aged GE and its constituents namely SAC and DADS in Alzheimer's transgenic model Tg2576. They indicated the involvement of cholesterol dependent mechanism of garlic and its constituents namely SAC and DADS by acting as HMG CoA reductase inhibitors. However, further structural studies are essential to elucidate the mechanisms by which garlic and its constituents inhibit fibril formation. While SAC could be important molecule for therapeutic development, the mechanism of anti-amyloidogenic activity is unknown, so a molecular approach to these interactions is now required.

Further studies, such as nuclear magnetic resonance (NMR) experiments are essential to reveal the exact structure-activity relationships for these compounds which exhibit anti-amyloidogenic and fibril destabilizing effects *in vitro*. Taken together, it is pertinent to consider that SAC could be useful as a chemical probe to understand molecular mechanisms of the cell death caused by the Aβ fibrils. Although the mechanism of this inhibition is still unclear, it is most plausible to speculate that SAC avidly binds to and obstructs the docking sites on Aβ fibrils or protofibrils that serve as seeds for fibril propagation to which monomeric Aβ is successively incorporated, thus inhibiting elongation of Aβ fibrils. Further, *in vitro* studies to examine the binding of SAC to Aβ in various states i.e., oligomers, protofibrils, and fibrils, will be needed.

4B.1. Results

4B.1.1. Analysis of Thio-T studies

In the present study, we advocated Thio-T studies to investigate the self-association kinetics of Aβ in presence of Cu^{2+} and its inhibition by CG. Thio-T, a fluorophore that showed greatly enhanced fluorescence on binding to Aβ fibrils (LeVine, 1993). Thioflavin-T is known to bind amyloid fibrils accompanied by a dramatic increase of fluorescence at around 482nm, when excited at 446nm. This makes Thio-T one of the most useful probes to detect the formation of fibrils. In the present study, we advocated Thio-T assay to investigate the aggregation kinetics of Aβ40 in presence of Cu^{2+} and CG. The kinetics of Aβ40 fibrillation are sigmoidal, defined by an initial lag phase, a subsequent growth phase in which Thio-T fluorescence increased, and a final equilibrium phase, where Thio-T fluorescence reached a plateau indicating the end of fibril formation. Thio-T fluorescence measurements were plotted as a function of time and fitted to a sigmoidal curve. This curve is consistent with the nucleation- dependent polymerization model (Jarrett and Lansbury, 1993; Naiki and Nakakuki, 1996).

In presence of Cu^{2+} (30μM), the kinetics of fibrillation was much faster, reflected in the decrease in lag time and a tremendous increase in the Thio-T binding signal in the transition phase (Figure 4B.1). Further, the limiting value was also reached much faster when compared to Aβ40 alone. This indicates that Cu^{2+} enhance Aβ fibril formation to a considerable extent. However, when Aβ-Cu^{2+} aggregation was monitored in presence of CG, the final equilibrium level decreased successively with increasing concentrations of CG viz. 0, 3, 5, 10, 20, 30 μM (Figure 4B.2). There was a dose-dependent inhibition of Cu^{2+} induced Aβ40 aggregation with increasing concentration of CG. A larger lag time without the fibril formation was observed in presence of CG interaction with Aβ- Cu^{2+}. For Aβ alone, a lag time of 1day was observed before the aggregation started as indicated by the increase in the Thio-T fluorescence emission, which reached equilibrium after 3 days and increased negligibly till 6 days. The kinetics of fibrillation was significantly lower, reflected in the increase in lag time to 4, 5 and 6 days at 3, 5, 10, 20 and 30 μM CG respectively. This indicates that CG delays Cu^{2+} Aβ fibril formation to a considerable

extent. The observations were confirmed repeating the experiment three times, where similar trend was observed (Figure 4B.2).

In order to determine whether CG could also be inhibitory when added after initial aggregation events, its effect on kinetics of disaggregation of Cu^{2+} induced Aβ preformed fibrils was evaluated. Cu^{2+} induced Aβ pre-formed fibrils, were mixed with 30 μM CG in a separate reaction mixture and incubation was continued for another 3 days at 37^0C. The Thio-T fluorescence intensity significantly decreased after addition of CG (Figure 4B.1).

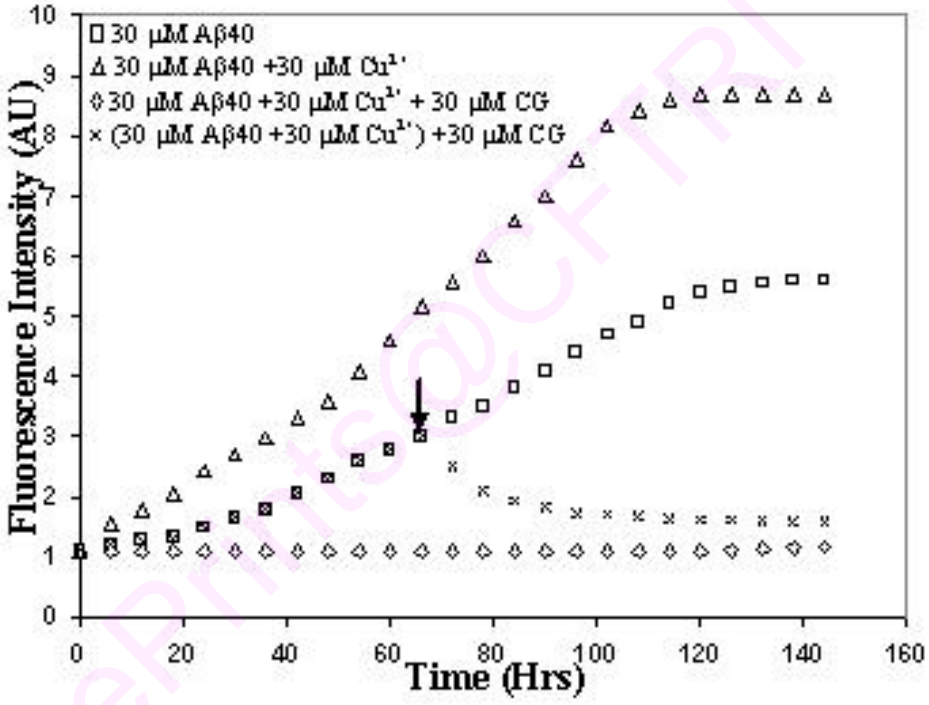


Figure 4B.1: Effect of CG on the kinetics of aggregation and disaggregation of Aβ(1-40) in presence of Cu^{2+} studied using Thio-T assay: The reaction mixtures containing (□) 30μM Aβ40, (Δ) 30μM Aβ40 + 30μM Cu^{2+} for 6 days at 37^0C, (◊) 30μM Aβ40 + 30μM Cu^{2+} + 30μM CG for 6 days at 37^0C, (×) preincubated 30μM Aβ40 in presence of 30μM Cu^{2+} +30μM CG. ↓: An arrow shown above indicates the time at which CG was introduced in the reaction mixture (i.e. after 3 days of Aβ incubation with Cu^{2+} at 37^0C). Points represent means of three independent experiments with S.D.± 0.5.

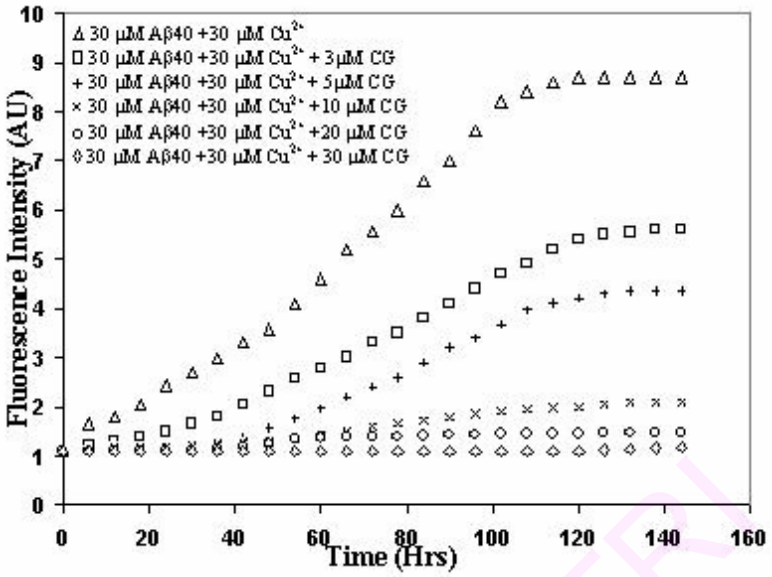


Figure 4B.2: Thio-T assay showing the effect of increasing concentrations of CG viz. 0, 3, 5, 10, 20, 30 μM on the kinetics of aggregation of Aβ(1-40) in presence of Cu^{2+}: The reaction mixtures were incubated at 37^0C for 6 days. Points represent means of three independent experiments with S.D.± 0.5.

The inhibitory effect of CG on Cu^{2+} induced Aβ aggregation was also quanitaively analyzed. Figure 4B.3 shows the best fits of the Eq (1) to the Thio-T fluorescence assay experimental data. As depicted in the figure, the curves are in good agreement with the experimental data. In case of curves representing Aβ and Aβ+Cu^{2+}, there is a sigmoidal transition with an initial lag phase, indicative of the involvement of intermediate species. It can be concluded from Figure 4B.3 that Aβ in presence of Cu^{2+} reaches limiting value faster than Aβ alone. However there is no sigmoidal time response curve on co-incubation of Aβ- Cu^{2+} complex with CG, as CG inhibits Aβ fibrillar formation.

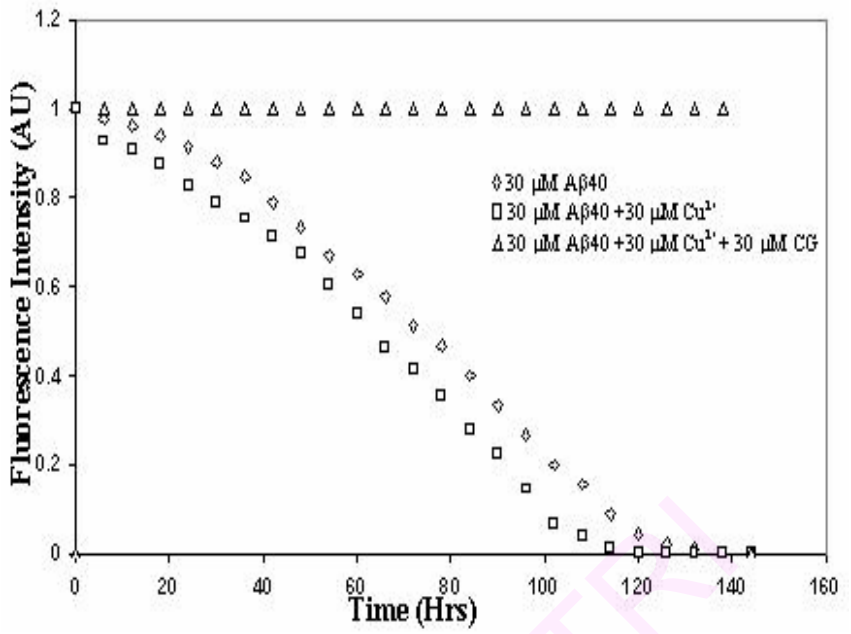


Figure 4B.3: Plots of the values [F(t)-F(∞)]/[F(0)-F(∞)] vs. elapsed time F(t) representing the ThT fluorescence emitted by fibrillar Aβ peptide: (◊) Aβ peptide (□) Aβ+Cu^{2+} (Δ) Aβ+ Cu^{2+}+ CG

4B.1.2. Transmission Electron Microscopy

TEM was used to confirm the inhibitory effects of CG on Cu^{2+} induced Aβ fibril formation and defibrillation effect on pre-formed Aβ fibrils (Figure 4B.4). Aβ40 (30 μM) incubated in presence of 30μM Cu^{2+} for 6 days at 37^0C formed extensive fibrils. The aggregates were seen as long, fibrillar forming large networks. Some small aggregates of variable size (more or like amorphous) were also seen in the image, however, the aggregates were predominantly fibrillar. The fibril formation reduced at 10 μM CG (data not shown), and more potent inhibition of fibril formation was seen at equimolar concentration of CG (30 μM). The number of fibrils were almost negligible and small amorphous aggregates were occasionally observed. Aβ pre-formed fibrils obtained after incubation with Cu^{2+} at 37°C for 3 days were incubated successively for another 3 days after addition of CG (30 μM). The fibrils formed had no branching and had less mature fibrillar morphology. Hence, CG limits fibril formation even when added halfway through the incubation time.

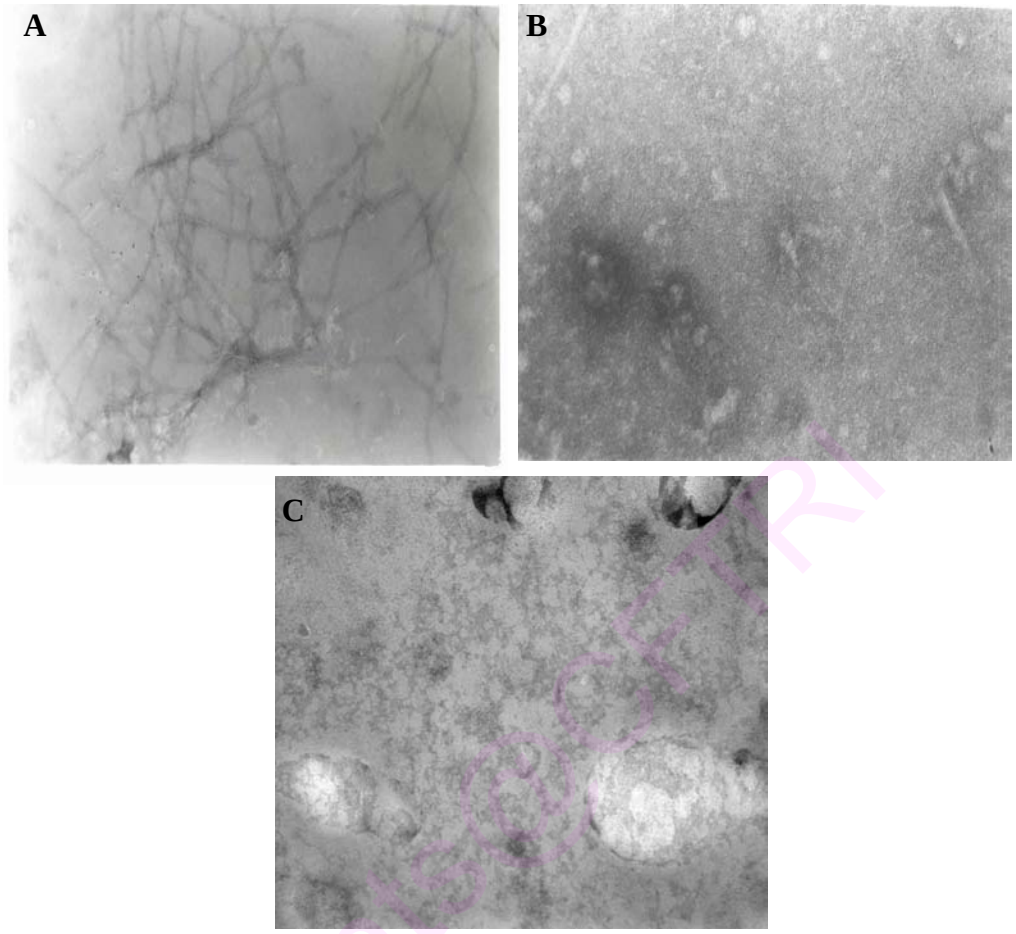


Figure 4B.4: Electron micrographs of Cu^{2+} induced Aβ40 fibrils in the presence and absence of CG: A) 30μM Aβ40 + 30μM Cu^{2+} incubated for 6 days at 37^0C; B) 30μM Aβ40 + 30μM Cu^{2+}+30μM CG incubated for 6 days at 37^0C; C) preincubated 30μM Aβ40 in presence of 30μM Cu^{2+} +30μM CG.

4B.1.3. SDS-Polyacrylamide Gel Electrophoresis

Further, Aβ incubated in presence of Cu^{2+} without the addition of CG showed oligomerization as viewed by the presence of higher molecular weight assemblies ranging from 10 to 45 kD. There was inhibition of Cu^{2+} induced Aβ oligomerization by CG as evidenced by a single band equivalent to that of 4kD. Thus it inhibits the formation of higher molecular weight assemblies and stabilizes the 4 kD soluble Aβ. Aβ-preformed fibrils got disaggregated mainly to monomeric Aβ on incubation with CG

(30μM), as evident by a major band corresponding to 4kD and a few minor bands of high molecular weight (Figure 4B.5).

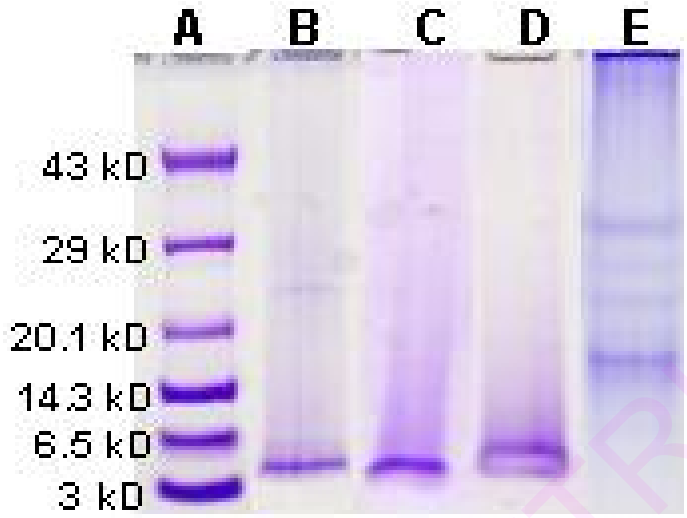


Figure 4B.5: SDS-PAGE of Cu^{2+} induced Aβ40 aggregation in presence and absence of CG: A) Molecular weight Marker (3-43kD); B) Fresh Aβ40; C) Aβ40 incubated for 6 days at 37^0C in presence of 30μM Cu^{2+}+ 30μM CG; D) Aβ40 (pre-incubated for 3 days with 30 μM Cu^{2+}) incubated for subsequent 3 days at 37^0C in presence of 30 μM CG; E) Aβ40 incubated for 6 days at 37^0C in presence of 30μM Cu^{2+}.

4B.1.4. Size exclusion High profile Liquid Chromatography

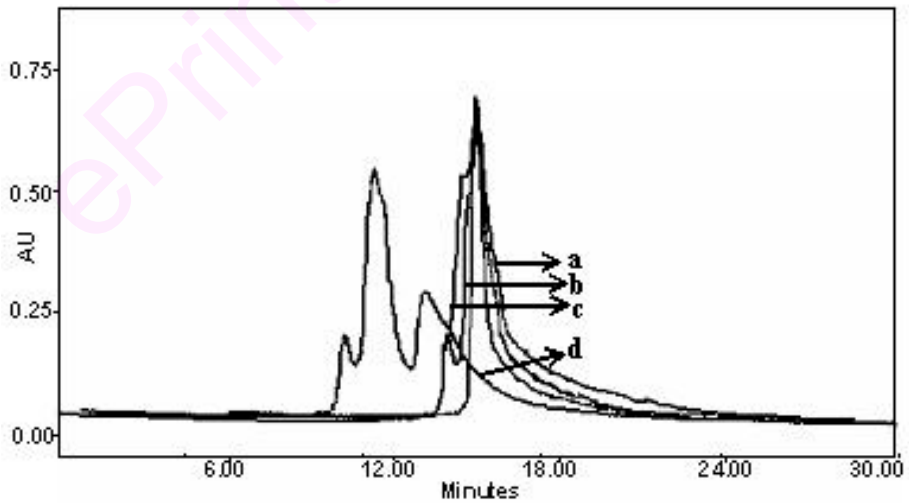


Figure 4B.6: Size exclusion-HPLC to study the association state of Aβ40 in presence of Cu^{2+} on incubation with CG: a) Freshly dissolved Aβ40; b) Aβ40 (pre-incubated with 30μM Cu^{2+} for 3 days) incubated for subsequent 3 days at 37^0C in presence of 30μM CG; c) Aβ40 incubated for 6 days at 37^0C in presence of 30μM CG and 30μM Cu^{2+}; d) Aβ40 incubated for 6 days at 37^0C in presence of 30μM Cu^{2+}.

Size exclusion HPLC was used to determine the association state of Aβ40 in presence of Cu^{2+} on incubation with CG. The incubated samples were centrifuged to remove precipitated aggregates, and the supernatant was run on SEC-HPLC. Chromatograms of incubated samples can be seen in Figure 4B.6. Aβ (30μM) incubated in presence of Cu^{2+} without CG appeared as multiple peaks, indicating the presence of Aβ in multimer state, whereas in presence of CG (30μM), the major peak obtained corresponded to that of soluble Aβ. The addition of CG (30μM) in the middle of the fibrillation reaction (after 3 days of incubation), as monitored by Thio-T, caused the peaks corresponding to the multimeric forms of Aβ to disappear. Hence, the predominant end product after defibrillation of preformed fibrils corresponded to that of soluble Aβ.

4B.1.5. Circular Dichroism Studies

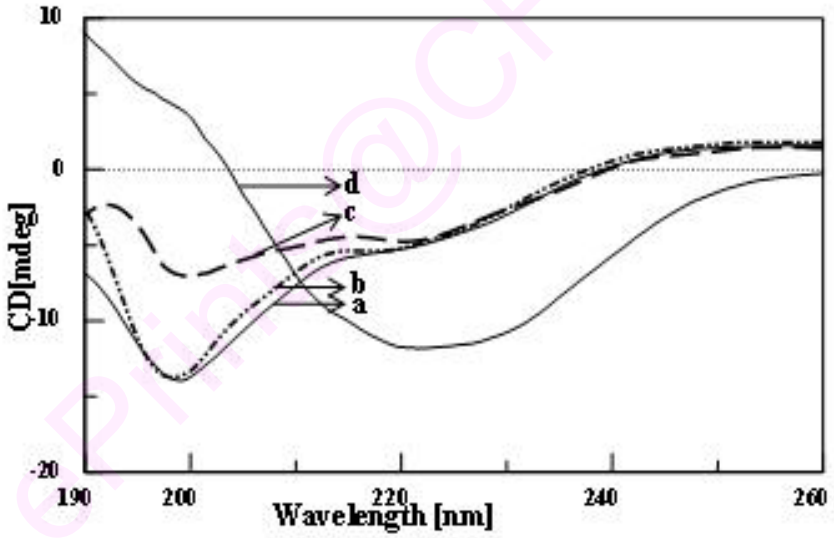


Figure 4B.7: CD spectra indicating the effect of CG binding on the secondary structure of Aβ-Cu^{2+} complex: a) Freshly dissolved Aβ40; b) Aβ40 incubated for 6 days at 37^0C in presence of 30μM Cu^{2+} and 30μM CG; c) Aβ40 (pre-incubated for 3 days with 30μM Cu^{2+}) incubated for subsequent 3 days at 37^0C in presence of 30μM CG; d) Aβ40 incubated for 6 days at 37^0C in presence of 30μM Cu^{2+}.

CD studies aimed to determine the effect of CG binding on the secondary structure of Aβ-Cu^{2+} complex spectra (Figure 4B.7). The CD spectrum of native Aβ40 was characterized by a strong negative CD band in the 195 to 200-nm region, indicative

of a random coil conformation. On incubation of Aβ with Cu^{2+} for 6 days at 37°C, the CD spectrum was characterized by a broad negative CD band with a characteristic minima at 220 nm, indicative of β-sheet conformation. On the other hand, when Aβ-Cu^{2+} was incubated in presence of CG under same conditions, the CD spectrum obtained had a broad negative band ranging from 198nm to 200nm, indicative of random coil conformation, similar to that of soluble Aβ. This spectrum clearly indicated that CG was capable of holding majority of Aβ in random coil. Further, Aβ pre-formed fibrils on incubation with CG for 3 days gave a CD spectrum characteristic of partially folded conformation. There was a significant decrease in the magnitude of negative band at 220 nm and appearance of a negative band at 198 nm, thus revealing a shift in the secondary structure of preformed Aβ fibrils towards partially folded conformation on binding to CG.

4B.1.6. Molecular modeling of Aβ- Cu^{2+}-CG interaction

The main purpose of the computational analysis of 3D structure and modeling is to ensure that the space and suitable residues for interaction are available at the binding site of Aβ. Firstly, the modeling studies readily resulted in the identification of four aminoacid residues suitable for accommodating Cu^{2+} (Figure 4B.8). The four residues namely His6, His12, His14 and Tyr10 have the probability of interaction with the Cu^{2+}. These molecular modeling results are in agreement with the results of Syme et al., (2004). Secondly, docking search in and around the Aβ40 structure resulted in precise orientation and positioning of the CG within a broader predicted binding site. The heat of formation of Aβ- Cu^{2+}-CG complex for docking was calculated to be 806954.2 kcal/mol. As expected for well-minimized models no bad contacts or clashes were observed. Thus the modeling results are consistent with the experimental findings.

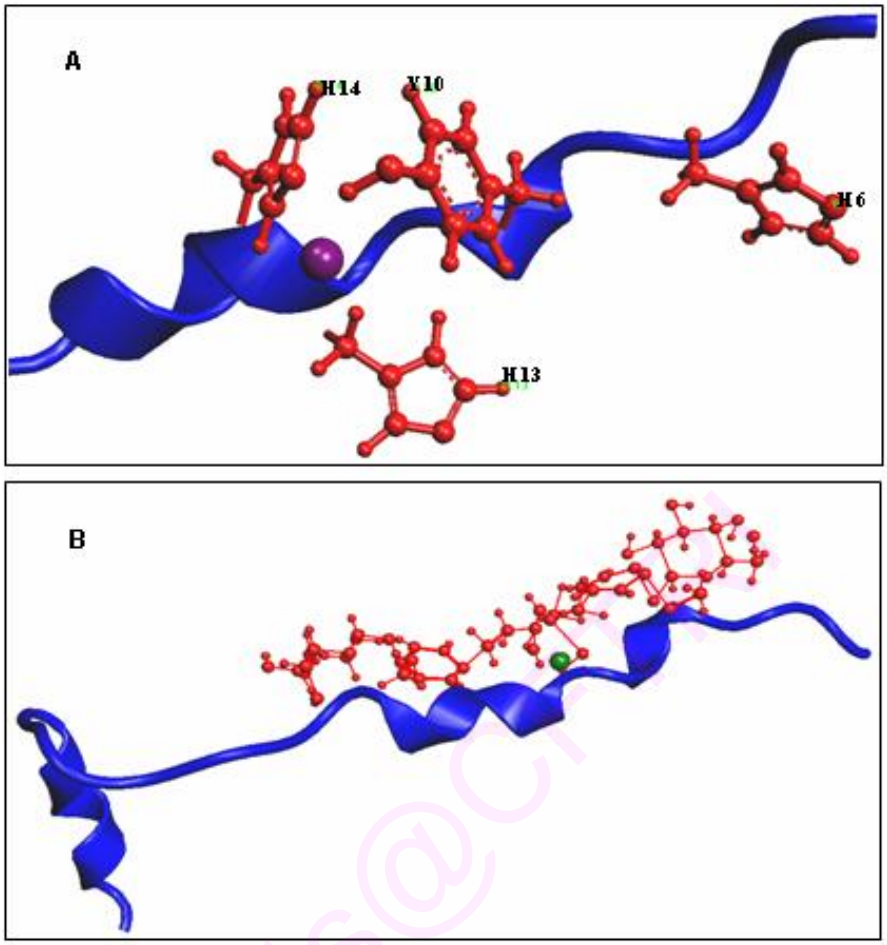


Figure 4B.8: Molecular modeling of Aβ- Cu^{2+}-CG interaction: A) The aminoacid residues (H14, Y10, H13, H6) involved in the binding of Cu^{2+} to Aβ40 have been highlighted. B) Molecular visualization of binding of CG with Cu^{2+}-Aβ40 complex.

4B.2. Discussion

The aggregation of Aβ is a key factor in the development of AD. It has been shown that physiological levels of metals such as Cu^{2+} and Zn^{2+} cause marked aggregation of Aβ (Bush et al., 1994). Metal chelators specific to Cu^{2+} and Zn^{2+} are known to reverse this Aβ aggregation process (Huang et al., 1997; Cherny et al., 1999). Further, neurotoxicity of Aβ is also linked to metal –induced oxidative damage observed in AD (Smith et al., 1997). Consequently, molecules with potent antioxidant and anti-inflammatory activities as well as properties of metal chelation would be best suited as therapeutic agents for AD. In particular, curcumin (diferulomethane), a low molecular weight molecule possess potent antioxidant and anti-inflammatory activities that has a

favorable toxicity profile and is under development and acts as a potential anti-fibrillogenic agent (Ringman et al 2004; Ono and Yamada, 2006, Dikshit et al., 2006; Frautschy et al., 2001; Lim et al., 2001; Ono et al., 2004; Kim et al., 2005). Yang et al., 2005 hypothesized that polar Aβ binding compounds like curcumin might be able to cross the blood brain barrier and bind to Aβ and related aggregates. It was reported that curcumin could bind amyloid to inhibit Aβ aggregation as well as fibril and oligomer formation with dosing at achievable levels (Yang et al., 2005). Besides above, curcumin chelation of both iron and copper has been proposed as one mechanism potentially contributing to amyloid reduction in animal models (Baum and Ng, 2004).

Curcumin has a structure similar to Congo red (Klunk et al., 1999) but with the charge replaced by polar groups like the brain permeable compound Chrysamine G (Klunk et al., 1999; Klunk et al., 1994). It is also similar to RS-0406, a novel compound selected from a screen of 113,000 compounds as a potent inhibitor of amyloid beta (Aβ) formation. Curcumin have two 4-hydroxy-3-methoxyphenyl rings or 3,4-dihydroxyphenyl rings symmetrically bound by a short hydrocarbon chain. Similarly the ferulic acid structure contains one 4-hydroxy-3-methoxyphenyl ring and has been identified as a degradation product of curcumin (Wang et al., 1997). The selection of curcumin-glucoside as a test compound was based on structural analogies with congo red, iododoxorubicin, curcumin. Although the molecular basis of curcumin as anti-amyloidogenic and fibril destabilizing in Aβ, is understood to a certain extent, it may be related to the propensity of these compounds to bind the fibrillogenic structure. Above all, curcumin is a potent antioxidant and an effective anti-inflammatory compound with anti-tumor activity (Zhao et al., 1989; Sreejayan and Rao 1997; Xu et al., 1998).

CG, has a basic structure of curcumin, with the 2 hydroxyl groups in phenolic rings replaced by glucose moieties, which augment the solubility of the molecule. Curc-gluc once enters the system is enzymatically degraded by glucosidases to mono and di-glucosides, making curcumin readily available for the interaction with Aβ. Alternatively this compact structure might be quite suitable for specifically binding to Aβ and subsequent destabilization of the β-sheet rich conformation in fibrils.

Brain levels of curcumin in the 0.1-1μM range are similar to those required to inhibit central nervous system AP-1 mediated transcription in vivo and related suppression of inducible nitric-oxide synthase and antioxidant activities. These doses can probably be achieved physiologically. Curc-gluc is rapidly glucorinidated after oral dosing so plasma levels remain low, despite high intake. High oral curcumin dosing appears safe (e.g. 4-8 g doses in human patients).

Our results demonstrate that CG inhibits Cu^{2+} induced Aβ aggregation and also causes disaggregation of preformed fibrils. Inhibition of Aβ fibril formation and destabilization of preformed fibrils by curcumin were also investigated previously (Yang et al., 2005). Ultra structural evidence, illustrates very fewer fibrils in CG treated Aβ pre-formed fibrils. The diketone and pairs of phenol and methoxy groups on the curcumin molecule might give it the ability to chelate cations (Ahsan et al., 1999; Araujo and Leon, 2001; Sreejayan and Rao, 1994). The β-diketone of curcumin readily exists in the keto-enol form (Pan et al., 1999) and perhaps this facilitates removal of metal ions from Aβ. There are some other drugs also which act by binding to metal ions. Infact, the anti-inflammatory action of curcumin, mediated by inhibition of NF-κB may also involve the possibility of binding of metals. The modification of curcumin to curcumin glucoside does not alter the diketone and methoxy groups which may bind metal ions, except for glucuronidation, which occupies both the phenolic groups (Ireson et al., 2001; Pan et al., 1999). Studies suggest that curcumin can bind metal ions such as copper and iron Araujo and Leon, 2001; Began et al., 1998; Began et al., 1999; Patro et al., 2002; Sreejayan and Rao, 1994). Baum and Ng, 2004 have spectrophotometrically quantified curcumin affinity for copper and iron, where each of these metal ions appeared to bind at least two curcumin molecules. They also showed that curcumin binding with metal has affinity in a range comparable to that of Aβ with Cu^{2+}(Baum and Ng, 2004). Hence, mechanism of curcumin binding to Aβ might be competition for or removal of metal from Aβ. Curcumin is reported to cross the blood brain barrier as mice injected intraperitoneally with 0.01 g curcumin per 100 g body weight had brain concentration of curcumin upto 0.3 μM (Pan et al., 1999). Hence, it can be said that the possible physiological effects of curcumin may be dependent upon a sufficient uptake into the brain. Thus, curcumin

might not only function as an antioxidant and inhibitor of Aβ fibril formation directly but also indirectly by chelating iron and copper to prevent Aβ aggregation. Hence we demonstrated that CG can prevent Cu^{2+} induced Aβ fibril formation at tolerable doses which is consistent with recent reports from Ono et al (2004) and Yang et al., (2006), even though mechanisms appear to be different.

Further studies, such as NMR experiments, are essential to reveal the exact structure-activity relationships for the better understanding of anti-fibrillogenic and fibril-destabilizing effects of these molecules on Aβ. The present study emphasizes the need for further research into the better understanding of the conformation dynamics, in particular stabilizing the monomeric species and mechanism of inhibition of oligomer formation, for more detailed studies at molecular level. Such information could be applicable to the design of drugs to curtail the fibril formation of β-sheet. Thus although in vivo studies will be necessary to establish the efficacy of the anti-aggregating and fibril destabilizing capacity of CG, our data strongly suggest that it might offer a useful therapeutic approach to AD.

CHAPTER 5:

Summary & Conclusion

5.1. Summary and Conclusion

AD is a progressive, degenerative disease of the brain, which causes thinking and memory to become seriously impaired. The symptoms include loss of memory, judgment and reasoning, and changes in mood, behavior and communication abilities. The histopathologic characteristic features seen in the brain of AD patients are the presence of SPs with Aβ accumulation, NFTs and cerebrovascular amyloid deposits. The etiology of AD has not been fully understood. Unproven hypotheses have included environmental toxins including metals, pesticides etc., head injury, inflammation, aging and genetic risk factors. A variety of mechanisms that are believed to cause accelerated neuronal death in AD have also been suggested, including oxidative stress, caspase activation, mitochondrial membrane depolarization, dysregulation of the cytosolic, mitochondrial and ER calcium homeostasis and excitotoxicity.

Despite progress in uncovering many of the factors that contribute to the etiology of the disease, the mechanism of the nerve cell death still remains unknown. It is very well accepted that conformational changes in the proteins namely Aβ play an important role in neurodegeneration in AD, however little is known about the DNA conformational alteration. A few earlier studies reported the nuclear localization of Aβ immunoreactivity. Moreover, there appeared to be an independent non-apoptotic relationship between DNA binding, DNA damage and Aβ in AD. Taken together, the above findings strongly link the Aβ and DNA damage.

Amyloid fibril formation is a process during which soluble proteins misfold and aggregate into fibrillar structures. In addition to the proposed genetic mutations or a polymorphism of presenilins, APP or Apo E, the post translational modifications of Aβ such as isomerization/ racemization are well speculated as enhancers for Aβ aggregation. Since racemization of amino acid residues in Aβ causes structural changes and functional inactivation or enhances the aggregation process in the modified proteins, this post-translational modification is suggested to be one of the progression factors in sporadic cases of AD. Further, the aggregation properties of Aβ strongly depend upon the aminoacid sequence and their stereospecificity. Racemization of different aminoacids in Aβ has varied effects on its aggregation kinetics. Previous studies have shown that all D-enantiomers of Aβ (25-35) and Aβ (1-42) exhibit similar biological properties to their L- counterparts. However, there

are no studies to understand the effect of complete racemization of aminoacids comprising Aβ (1-40) on its DNA nicking and aggregation properties.

Further, the disease modifying treatment of AD involves reduction in Aβ aggregation and toxicity. Therefore, many therapeutic efforts are targeted at reducing Aβ production, increasing Aβ clearance and inhibiting Aβ aggregation. A number of antioxidants have been reported to inhibit the formation and extension of Aβ fibrils, as well as to destabilize preformed fibrillar Aβ in vitro. Guided with this precept, a search for the natural inhibitors of fibrillogenesis such as, bioavailable components of garlic and curcumin could be realistic to clinical practice. Garlic and its components were demonstrated to have anti-amyloidogenic, anti-inflammatory and anti-tangle effects in Alzheimer's transgenic model Tg2576. Further, severe dysregulation of metal ion homeostasis has been reported in AD. Increased concentrations of metals like Cu^{2+} are detected in SPs in AD, where it is known to bind and induce Aβ aggregation. Studies also suggested that curcumin can bind metal ions such as copper and iron. It is also known to significantly reduce levels of brain amyloid, plaques and also inhibit the formation of Aβ oligomers and fibrils *in vitro*. However, there are limited studies to show the effect of Garlic, its component SAC and curcumin on modulation of Aβ aggregation.

The present work is focused on

Objectives:

a) To study the role of amino acid racemization on aggregation kinetics of Aβ(1-40).

b) To understand the role of stereo-specificity and modified aminoacids on Aβ (1- 40) interaction with DNA.

c) To study the role of garlic active components and other selected dietary molecules on modulation of conformation and aggregation of Aβ (1-40).

Chapter 1: General Introduction:

1. AD is one of the most common forms of senile dementia and Aβ deposition is a hallmark feature of its pathogenesis.

2. AD is usually classified into early-onset and late-onset dementia and also into familial and sporadic forms according to the genetic background.

3. The genetic risk factors of AD include mutations in the genes for APP on chromosome 21, PS-1 on chromosome 14, and PS-2 on chromosome1.

4. The environmental risk factors include metals, head injury, long-term depression, high-fat diet, oxidative stress, inflammation, aging, smoking, lack of exercise, alcohol, pesticides etc.

5. Large extracellular Aβ plaques and tau-containing intraneuronal NFTs characterize AD from a histopathologic perspective.

6. The most leading hypothesis regarding the cause of AD identifies the cytotoxic species as an intermediate misfolded form of Aβ, neither a soluble monomer nor a mature aggregated polymer but an oligomeric species.

7. Aβ peptides are reported to trigger the degeneration of neuronal cells via activation of both an apoptotic as well as non-apoptotic pathway.

8. DNA fragmentation could be one of the crucial steps in B→Z DNA topological transition observed in AD brain.

9. Recent data supported the concept of intracellular existence and toxicity of Aβ. These studies suggested that Aβ-dependent toxicity could occur before significant extracellular accumulation.

10. Various reports highlighted the intranuclear localization of Aβ in the vicinity of DNA in the AD brain. They proposed that intranuclear Aβ might have role in inducing DNA damage by direct or indirect mechanisms.

11. Racemization of aminoacid residues in Aβ causes structural and functional changes, this post-translational modification is suggested to be one of the progression factors in sporadic cases of AD.

12. Compounds that hinder the prevention of Aβ aggregation into multimeric species are most effective as they stop the protein polymerization prior to the formation of the primary cytotoxic moieties.

Chapter 2: Role of amino acid stereospecificity in Aβ aggregation

1. This study is an attempt to understand how changing the stereospecificity of the amino acids comprising Aβ influences the characteristics of Aβ aggregation parameters.

2. The CD spectrum of Aβ(1-40) all L had a strong negative band at 198 nm region, indicative of random coil conformation. However, the CD spectrum of Aβ(1-40) all-D was a mirror image of Aβ(1-40) all L-spectrum.

3. Thio-T assay showed differential aggregation pattern of both Aβ enantiomers. Both initial and final Thio-T values are high in case of Aβ(1-40) all-L. However, Aβ(1-40) all-D showed a sharper increase during transition phase.

4. TEM studies showed that both L and D enantiomers of Aβ(1-40) formed insoluble fibrillar aggregates. Aβ(1-40) all-L gave rise to longer fibrils with branching and D-form formed shorter fibrils with no branching.

5. Intrinsic Tyrosine fluorescence intensity investigated the differences in conformation of Aβ40 enantiomers assembly. The conformational changes take place in both Aβ(1-40) all-L and Aβ(1-40) all-D but relatively slower in Aβ(1-40) all-D.

6. Acrylamide fluorescence quenching indicated that the tyrosine residues of natively unfolded Aβ enantiomers were partially protected from quencher molecules. However, the degree of quenching induced in Aβ(1-40) all-D by acrylamide is comparatively lesser.

7. ANS binding studies showed that with aging there was induction of blue shift (~20 nm) in ANS λ_{max} in Aβ enantiomers but formation of partially folded conformation with solvent exposed hydrophobic patches was much quicker in Aβ(1-40) all-L when compared to Aβ(1-40) all-D.

8. The data suggest that the aggregation and folding parameters of Aβ are stereospecific.

Chapter3: Differential DNA nicking activity of L- and D- enantiomers of Aβ (1-40)

1. The neuronal localization of Aβ immunoreactivity in apoptotic nuclei in the vicinity of DNA/ chromatin of the hippocampal neurons in human AD brain has been reported and various Aβ fragments are also known to effectively bind and alter the conformation of DNA.

2. In this study, we characterized the stereospecific based binding and DNA nicking activity of soluble and aggregated forms of both Aβ (1-40) all L and Aβ (1-40) all D.

3. CD studies revealed that Aβ(1-40) all-L induced ψ -DNA conformation, however, altered B-DNA was resulted by the interaction of Aβ(1-40) all D with Sc DNA.

4. Both Aβ40 enantiomers have DNA nicking activity like nucleases in a concentration and time-dependent manner, but D-enantiomer of Aβ40 was more

potential in nicking ScDNA when compared to L-enantiomer of Aβ40 as observed in Agarose Gel elctrophoresis and Nick translation Assay.

5. Both Mg^{2+} and Ca^{2+} enhanced the DNA nicking activity of Aβ(1-40) all-L and Aβ(1-40) all D, whereas Zn inhibited DNA nicking activity of both Aβ40 enantiomers.

6. The maximum DNA nicking activity was observed at 12 hrs of Aβ peptide aging and the nicking activity of both Aβ40L and Aβ40D decreased after 12 hrs of aging. Both Aβ40L and Aβ40D in aggregated state had comparatively less nicking activity when compared to freshly dissolved ones.

7. Both Aβ40L and Aβ40D only nicked ssc DNA and did not have an effect on dscDNA, hence evidencing that Aβ40 enantiomers nicking is single stranded specific. Moreover Nick translation assay showed that the appearance of DSBs was due to the result of cumulative single strand breaks in ScDNA.

8. The nuclease inhibitors like ATA and DEPC abolished DNA nicking activity of Aβ peptides. Moreover, Aβ40 showed sequence homology with many other DNA binding proteins and nucleases. Hence, we hypothesized that Aβ may induce toxicity through an independent non-apoptotic mechanism by behaving like a nuclease.

Chapter 4: Anti-amyloidogenic activity of Garlic, its components and curcumin derivative on Aβ(1-40)

The dietary components like garlic, SAC and curcumin are known to have biological activities like anti-amyloidogenic, anti-inflammatory effects. But mechanistic studies are not clearly understood.

Part A

1. Thio-T assay and TEM studies showed there was a dose-dependent inhibition of Aβ40 aggregation and disaggregation of preformed fibrils with increasing w/w ratio of FGE. The inhibition of Aβ aggregation in presence of BGE eliminated the possibility of Aβ degradation by proteases present in garlic.

2. A component of garlic, SAC also showed inhibitory effect on Aβ aggregation and disaggregation of Aβ preformed fibrils as revealed by both Thio-T assay and TEM.

3. Both FGE and BGE inhibited oligomerization of Aβ as evidenced by a single band equivalent to that of 4kD in SDS-PAGE. SAC also inhibits the formation of higher molecular weight assemblies of Aβ.

4. SE-HPLC showed that in presence of SAC, the major peak obtained corresponded to that of soluble Aβ. The addition of SAC in the middle of the fibrillation reaction, caused the peaks corresponding to the multimeric forms of Aβ to disappear.

5. When Aβ was incubated in presence of SAC, the CD spectrum obtained had a broad negative band ranging from 198nm to 200nm, along with another shorter negative band in the 220 to 225 nm region. This indicated that SAC was capable of holding majority of Aβ in random coil.

6. Fluorescence quenching measurements revealed that there was a gradual decrease in the fluorescence intensity on addition of SAC indicating burying of the tyrosine in the core of the newly formed conformation of Aβ.

7. The docking search in and around the Aβ40 structure readily resulted in the identification of a site suitable for accommodating SAC. SAC has the possibility of interaction with the positively charged Gln^{15}-Lys^{16} segment in Aβ.

Part B

8. CG inhibited Cu^{2+} induced Aβ aggregation and also causes disaggregation of preformed fibrils in a concentration dependent manner as revealed by Thio-T assay and TEM.

9. CG inhibited the formation of higher molecular weight assemblies and stabilizes the 4 kD soluble Aβ in presence of Cu^{2+} as shown by SDS-PAGE.

10. The predominant end product after incubation of soluble Aβ with CG and in defibrillation process of preformed fibrils in presence of Cu^{2+} corresponded to that of soluble Aβ.

11. In presence of CG, majority of Aβ on incubation with Cu^{2+} remained in random coil conformation as studied by circular dichroism.

12. The 3D structure of Aβ-Cu^{2+}-CG complex was also predicted employing automated docking studies.

5.2. Conclusion

The data suggested that the aggregation and folding parameters of Aβ were stereospecific. The differential aggregation pattern of both Aβ(1-40) all-L and all-D was revealed by both Thio-T pattern and TEM. Both enantiomers formed insoluble aggregates as a function of time with different fibrillar morphology. The monitoring

of intrinsic tyrosine fluorescence, acrylamide quenching, ANS binding studies demonstrated that L-enantiomer of Aβ(1-40) was more prone for aggregation when compared to that of D-enantiomer. This study clearly showed that the aggregation property of Aβ is coded in its sequence and depends upon the amino acid sequence and their stereospecificity.

Further, the two enantiomers of Aβ40 altered conformation of ScDNA differently. The ψ -DNA conformation induced by Aβ40L was observed to be similar in conformation to Z -DNA. The altered B-DNA resulted by the interaction of Aβ40D may also have its basic characteristics altered when compared to B-DNA. The D-enantiomer of Aβ40 was more potential in nicking ScDNA when compared to L-enantiomer of Aβ40. SSBs formed were more compared to DSBs, indicating that DSBs were formed as a result of cumulative SSBs in ScDNA. Moreover, both Aβ40L and D showed enhanced DNA nicking activity in presence of Mg^{2+} and Ca^{2+} whereas Zn inhibited Aβ40L/D from nicking DNA. The nuclease inhibitors like ATA and DEPC abolished DNA nicking activity of Aβ peptides. Hence, it can be hypothesized that Aβ may induce toxicity through an independent non-apoptotic mechanism by behaving like a nuclease.

There was a dose-dependent inhibition of Aβ40 aggregation with increasing w/w ratio of FGE. The inhibition of Aβ aggregation in presence of BGE eliminated the possibility of Aβ degradation by proteases present in garlic. The extract could even cause disaggregation of preformed fibrils in solution. Further, a component of garlic, SAC also showed inhibitory effect on Aβ aggregation and disaggregation of preformed fibrils. There is induction of partially folded conformation in Aβ on binding to SAC. Further, the results demonstrated that CG inhibited Cu^{2+} induced Aβ aggregation and also caused disaggregation of preformed fibrils. There was disappearance of Cu^{2+} induced large fibrillar networks of Aβ in presence of CG. It inhibited the formation of higher molecular weight assemblies and in presence of CG, majority of Aβ remained in random coil conformation. The 3D models of Aβ –SAC and Aβ-Cu^{2+}-CG complex were also predicted employing automated docking studies. Hence, garlic and curcumin could be used as an important bio-source for the development of potential anti-amyloidogenic agents.

CHAPTER 5

Bibliography

6.1. Bibliography

1. Abramov A.Y., Canevari L., Duchen M.R., Calcium signals induced by amyloid beta peptide and their consequences in neurons and astrocytes in culture. Biochim Biophys Acta. 1742 (2004) 81-87.
2. Aggarwal B.B., Sundaram C., Malani N., Ichikawa H., Curcumin: the Indian solid gold. Adv Exp Med Biol. 595 (2007) 1-75. Review.
3. Ahn B.W., Song D.U., Jung Y.D., Chay K.O., Chung M.A., Yang S.Y., Shin B.A., Detection of β-Amyloid peptide aggregation using DNA electrophoresis. Anal. Biochem. 284 (2000) 401-405.
4. Ahsan H., Parveen N., Khan N.U., Hadi S.M., Pro-oxidant, anti-oxidant and cleavage activities on DNA of curcumin and its derivatives demethoxycurcumin and bisdemethoxycurcumin. Chemico-Biol Interact. 121 (1999) 161-175.
5. Aigelsreiter A., Janig E., Stumptner C., Fuchsbichler A., Zatloukal K., Denk H., How a cell deals with abnormal proteins. Pathogenetic mechanisms in protein aggregation diseases. Pathobiol. 74 (2007) 145-158.
6. Akama K.T., Albanese C., Pestell R.G., Van Eldik L.J., Amyloid-beta peptide stimulates nitric oxide production in astrocytes through an NFkappaB-dependent mechanism. Proc Natl Acad Sci (USA) 95 (1998) 5795–5800.
7. Alberts B., Bray D., Lewis J., Raff M., Roberts K., Watson J.D., Molecular biology of the cell. Garland, New York, (1983) p. 286.
8. Alzheimer A., Uber eine eignartige erkrankung der birnrinde. All Z Psychiat. 64 (1907) 146-148.
9. Anderson A.J., Pike C.J., Cotman C.W., Differential induction of immediate early gene proteins in cultured neurons by β-amyloid (Abeta): association of c-Jun with Aβ-induced apoptosis. J. Neurochem. 65 (1995) 1487-1498.
10. Anitha S., Rao K.S.J., Latha K.S., Viswamitra M.A., First evidence to show the topological change of DNA from B-DNA to Z-DNA conformation in the hippocampus of Alzheimer's brain. J. Neurol. Med. 2 (2002) 287-295.

11. Anitha S., Stein R., Jagannatha Rao K.S., Latha K.S., Viswamitra M.A., A link between apoptotic DNA damage and DNA topology in Alzheimer's disease brain: a hypothesis. Alz. Rep. 4 (2001) 121-131.
12. Araujo C.A.C. and Leon L.L., Biological Activities of Curcuma longa L., Mem Inst Oswaldo Cruz. Rio de Janeiro 96 (2001) 723–728.
13. Arends M..J., Morris R.G., Wyllie A.H., Apoptosis. The role of the endonuclease. Am. J. Pathol. 136 (1990) 593-608.
14. Arispe N., Pollard H.B., Rojas E., Giant multilevel cation channels formed by Alzheimer disease amyloid beta-protein [A beta P-(1-40)] in bilayer membranes. Proc Natl Acad Sci. U S A. 90 (1993) 10573-10577.
15. Armstrong R.A., Plaques and tangles and the pathogenesis of Alzheimer's disease. Folia Neuropathol. 44 (2006) 1-11.
16. Artavanis-Tsakonas S., Rand M.D., Lake R.J., Notch signaling: cell fate control and signal integration in development. Science 284 (1999) 770-776.
17. Atwood C.S., Huang X., Moir R.D., Bacarra N.M., Romano D., Tanzi R.E., Bush A.I., Dramatic aggregation of Alzheimer Ab by Cu(II) is induced by conditions representing physiological acidosis. J Biol Chem. 273 (1998) 12817–12826.
18. Atwood C.S., Huang X., Moir R.D., Smith M.A., Tanzi R.E., Roher A.E., Bush A.I., Perry G., Neuroinflammatory responses in the Alzheimer's disease brain promote the oxidative post-translational modification of amyloid deposits. In: Iqbal K, Sisodia SS, Winblad B, editors. Alzheimer's disease: advances in etiology, pathogenesis and therapeutics. Chichester, UK: Wiley, (2001) 341–361.
19. Atwood C.S., Scarpa R.C. and Huang X. Moir R.D., Jones W.D., Fairlie D.P., Tanzi R.E., Bush A.I., Characterization of Copper interactions with Alzheimer Amyloid β Peptides: Identification of an Attomolar-Affinity Copper Binding Site on Amyloid β1–42, J Neurochem. 75 (2000) 1219–1233.
20. Augustinack J., Schneider A., Mandelkow E., Hyman B., Specific tau phosphorylation sites correlate with severity of neuronal cytopathology in Alzheimer's disease. Acta Neuropathol. (Berl) 103 (2002) 26-35.

21. Auwera Van der I., Wera S., Van Leuven F., Henderson S.T., A ketogenic diet reduces amyloid beta 40 and 42 in a mouse model of Alzheimer's disease. Nutr Metab. (Lond) 2 (2005) 28.
22. Bahr B.A., Hoffman K.B., Yang A.J., Hess U.S., Glabe C.G. and Lynch G., Amyloid beta protein is internalized selectively by hippocampal field CA1 and causes neurons to accumulate amyloidogenic carboxyterminal fragments of the amyloid precursor protein. J. Comp. Neurol. 397 (1998) 139-147.
23. Bala K., Tripathy B. C. and Sharma D., Neuroprotective and antiageing effects of curcumin in aged rat brain regions. Biogerontology 7 (2006) 81–89.
24. Bano S., Parihar M.S., Reduction of lipid peroxidation in different brain regions by a combination of α-tocopherol and ascorbic acid. J Neural Transm. 104 (1997) 1277-1286.
25. Barghorn S., Nimmrich V., Striebinger A., Krantz C., Keller P., Janson B., Bahr M., Schmidt M., Bitner R.S., Harlan J., Barlow E., Ebert U., Hillen H., Globular amyloid beta-peptide oligomer- a homogenous and stable neuropathological protein in Alzheimer's disease. J Neurochem. 95 (2005) 834-847.
26. Barinaga M., Death gives birth to the nervous system. But how? Science 263 (1994) 754-756.
27. Barnes N.Y., Li L., Yoshikawa K., Schwartz L.M., Oppenheim R.W., Milligan C.E., Increased production of amyloid precursor protein provides a substrate for caspase-3 in dying motoneurons. J. Neurosci. 18 (1998) 5869-5880.
28. Baron P., Galimberti D., Meda L., Scarpini E., Conti G., Cogiamanian F., Scarlato G., Production of IL-6 by human myoblasts stimulated with Abeta: relevance in the pathogenesis of IBM. Neurology 57(2001) 1561-1565.
29. Barrow C.J. and Zagorski M.G., Solution structures of beta peptide and its constituent fragments: relation to amyloid deposition. Science 253 (1991) 179-182.
30. Barrow C.J., Yasuda A., Kenny P.T., Zagorski M.G., Solution conformations and aggregational properties of synthetic amyloid beta-peptides of Alzheimer's disease. Analysis of circular dichroism spectra. J Mol Biol. 225 (1992) 1075-1093.

31. Barzilay G., Mol C.D., Robson C.N., Walker L.J., Cunningham R.P., Tainer J.A., Hickson I.D., Identification of critical active-site residues in the multifunctional human DNA repair enzyme HAP1. Nat. Struct. Biol. 2 (1995) 561-568.
32. Bauer W.R., Crick F.H.C., White H., Supercoiled DNA. Sci. Am. 16 (1980) 243–245.
33. Baum L. and Ng Alex., Curcumin interaction with copper and iron suggests one possible mechanism of action in Alzheimer's disease animal models. J. Alz. Dis. 6 (2004) 367-377.
34. Began G., Sudharshan E., Appu Rao A.G., Inhibition of lipoxygenase 1 by phosphatidylcholine micelles-bound curcumin. Lipids. 33 (1998) 1223-1228.
35. Began G., Sudharshan E., Udaya Sankar K., Appu Rao A.G., Interaction of curcumin with phosphatidylcholine: A spectrofluorometric study. J Agric Food Chem. 47 (1999) 4992-4997.
36. Behl A., Davis J., Cole G.M., Shubert D., Vitamin E protects nerve cells from amyloid-β protein toxicity. Biochem Bipohys Res Commun. 186 (1992) 944–950.
37. Behl C., Holsboer F., The female sex hormone estrogen as a neuroprotectant. Tredns Pharmacol Sci. 20 (1999) 441-444.
38. Bentahir M., Nyabi O., Verhamme J., Tolia A., Horré K., Wiltfang J., Esselmann H., De Strooper B., Presenilin clinical mutations can affect γ-secretase activity by different mechanisms. J. Neurochem. 96 (2006) 732-742.
39. Bera A., Roche A.C., Nandi P.K., Bending and unwinding of nucleic acid by prion protein. Biochemistry 46 (2007) 1320-1328.
40. Bergmeyer H.U., Methods of enzymatic analysis Verlag Chemie. Weinheim, Germany 6 (1984) 97-98.
41. Bertram L., Tanzi R.E., The current status of Alzheimer's disease genetics: what do we tell patients? Pharmacol Res. 50 (2004) 385-396.
42. Bieler S., Soto C., Beta-sheet breakers for Alzheimer's disease therapy. Curr Drug Targets. 6 (2004) 553-558. Review.
43. Billings L.M., Oddo S., Green K.N., McGaugh J.L. and Laferla F.M., Intraneuronal Abeta causes the onset of early Alzheimer's disease-related cognitive deficits in transgenic mice. Neuron 45 (2005) 675-688.

44. Binder L.I., Frankfurter A., Rebhun L.I., The distribution of tau in the mammalian central nervous system. J Cell Biol. 101 (1985) 1371-1378.
45. Blanchard B.J., Chen A., Rozeboom L.M., Stafford K.A., Weigele P., Ingram V.M., Efficient reversal of Alzheimer's disease fibril formation and elimination of neurotoxicity by a small molecule. Proc Natl Acad Sci. USA 101 (2004) 14326-14332.
46. Blanchard B.J., Hiniker A.E., Lu C.C., Margolin Y., Yu A.S., Ingram V.M., Elimination of Amyloid beta neurotoxicity. J Alzheimers Dis. 2 (2000) 137-149.
47. Block E. The chemistry of garlic and onions. Sci Am. 252 (1985) 114-119.
48. Bonomo S.M., Rigamonti A.E., Giunta M., Galimberti D., Guaita A., Gagliano M.G., Müller E.E., Cella S.G., Menopausal transition: A possible risk factor for brain pathologic events. Neurobiol Aging. (2007) Epub ahead of print.
49. Borek C., Garlic reduces dementia and heart-disease risk, J. Nutr. 136 (2006) 810S-812S. Review.
50. Braak H., Braak E., Neuropathological stageing of Alzheimer's-related changes. Acta Neuropathol. 82 (1991) 239–259.
51. Brion J.P., Flament-Durand J., Dustin P., Alzheimer's disease and tau proteins. Lancet 2 (1986) 1098.
52. Buckig A., Tikkanen R., Herzog V., Schmitz A., Cytosolic and nuclear aggregation of the amyloid beta-peptide following its expression in the endoplasmic reticulum. Histochem Cell Biol. 118 (2002) 353-360.
53. Burdick D., Soreghan B., Kwon M., Kosmoski J., Knauer M., Henschen A Yates J., Cotman C., Glabe C., Assembly and aggregation properties of synthetic Alzheimer's A4/beta amyloid peptide analogs. J Biol Chem. 267 (1992) 546–554.
54. Busciglio J., Lorenzo A., Yeh J., Yankner B.A., β-Amyloid fibrils induces tau phosphorylation and loss of microtubule binding. Neuron 14 (1995) 879-888.
55. Bush A.I., Pettingell W.H., Multhaup G., Paradis M.D., Vonsattel J.P., Gusella J.F., Beyreuther K., Masters C.L.,Tanzi R.E., Rapid induction of Alzheimer A beta amyloid formation by zinc. Science. 265 (1994) 1464-1467.

56. Butterfield D.A., Drake J., Pocernich C., Castenga A., Evidence of oxidative damage in Alzheimer's disease brain; central role for amyloid beta-peptide. Trends Mol Med. 7 (2001) 548-554.

57. Butterfield D.A., Martin L., Carney J.M., Hensley K., Aβ (25–35) peptide displays H_2O_2-like reactivity towards aqueous Fe^{2+}, nitroxide spin probes, and synaptosomal membrane proteins. Life Sci. 58 (1996) 217–228.

58. Byrne E., Does mitochondrial chain dysfunction have a role in common neurodegenerative disorders? J Clin Neurosci. 29 (2002) 222-230.

59. Cacabelos R., Genomic characterization of Alzheimer's disease and genotype-related phenotypic analysis of biological markers in dementia. Pharmacogenom. 5 (2004) 1049-1115.

60. Campion D., Brice A., Hannequin D., Charbonnier F., Dubois B., Martin C., Michon A., Penet C., Bellis M., Calenda A., Martinez M., Agid Y., Clerget-Darpoux F., Frebourg T., The French Alzheimer's disease study group: No founder effect in three novel Alzheimer's disease families with APP 717 Val-Ile mutation. J Med Genet. 33 (1996) 661-664.

61. Cao X., Sudhof T.C., A transcriptionally [correction of transcriptively] active complex of APP with Fe65 and histone acetyltransferase Tip60. Science 293, (2001) 115-120.

62. Casley C.S., Canevari L., Land J.M., Clark J.B., Sharpe M.A., Beta –amyloid inhibits integrated mitochondrial respiration and key enzyme activities. J. Neurochem. 80 (2002) 91-100.

63. Castellani R.J., Harris P.L., Sayre L.M., Fujii J., Taniguchi N., Vitek M.P., Founds H., Atwood C.S., Perry G., Smith M.A. Active glycation in neurofibrillary pathology of Alzheimer disease: N(epsilon)- (carv\boxymethyl) lysine and hexitol-lysine. Free Radic Biol Med. 31 (2001) 175-180.

64. Castenga A., Thongboonkerd V., Klein J.B., Lynn B., Markesbery W.R., Butterfield D.A., Proteomic identification of nitrated proteins in Alzheimer's disease brain. J Neurochem 85 (2003) 1394-1401.

65. Castillo G.M., Lukito W., Peskind E., Raskind M., Kirschner D.A., Yee A.G., Snow A.D., Laminin inhibition of beta-amyloid protein (Abeta) fibrillogenesis

and identification of an Abeta binding site localized to the globular domain repeats on the laminin a chain. J Neurosci Res. 62 (2000) 451-462.

66. Cavallito C.J., Bailey J.H., Allicin, the antibacterial principle of Allium sativum. I. Isolation, physical properties and antibacterial action. J. Am. Chem. Soc. 66 (1944) 1950.
67. Champ P.C., Maurice S., Vargason J.M., Camp T., Ho P.S., Distributions of Z-DNA and nuclear factor I in human chromosome 22: a model for coupled transcriptional regulation. Nucleic Acids Res. 32 (2004) 6501-6510.
68. Champion C.S.K.D., Rajan M.T., Rao K.S.J., Viswamitra M.A., Interaction of Co, Mn, Mg, and Al with d(GCCATGGC) and d(CCGGGCCCGG): a spectroscopic study. Cell Mol Life Sci. 54 (1998) 488–496.
69. Chan S.L., Culmsee C., Haughey N., Klapper W., Mattson M.P., Presenilin-1 mutations sensitize neurons to DNA damage-induced death by a mechanism involving perturbed calcium homeostasis and activation of calpains and caspase-12. Neurobiol. Dis. 11 (2002) 2-19.
70. Chandak G.R., Sridevi M.U., Vas C.J., Paniker D.M., Sing L., Apolipoprotein E and presenilin-1 allelic variation and Alzheimer's disease in India. Hum Biol. 74 (2002) 683-693.
71. Chandra V., Ganguli M., Pandav R., Johnston J., Belle S., DeKosky S.T., Prevalence of Alzheimer's disease and other dementias in rural India: the Indo-US study. Neurology 51 (1998) 1000-1008.
72. Chatterjee B., Rao G.R.K., Superhelical density of goat mitochondrial DNA: Fluorescent studies. Indian J. Biochem. Biophys. 31 (1994) 77-79.
73. Chauhan N.B., Effect of aged garlic extract on APP processing and tau phosphorylation in Alzheimer's transgenic model Tg2576. J. Ethnopharmacol. 108 (2006) 385-394.
74. Chauhan N.B., Sandoval J., Amelioration of early cognitive deficits by aged garlic extract in Alzheimer's transgenic mice. Phytother Res. 21 (2007) 629-640.
75. Chen F., Hasegawa H., Schmitt-Ulms G., Kawarai T., Bohm C., Katayama T., Gu Y., Sanjo N., Glista M., Rogaeva E., Wakutani Y., Pardossi-Piquard R., Ruan X., Tandon A., Checler F., Marambaud P., Hansen K., Westaway D., St George-

Hyslop P., Fraser P., TMP21 is a presenilin complex component that modulates gamma-secretase but not epsilon-secretase activity. Nature 440 (2006) 1208-1212.

76. Chen S.Y., Wright J.W., Barnes C.D., The neurochemical and behavioral effects of beta-amyloid peptide (25-35). Brain Res. 720 (1996) 54-60.
77. Cheng A.L., Hsu C.H., Lin J. K., Hsu M.M., Ho Y.F., Shen T.S., Ko J.Y., Lin J.T., Lin B.R., Ming-Shiang W., Yu H.S., Jee S.H., Chen G.S., Chen T.M., Chen C.A., Lai M.K., Pu Y.S., Pan M.H., Wang Y.J., Tsai C.C., Hsieh C.Y., Phase I clinical trial of curcumin, a chemopreventive agent, in patients with high-risk or pre-malignant lesions. Anticancer Res. 21 (2001) 2895–2900.
78. Cherny D., Hoyer W., Subramaniam V., Jovin T.M., Double stranded DNA stimulates the fibrillation of α-synuclein *in vitro* and is associated with the mature fibrils: An electron microscopy study. J. Mol. Biol. 344 (2004) 929-938.
79. Cherny D.I., Striker G., Subramaniam V., Jett S.D., Palecek E., Jovin T.M., DNA bending due to specific p53 and p53 core domain DNA interactions visualized by electron microscopy. J. Mol. Biol. 294 (1999) 1015-1026.
80. Cherny R.A., Atwood C.S., Xilinas M.E., Gray D.N., Jones W.D., McLean C.A., Barnham K.J., Volitakis I., Fraser F.W., Kim Y., Huang X., Goldstein L.E., Moir R.D., Lim J.T., Beyreuther K., Zheng H., Tanzi R.E., Masters C.L. and Bush A.I., Treatment with a copper-zinc chelator markedly and rapidly inhibits β- amyloid accumulation in Alzheimer's disease transgenic mice. Neuron. 30 (2001) 665–676.
81. Cherny R.A., Legg J.T., McLean C.A., Fairlie D.P., Huang X., Atwood C.S., Beyreuther K., Tanzi R.E., Masters C.L., Bush A.I., Aqueous Dissolution of Alzheimer's Disease Aβ Amyloid Deposits by Biometal Depletion, J Biol Chem. 274 (1999) 23223–23228.
82. Choi D.W., Calcium-mediated neurotoxicity: relationship to specific channel types and role in ischaemic damage. Trends Neurosci. 10 (1988) 465-469.
83. Citron M., Secretases as targets for the treatment of Alzheimer's disease. Mol Med Today 6 (2000) 392-397.
84. Citron M., Westaway D., Xia W., Carlson G., Diehl T., Levesque G., Johnson-Wood K., Lee M., Seubert P., Davis A., Kholodenko D., Motter R., Sherrington

R., Perry B., Yao H., Strome R., Lieberburg I., Rommens J., Kim S., Schenk D., Fraser P., St George Hyslop P., Selkoe DJ., Mutant presenilins of Alzheimer's disease increase production of 42-residue amyloid β-protein in both transfected cells and transgenic mice. Nature medicine 3 (1997) 67-72.

85. Clearly M.L., Smith S.D., Sklar J., Cloning and structural analysis of cDNAs for bcl-2 and a hybrid cl-2/immunoglobulin transcript resulting from the t (14;18) translocation. Cell 47 (1986) 19-28.
86. Cohen G.M., Caspases: the executioners of apoptosis. Biochem J. 326 (1997) 1-16.
87. Cole G.M., Morihara T., Lim G.P., Yang F., Begum A., Frautschy S.A., NSAID and antioxidant prevention of Alzheimer's disease: lessons from in vitro and animal models. Ann. N. Y. Acad. Sci. 1035 (2004) 68–84.
88. Colurso G.J., Nilson J.E., Vervoort L.G., Quantitative assessment of DNA fragmentation and beta-amyloid deposition in insular cortex and midfrontal gyrus from patients with Alzheimer's disease. Life Sci. 73 (2003) 1795-1803.
89. Cook D.G., Forman M.S., Sung J.C., Leight S., Kolson D.L., Iwatsubo T., Lee V.M., Doms R.W., Alzheimer's A beta (1-42) is generated in the endoplasmic reticulum/ intermediate compartment of NT2N cells. Nat. Med. 5 (1997) 560-564.
90. Cooke J.R., McKie E.A., Ward J.M., Keshavarz-Moore E., Impact of intrinsic DNA structure on processing of plasmids for gene therapy and DNA vaccines. J Biotechnol. 114 (2004) 239-254.
91. Corces V.G., Manso R., DeLaTorre J., Avila J., Nasr A., Wiche G., Effects of DNA on microtubule assembly. Eur J Biochem. 105 (1980) 7-16.
92. Corder E.H., Saunders A.M., Strittmatter W.J., Schmechel D.E., Gaskell P.C., Small G.W., Roses A.D., Haines J.L., Pericak-Vance M.A., Gene dose of apolipoprotein E type e4 allele and the risk of Alzheimer's disease in late onset families. Science 261 (1993) 921-923.
93. Cotman C.W., Anderson A.J., A potential role for apoptosis in neurodegeneration and Alzheimer's disease. Mol Neurobiol. 10 (1995) 19-45.
94. Cotman C.W., Pike C.J., Copani A., β-Amyloid neurotoxicity: a discussion of in vitro findings. Neurobiol Aging 13 (1992) 587–590.

95. Cotman C.W., Whittemore E.R., Watt J.A., Anderson A.J., Loo D.T., Possible role of apoptosis in Alzheimer's disease. Ann N Y Acad Sci. 747 (1994) 36-49.
96. Cottrell D.A., Blakely E.L., Johnson M.A., Ince P.G., Turnbull D.M., Mitochondrial enzyme deficient hippocampal neurons and choroidal cells in AD. Neurology 57 (2001) 260-264.
97. Crapper McLachlan D.R., Quittkat S., De Boni U., Altered chromatin conformation in Alzheimer's disease. Brain 102 (1979) 483–495.
98. Crapper McLachlan D.R., Bergeron C., Smith J.E., Boomer D., Rifat S.L., Risk for neuropathologically confirmed Alzheimer's disease and residual aluminium in municipal drinking water employing weighted residential histories. Neurology. 46 (1996) 401–405.
99. Crapper McLachlan D.R., Dalton A.J., Kruck T.P., Bell M.Y., Smith W.L., Kalow W., Andrews D.F., Intramuscular desferrioxamine in patients with Alzheimer's disease. Lancet 337 (1991) 1304-1308.
100. Crapper McLachlan D.R., Kruck T.P., Lukiw W.J., Krishnan S.S., Would decreased aluminum ingestion reduce the incidence of Alzheimer's disease? CMAJ. 145 (1991) 793-804.
101. Crass P., Smith M.A., Richey P.L., Siedlak S.L., Mulvihill P., Perry G., Extracellular neurofibrillary tangles reflect neuronal loss and provide further evidence of extensive protein cross-linking in Alzheimer disease. Acta Neuropathol. 89 (1995) 291-295.
102. Cribbs D.H., Pike C.J., Weinstein S.L., Velazquez P., Cotman C.W., All-D-enantiomers of beta-amyloid exhibit similar biological properties to all-L-beta-amyloids. J. Biol. Chem. 272 (1997) 7431-7436.
103. Cuajungco M.P., Faget K.Y., Zinc takes the center stage: its paradoxical role in Alzheimer's disease. Brain Res Brain Res Rev. 41 (2003) 44-56.
104. Cuajungco M.P., Goldstein L.E., Nunomura A., Smith M.A., Lim J.T., Atwood C.S., Huang X., Farrag Y.W., Perry G., Bush A.I., Evidence that the Beta amyloid plaques of Alzheimer's disease represent the redox silencing and entombment of Aβ by Zinc. J Biol Chem. 275 (2000) 19439-19442.

105. Cuello A.C., Intracellular and extracellular Abeta, a tale of two neuropathologies. Brain Pathol. 15 (2005) 66-71.
106. Cummings J.L. and Cole G., Alzheimer disease, JAMA. 287 (2002) 2335–2338.
107. D'Andrea M.R., Nagele R.G., Wang H.Y., Peterson P.A. and Lee D.H. Evidence that neurons accumulating amyloid can undergo lysis to form amyloid plaques in Alzheimer's disease. Histopathology 38 (2001) 120-134.
108. Danielsson J., Andersson A., Jarvet J., Graslund A.,. 15N relaxation study of the amyloid beta-peptide: structural propensities and persistence length. Magn Reson Chem. 44 Spec No. (2006) S114-S121.
109. Davies S.W., Turmaine M., Cozens B.A., DiFiglia M., Sharp A.H., Ross C.A., Scherzinger E., Wanker E.E., Mangiarini L., Bates G.P., Formation of neuronal intranuclear inclusions underlies the neurological dysfunction in mice transgenic for the HD mutation. Cell 90 (1997) 537-548.
110. De Felice F.G., Velasco P.T., Lambert M.P., Viola K., Fernandez S.J., Ferreira S.T., Klein W.L., Aβ oligomers induce neuronal oxidative stress through an NMDA receptor-dependent mechanism that is blocked by the Alzheimer's drug memantine. J. Biol. Chem. 282 (2007) 11590-11601.
111. De Strooper B., Annaert W., Proteolytic processing and cell biological functions of the amyloid precursor protein. J Cell Sci. 113 (Pt 11) (2000) 1857-1870.
112. DeGiorgio L.A., DeGiorgio N., Milner T.A., Conti B., Volpe B.T., Neurotoxic APP C-terminal and beta-amyloid domains colocalize in the nuclei of substantia nigra pars reticulata neurons undergoing delayed degeneration. Brain Res. 874 (2000) 137-146.
113. Deloncle R., Guillard O., Mechanism of Alzheimer's disease: arguments for a neurotransmitter-aluminium complex implication. Neurochem. Res. 15 (1990) 1239-1245.
114. Deng G., Wu R., Terminal transferase: use in the tailing of DNA and for in vitro mutagenesis. Methods Enzymol. 100 (1983) 96-116.
115. Deshpande A., Mina E., Glabe C., Busciglio J., Different conformations of amyloid beta induce neurotoxicity by distinct mechanisms in human cortical neurons. J. Neurosci. 26 (2006) 6011-6018.

116. Dewar M.J.S., Zoebisch E.G., Healy E.F. Stewart J.J.P. AM1: A general purpose quantum mechanical molecular model. J Am Chem Soc. 107 (1985) 3902-3909.

117. DiFiglia M., Sapp E., Chase K.O., Davies S.W., Bates G.P., Vonsattel J.P., Aronin N., Aggregation of huntingtin in neuronal intranuclear inclusions and dystrophic neurites in brain. Science 277 (1997) 1990-1993.

118. Dikalov S.I., Vitek M.P., Maples K.R., Mason R.P., Amyloid-β-peptides do not form peptide-derived free radicals spontaneously, but enhance metal-catalyzed oxidation of hydroxylamines to nitroxides. J Biol Chem. 274 (1999) 9392–9399.

119. Dikshit P., Goswami A., Mishra A., Nukina N., Jana N.R., Curcumin enhances the polyglutamine-expanded truncated N-terminal huntingtin-induced cell death by promoting proteasomal malfunction. Biochem Biophys Res Commun. 342 (2006) 1323-1328.

120. Divry P., Etude histo-chimique des plaques seniles. J Belge Neurol Psychiat 27 (1927) 643-657.

121. Doraiswamy P.M., Finefrock A.E., Metals in our minds: therapeutic implications for neurodegenerative disorders. Lancet Neurol. 3 (2004) 431-434.

122. Dosunmu R., Wu J., Basha M.R., Zawia N.H., Environmental and dietary risk factors in Alzheimer's disease. Expert Rev Neurother. 7(2007) 887-900. Review.

123. Duan J., Nilsson L., Effect of Zn^{2+} on DNA recognition and stability of the p53 DNA-binding domain. Biochemistry. 45 (2006) 7483-7492.

124. Durell S.R., Guy H.R., Arispe N., Rojas E., Pollard H.B., Theoretical models of the ion channel structure of amyloid beta-protein. Biophys J. 67 (1994) 2137-2145.

125. Dyrks T., Dyrks E., Hartmann T., Masters C., Beyreuther K., Amyloidogenicity of βA4 and β A4-bearing amyloid protein precursor fragments by metal-catalyzed oxidation. J.Biol. Chem. 267 (1992) 18210-18217.

126. Earnshaw W.C., Apoptosis. A cellular poison cupboard. Nature 397 (1999) 387-389.

127. Echeverria V., Ducatenzeiler A., Dowd E., Janne J., Grant S.M., Szyf M., Wandosell F., Avila J., Grimm H., Dunnett S.B., Hartmann T., Alhonen L., Cuello A.C., Altered mitogen-activated protein kinase signaling, tau

hyperphosphorylation and mild spatial learning dysfunction in transgenic rats expressing the beta-amyloid peptide intracellularly in hippocampal and cortical neurons. Neuroscience 129 (2004) 583-592.

128. Ehehalt R., Keller P., Haass C., Thiele C., Simons K., Amyloidogenic processing of the Alzheimer beta-amyloid precursor protein depends on lipid rafts. J Cell Biol. 160 (2003) 113-123.
129. Espeseth A.S., Xu M., Huang Q., Coburn C.A., Jones K.L., Ferrer M., Zuck P.D., Strulovici B., Price E.A., Wu G., Wolfe A.L., Lineberger J.E., Sardana M., Tugusheva K., Pietrak B.L., Crouthamel M.C., Lai M.T., Dodson E.C., Bazzo R., Shi X.P., Simon A.J., Li Y., Hazuda D.J., Compounds that bind APP and inhibit Abeta processing in vitro suggest a novel approach to Alzheimer disease therapeutics. J Biol Chem. 280 (2005) 17792-17797.
130. Esposito L., Gan L., Yu G.Q., Essrich C., Mucke L., Intracellularly generated amyloid-beta peptide counteracts the antiapoptotic function of its precursor protein and primes proapoptotic pathways for activation by other insults in neuroblastoma cells. J Neurochem. 91 (2004) 1260-1274.
131. Fenwick G.R., Hanley A.B., The genus Allium--Part 1. Crit Rev Food Sci Nutr. 22 (1985) 199-271. Review.
132. Findeis M.A., Musso G.M., Arico-Muendel C.C., Benjamin H.W., Hundal A.M., Lee J.J., Chin J., Kelley M., Wakefield J., Hayward N.J., Molineaux S.M., Modified-peptide inhibitors of amyloid beta-peptide polymerization. Biochemistry. 38 (1999) 6791-6800.
133. Finder V.H., Glockshuber R., Amyloid-beta aggregation. Neurodegener Dis. 4 (2007) 13-27.
134. Fisher G.H., D'Aniello A., Vetere A., Padula L., Cusano G.P., Man E.H., Free D-aspartate and D-alanine in normal and Alzheimer brain. Brain Res. Bull. 26 (1991) 983-985.
135. Folstein M.F., Folstein S.E., McHugh P.R., "Mini-mental state". A practical method for grading the cognitive state of patients for the clinician. J Psychiatr Res. 12 (1975) 189-198.

136. Forloni G., Bugiani O., Tagliavini F., Salmona M., Apoptosis-mediated neurotoxicity induced by beta-amyloid and PrP fragments. Mol. Chem. Neuropathol. 28 (1996) 163-171.

137. Forloni G., Colombo L., Girola L., Tagliavini F., Salmona M., Anti-amyloidogenic activity of tetracyclines: studies in vitro. FEBS Lett. 487 (2001) 404-407.

138. Frank R.A., Galasko D., Hampel H., Hardy J., Leon M.J., Mehta P.D., Rogers J., Siemers E., Trojanowski J.Q., Biological markers for therapeutic trials in Alzheimer's disease proceedings of the biological markers working group: NIA on neuroimaging in Alzheimer's disease. Neurobiol Aging. 24 (2003) 521-536.

139. Frankowski H., Missotten M., Fernandez P.A., Martinou I., Michel P., Sadoul R., Martinou J.C., Function and expression of Bcl-x gene in the developing and adult nervous system. Neuroreport 6 (1995) 1917-1921.

140. Frautschy S.A., Hu W., Kim P., Miller S.A., Chu T., Harris-White M.E., Cole G.M., Phenolic anti-inflammatory antioxidant reversal of Aβ-induced cognitive deficits and neuropathology. Neurobiol Aging 22 (2001) 993-1005.

141. Frederickson C.J., Neurobiology of zinc and zinc-containing neurons. Int. Rev. Neurobiol. 31 (1989) 145-238.

142. Gabbita S.P., Lovell M.A., Markesbery W.R., Increased nuclear DNA oxidation in the brain in Alzheimer's disease. J Neurochem. 71 (2001) 2034-2040.

143. Garcia I., Martinou I., Tsujimoto Y., Martinou J.C., Prevention of programmed cell death of sympathetic neurons by the bcl-2 protooncogene. Science 258 (1992) 302-304.

144. Garcia-Alloza M., Borrelli L.A., Rozkalne A., Hyman B.T., Bacskai B.J., Curcumin labels amyloid pathology in vivo, disrupts existing plaques, and partially restores distorted neurites in an Alzheimer mouse model. J Neurochem. 102 (2007) 1095-1104.

145. Garner M.M., Felsenfeld G., Effect of Z-DNA on nucleosome placement. J Mol Biol. 196 (1987) 581-590.

146. Gasparini L., Netzer W.J., Greengard P., Xu H., Does insulin dysfunction play a role in Alzheimer's disease. Trends Pharmacol Sci. 23 (2002) 288-293.

147. Geddes J.W., Anderson K.J., Cotman C.W., Senile plaques as aberrant sprout-stimulating structures. Exp Neurol. 94 (1986) 767–776.

148. Geiger T., Clarke S., Deamidation, isomerization, and racemization at asparaginyl and aspartyl residues in peptides. Succinimide-linked reactions that contribute to protein degradation. J. Biol. Chem. 262 (1987) 785-794.

149. Gervais F.G., Xu D., Robertson G.S., Vaillancourt J.P., Zhu Y., Huang J., LeBlanc A., Smith D., Rigby M., Shearman M.S., Clarke E.E., Zheng H., Van Der Ploeg L.H., Ruffolo S.C., Thornberry N.A., Xanthoudakis S., Zamboni R.J., Roy S., Nicholson D.W., Involvement of caspases in proteolytic cleavage of Alzheimer's amyloid-beta precursor protein and amyloidogenic A beta peptide formation. Cell 97 (1999) 395-406.

150. Geula C., Wu C.K., Saroff D., Lorenzo A., Yuan M., Yankner B.A., Aging renders the brain vulnerable to amyloid β-protein neurotoxicity. Nature Med. 4 (1998) 827-831.

151. Ghanta J., Shen C.L., Kiessling L.L., Murphy R.M., A strategy for designing inhibitors of beta-amyloid toxicity. J Biol Chem. 271(1996) 29525-29528.

152. Ginsberg S.D., Crino P.B., Hemby S.E., Weingarten J.A., Lee V.M., Eberwine J.H., Trojanowski J.Q., Predominance of neuronal mRNAs in individual Alzheimer’s disease senile plaques. Ann. Neurol. 45 (1999) 174-181.

153. Giovannelli L., Scali C., Faussone-Pellegrini M.S., Pepeu G., Casamenti F., Long-term changes in the aggregation state and toxic effects of beta-amyloid injected into the rat brain. Neurosci. 87 (1998) 349-357.

154. Glenner G.G., Wong C.W., Alzheimer’s disease: initial report of the purification and characterization of a novel cerebrovascular amyloid protein. Biochem Biophys Res Comm. 120 (1984) 885-890.

155. Goate A., Chartier-Harlin M.C., Mullan M., Brown J., Crawford F., Fidani L., Giuffra L., Haynes A., Irving N., James L., Segregation of a missense mutation in the amyloid precursor protein gene with familial Alzheimer's disease. Nature. 349 (1991) 704-706.

156. Goedert M., Klug A., Crowther R.A., Tau protein, the paired helical filament and Alzheimer's disease. J Alzheimers Dis. 9 (2006) 195-207.

157. Gomez-Isla T., Hollister R., West H., Mui S., Growdon J.H., Petersen R.C. Parisi JE, Hyman BT. et al., Neuronal loss correlates with but exceeds neurofibrillary tangles in Alzheimer's disease. Ann. Neurology 41 (1997) 17-24.
158. Gonzales C., Martin T., Cacho J., Breñas M.T., Arroyo T., García-Berrocal B., Navajo J.A., González-Buitrago J.M., Serum zinc, copper, insulin and lipids in Alzheimer's disease epsilon 4 apolipoprotein E allele carriers. Eur J Clin Invest. 29 (1999) 637-642.
159. Gonzalez G.R., Haxo R.S., Schleich T., Mechanism of Action of Polymeric Aurintricarboxylic Acid, a Potent Inhibitor of Protein-Nucleic Acid Interactions. Biochemistry 19 (1980) 4299-4303.
160. Govaerts L., Schoenen J., Bouhy D., Pathogenesis of Alzheimer's disease: molecular and cellular mechanisms. Rev. Med. Liege. 62 (2007) 209-216.
161. Graever M.B., Kosel S., Egensperger R., Banati R.B., Muler U., Bise K., Hoff P., Moller H.J., Fujisawa K., Mehraein P., Rediscovery of the case described by Alois Alzheimer in 1911: historical, histological and molecular genetic analysis. Neurogenet. 1 (1997) 73.
162. Grant S.M., Ducatenzeiler A., Szyl M., Cuello A.C., Aβ immunoreactive material is present in all intracellular compartments in transfected neuronally differentiated, P19 expressing the human amyloid β- protein precursor. J Alzheimer's Dis. 2 (2000) 207-222.
163. Gray D.M., Taylor T.N., Lang D., Dehydrated circular DNA: circular dichroism of molecules in ethanol solutions. Biopolymers 17 (1978) 1–45.
164. Gray E.G., Paula-Barbosa M., Roher A., Alzheimer's disease: paired helical filaments and cytomembranes. Neuropathol Appl Neurobiol. 13 (1987) 91-110.
165. Greenwood J.A., Johnson G.W., Localization and insitu phosphorylation state of nucleolar tau. Exp Cell Res. 220 (1995) 332-337.
166. Gregory G.C., Halliday G.M., What is the dominant Abeta species in human brain tissue? A review. Neurotox. Res. 7 (2005) 29-41.
167. Grundke-Iqbal I., Iqbal K., Quinlan M., Tung Y.C., Zaidid M.S., Wisniewski H.M., Microtubule-associated protein tau. A compartment of Alzheimer paired helical filaments. J Biol Chem. 261 (1986) 6084-6089.

168. Grundke-Iqbal I., Vorbrodt A.W., Iqbal K., Tung Y.C., Wang G.P., Wisniewski H.M., Microtubule- associated polypeptide tau are altered in Alzheimer paired helical filaments. Brain Res. 464 (1988) 43-52.

169. Guillaume D., Bertrand P., Dea D., Davignon J., Poirier J., Apolipoprotein E and low-density lipoprotein binding and internalization in primary cultures of rat astrocytes: isoform-specific alterations. J Neurochem. 66 (1996) 2410-2418.

170. Gupta V.B., Anitha S., Hegde M.L., Zecca L., Garruto R.M., Ravid R., Stein R., Shankar S., Shanmugavelu P., Rao K.S.J., Aluminum in Alzheimer's disease: Are we at a cross roads. Cell Mol Life Sci. 62 (2005) 143-158.

171. Gupta V.B., Hegde M.L., Rao K.S.J., Role of protein conformational dynamics and DNA integrity in relevance to neuronal cell death in neurodegeneration. Curr. Alzheimer. Res. 3 (2006) 297-309.

172. Gureje O., Ogunniyi A., Baiyewu O., Price B., Unverzagt F. W., Evans R. M., Smith-Gamble V., Lane K. A., Gao S, Hall K. S., Hendrie H. C., Murrell J. R. APOE epsilon4 is not associated with Alzheimer's disease in elderly Nigerians. Annals of Neurology. 59 (2006) 182-185.

173. Haass C., Scholossmacher M.G., Hung A.Y., Vigo-Pelfrey C., Mellon A., Ostaszewski B.L., Lieberburg I., Koo E.H., Schenk D., Teplow D.B., Selkoe D.J., Amyloid beta-peptide is produced by cultured cells during normal metabolism. Nature. 359 (1992) 322-325.

174. Hanlon S., Brudno S., Wu T.T., Wolf B., Structural transitions of deoxyribonucleic acid in aqueous electrolyte solutions: reference spectra of conformational limits. Biochemistry. 14 (1975) 1648–1660.

175. Haque N., Tanaka T., Iqbal K., Grundke-Iqbal I., Regulation of expression, phosphorylation and biological activity of tau during differentiation in SY5Y cells. Brain Res. 838 (1999) 69-77.

176. Hara T., Nakamura K., Matsui M., Yamamoto A., Nakahara Y., Migishima R. S., Yokoyama M., Mishima K., Saito I., Okano H., Mizushima N. Suppression of basal autophagy in neural cells causes neurodegenerative disease in mice. Nature. 441 (2006) 885-889.

177. Hardy J., Amyloid, the presenilins and Alzheimer's disease. Trends Neurosci. 20 (1997) 154-159.

178. Hardy J., Mullan M., Alzheimer's disease. In search of the soluble. Nature. 359 (1992) 268-269.

179. Harper J.D., Lieber C.M., Lansbury Jr. P.T., Atomic microscopic imaging of seeded fibril formation and fibril branching by the Alzheimer's disease amyloid-beta protein. Chem. Biol. 4 (1997) 951-959.

180. Harrison R.S., Sharpe P.C., Singh Y., Fairlie D.P., Amyloid peptides and proteins in review. Rev. Physiol. Biochem. Pharmacol. 159 (2008) 1-77.

181. Hartmann T., Bieger S.C., Bruhl B., Tienari P.J., Ida N., Allsop D., Roberts G.W., Masters C.L., Dotti C.G., Unsicker K., Beyreuther K., Distinct sites of intracellular production for Alzheimer's disease A beta40/42 amyloid peptides. Nat Med. 3 (1997) 1016-1020.

182. Hartter D.E., Barnea A., Evidence for release of copper in the brain: depolarization-induced release of newly taken-up 67 copper. Synapse. 2 (1988) 412-415.

183. Hartwig A., Role of magnesium in genomic stability. Mutat. Res. 475 (2001) 113-121.

184. Hashimoto M., Rockenstein E., Crews L., Masliah E., Role of protein aggregation in mitochondrial dysfunction and neurodegeneration in Alzheimer's and Parkinson's diseases.". Neuromolecular Med. 4 (2003) 21-36.

185. Hegde M.L. and Jagannatha Rao K.S., Challenges and complexities of α-synuclein toxicity: new postulates in unfolding the mystery associated with Parkinson's disease. Arch. Biochem. Biophys. 418 (2003) 169-178.

186. Hegde M.L., Anitha S., Latha K.S., Mustak M.S., Stein R., Ravid R., Rao K.S., First evidence for helical transitions in supercoiled DNA by amyloid β peptide (1-42) and Aluminium: A new insight in understanding Alzheimer's disease. J Mol. Neurosci. 22 (2004) 19-31.

187. Hegde M.L., Rao K.S. J., DNA induces folding in α-synuclein: Understanding the mechanism using chaperone property of osmolytes. Arch. Biochem. Biophys. 464 (2007) 57-69.

188. Henderson A.S., The risk factors for Alzheimer's disease: a review and a hypothesis. Acta. Psychiatr. Scand. 78 (1988) 257-275.
189. Henderson V.W., Estrogen, cognition, and a woman's risk of Alzheimer's disease. Am J Med. 103 (1997) 11S-18S.
190. Henderson V.W., Hormone therapy and Alzheimer's disease: benefit or harm? Exper Opin Pharmacother. 5 (2004) 389-406.
191. Hensley K., Carney J.M., Mattson M.P., Aksenova M., Harris M., Wu J.F., Floyd R.A., Butterfield D.A., A model for beta-amyloid aggregation and neurotoxicity based on free radical generation by the peptide: relevance to Alzheimer disease. Proc Natl Acad Sci. (USA) 91 (1994) 3270–3274.
192. Herbert A., Rich A., Left-handed Z-DNA: structure and function. Genetica. 106 (1999) 37-47.
193. Hock C., Konietzko U., Papassotiropolous A., Wallmer A., Strelfer J., Von Rotz R.C., Davey G., Moritz E., Nitsch R.M., Generation of antibodies specific for β-amyloid by vaccination of patients with Alzheimer's disease. Nature Med. 8 (2002) 1270-1275.
194. Hockenbery D.M., Bcl-2, a novel regulator of cell death. Bioessays. 17 (1995) 631-638.
195. Holcomb L., Gordon M.N., McGowan E., Yu X., Benkovic S., Jantzen P., Wright K., Saad I., Mueller R., Morgan D., Sanders S., Zehr C., O'Campo K., Hardy J., Prada C.M., Eckman C., Younkin S., Hsiao K., Duff K., Accelerated Alzheimer-type phenotype in transgenic mice carrying both mutant amyloid precursor protein and presenilin 1 transgenes. Nat. Med. 4 (1998) 97-100.
196. Holt P.R., Katz S., Kirshoff R., Curcumin therapy in inflammatory bowel disease: a pilot study. Dig. Dis. Sci. 50 (2005) 2191–2193.
197. Howlett D., Cutler P., Heales S., Camilleri P., Hemin and related porphyrins inhibit beta-amyloid aggregation. FEBS Lett. 417 (1997) 249-251.
198. Howlett D.R., Perry A.E., Godfrey F., Swatton J.E., Jennings K.H., Spitzfaden C., Wadsworth H., Wood S.J., Markwell R.E., Inhibition of fibril formation in beta-amyloid peptide by a novel series of benzofurans. Biochem J. 340 (1999) 283-289.

199. Hua Q. and He R.Q., Tau could protect DNA double helix structure. Biochem Biophys Acta. 36763 (2002a) 1-7.

200. Hua Q., He R.Q., Effect of phosphorylation and aggregation on tau binding to DNA. Protein Pep Lett. 9 (2002b) 249-357.

201. Hua Q., He R.Q., Human neuronal tau promoting the melting temperature of DNA. Clin Sci Bull. 45 (2000) 999-1001.

202. Huang H.M., Ou H.C. and Hsieh S.J., Antioxidants prevent amyloid peptide-induced apoptosis and alteration of calcium homeostasis in cultured cortical neurons. Life Sci. 66 (2000) 1879-1892.

203. Huang X., Atwood C.S., Hartshorn M.A., Multhaup G., Goldstein L.E., Scarpa R.C., Cuajungco M.P., Gray D.N., Lim J., Moir R.D., Tanzi R.E., Bush A.I., The Aβ peptide of Alzheimer's disease directly produces hydrogen peroxide through metal ion reduction. Biochemistry. 38 (1999) 7609–7616.

204. Huang X., Atwood C.S., Moir R.D., Hartshorn M.A., Vonsattel J.P., Tanzi R.E., Bush A.I., Zinc-induced Alzheimer's Abeta1-40 aggregation is mediated by conformational factors. J.Biol. Chem. 272 (1997) 26464-26470.

205. Huang X., Cuajugco M.P. and Atwood C.S., Moir R.D., Tanzi R.E., Bush A.I., Alzheimer’s disease, beta-amyloid protein and zinc. J. Nutr. 130 (2000) 1488S-1492S.

206. Hutton M., Busfield F., Wragg M., Crook R., Perez-Tur J., Clark R.F., Prihar G., Talbot C., Phillips H., Wright K., Baker M., Lendon C., Duff K., Martinez A., Houlden H., Nichols A., Karran E., Roberts G., Roques P., Rossor M., Venter J.C., Adams M.D., Cline R.T., Phillips C.A., Fuldner R.A., Hardy J., Goate A., Complete analysis of the presenilin 1 gene in early onset Alzheimer’s disease. NeuroReport 7 (1996) 801-805.

207. Hyman B.T., West H.L., Rebeck G.W., Buldyrev S.V., Mantegna R.N., Ukleja M., Havlin S., Stanley H.E., Quantitative analysis of senile plaques in Alzheimer’s disease: Observation of log-normal size distribution and molecular epidemiology of differences associated with ApoE genotype and trisomy 21 (Down syndrome). Proc Natl Acad Sci. (USA) 92 (1995) 3586-3590.

208. Iijima K., Liu H.P., Chiang A.S., Hearn S.A., Konsolaki M., Zhong Y., Dissecting the pathological effects of human Abeta40 and Abeta42 in Drosophila: a potential model for Alzheimer's disease. Proc Natl Acad Sci. (USA) 101 (2004) 6623-6628.
209. Imada O., Toxicity aspects of garlic. In: First World Congress on the Health Significance of Garlic and Garlic constituents. Nutrition International, Irvine, CA, (1990) 47.
210. Imai J., Ide N., Nagae S., Moriguchi T., Matsuura H., Itakura Y., Antioxidant and radical scavenging effects of aged garlic extract and its constituents. Planta Med. 60 (1994) 417-420.
211. Imai T., Kosuge Y., Ishige K., Ito Y., Amyloid beta-protein potentiates tunicamycin-induced neuronal death in organotypic hippocampal slice cultures. Neuroscience. 147 (2007) 639-651.
212. Ireson C., Orr S., Jones D.J.L., Verschoyle R., Lim C.K., Luo J.L., Howells L., Plummer S., Jukes R., Williams M., Steward W.P., Gescher A., Characterization of metabolites of the chemopreventive agent curcumin in human and rat hepatocytes and in the rat in vivo, and evaluation of their ability to inhibit phorbol ester-induced Prostaglandin E2 Production. Cancer Res. 61 (2001) 1058-1064.
213. Ishige K., Takagi N., Imai T., Rausch W.D., Kosuge Y., Kihara T., Kusama-Eguchi K., Ikeda H., Cools A.R., Waddington J.L., Koshikawa N., Ito Y.J., Role of caspase-12 in amyloid beta-peptide-induced toxicity in organotypic hippocampal slices cultured for long periods. Pharmacol Sci. 104 (2007) 46-55.
214. Ito Y., Kosuge Y., Sakikubo T., Horie K., Ishikawa N., Obokata N., Yokoyama E., Yamashina K., Yamamoto M., Saito H., Arakawa M., Ishige K., Protective effect of S-allyl-L-cysteine, a garlic compound, on amyloid beta-protein-induced cell death in nerve growth factor-differentiated PC12 cells. Neurosci Res. 46 (2003) 119-125.
215. Jackson R., McNeil B., Taylor C., Holl G., Ruff D., Gwebu E.T., Effect of aged garlic extract on caspase-3 activity, in vitro. Nutr Neurosci. 5 (2002) 287-290.
216. Jacobsen M.D., Weil M., Raff M.C., Role of Ced-3/ICE-family proteases in staurosporine-induced programmed cell death. J Cell Biol. 133 (1996) 1041-1051.

217. Jarrett J.T., Lansbury Jr. P.T., Seeding "one-dimensional crystallization" of amyloid: a pathogenic mechanism in Alzheimer's disease and scrapie? Cell. 73 (1993) 1055–1058.

218. Johnstone E.M., Babbey L.E., Stephenson D., Paul D.C., Santerre R.F., Clemens J.A., Williams D.C., Little S.P., Nuclear and cytoplasmic localization of the beta-amyloid peptide (1-43) in transfected 293 cells. Biochem Biophys Res Commun. 220 (1996) 710-718.

219. Jorm A.F., Van Duijn C.M., Chandra V., Fratiglioni L., Graves A.B., Heyman A., Kokmen E., Kondo K., Mortimer J., Rocca W.A., Shalat S.L., Soininen H., The Eurodem risk factors research group: Psychiatric history and related exposures as risk factors for Alzheimer's disease: a collaborative re-analysis of case-control studies. Int J Epidemiol. 20 (1991) S43-S47.

220. Jung K.K., Lee H.S., Cho J.Y., Shin W.C., Rhee M.H., Kim T.G., Kang J.H., Kim S.H., Hong S. and Kang S.Y., Inhibitory effect of curcumin on nitric oxide production from lipopolysaccharide- activated primary microglia. Life Sci. 79 (2006) 2022–2031.

221. Kadioglu E., Sardas A., Aslan S., Isik E., East Karakaya A. Detection of oxidative damage in lymphocytes of patients with Alzheimer's disease. Biomarkers. 9 (2004) 203-209.

222. Kanapathipillai M., Lentzen G., Sierks M., Park C.B., Ectoine and hydroxyectoine inhibit aggregation and neurotoxicity of Alzheimer's beta-amyloid. FEBS Lett. 579 (2005) 4775-4780.

223. Kaneko I., Morimoto K., Kubo T., Drastic neuronal loss in vivo by beta-amyloid racemized at Ser (26) residue: conversion of non-toxic [D-Ser (26)] beta amyloid 1-40 to toxic and proteinase-resistant fragments. Neuroscience. 104 (2001) 1003-1011.

224. Kanezawa A., Nakagawa S., Sumiyoshi H., Masamoto K., Harada H., Nakagami S., Date S., Yokota A., Nishikawa M., Fuwa T., General toxicity tests of garlic extract preparation (Kyoleopin) containing vitamins. Oyo yakuri. 27 (1984) 909-929.

225. Kang J., Lemaire H.G., Unterbeck A., Salbaum J.M., Masters C.L., Grzescnik K.H., Multhaup G., Beyreuther K., Muller-Hill B., The precursor of Alzheimer's disease amyloid A4 protein resembles a cell-surface receptor. Nature. 325 (1987) 733-736.

226. Katzman R., A neurologist's view of Alzheimer's disease and dementia. Int Psychogeriatry. 16 (2004) 259-273.

227. Kawarabayashi T., Younkin L.H., Saido T.C., Shoji M., Ashe K.H., Younkin S.G., Age-dependent changes in brain, CSF, and plasma amyloid (beta) protein in the Tg2576 transgenic mouse model of Alzheimer's disease. J. Neurosci. 21 (2001) 372-381.

228. Kim H., Park B.S., Lee K.G., Choi C.Y., Jang S.S., Kim Y.H., Lee S.E., Effects of naturally occurring compounds on fibril formation and oxidative stress of beta-amyloid. J Agric Food Chem. 53 (2005) 8537-8541.

229. Kim H.Y., Park E.J., Joe E.H., Jou I. Curcumin suppresses Janus kinase-STAT inflammatory signaling through activation of Src homology 2 domain-containing tyrosine phosphatase 2 in brain microglia. J. Immunol. 171 (2003) 6072–6079.

230. Kimberly W.T., Zheng J.B., Guenette S.Y., Selkoe D.J., The intracellular domain of the beta-amyloid precursor protein is stabilized by Fe65 and translocates to the nucleus in a notch-like manner. J. Biol. Chem. 276 (2001) 40288-40292.

231. Klafki H.W., Staufenbiel M., Kornhuber J., Wiltfang J., Therapeutic approaches to Alzheimer's disease. Brain. 129 (2006) 2840-2855.

232. Klunk W.E., Debnath M.L., Pettegrew J.W., Development of small molecule probes for the beta-amyloid protein of Alzheimer's disease. Neurobiol Aging. 15 (1994) 691-698.

233. Klunk W.E., Jacob R.F., Mason R.P., Quantifying amyloid beta-peptide (Abeta) aggregation using the Congo red-Abeta (CR-abeta) spectrophotometric assay. Anal Biochem. 266 (1999) 66-76.

234. Knauer M.F., Soreghan B., Burdick D., Kosmoski J. and Glabe C.G. Intracellular accumulation and resistance to degradation of the Alzheimer amyloid A4/ beta protein. Proc. Natl. Acad. Sci. USA 89 (1992) 7437-7441.

235. Kokubo H., Kayed R., Glabe C.G., Yamaguchi H., Soluble Abeta oligomers ultrastructurally localize to cell processes and might be related to synaptic dysfunction in Alzheimer's disease brain. Brain Res 1031 (2005) 222-228.
236. Koo E.H., Squazzo S.L., Evidence that production and release of amyloid beta-protein involves the endocytic pathway. J. Biol. Chem. 269 (1994) 17386-17389.
237. Kosik K.S., Joachim C.L., Selkoe D.J., Microtubule-associated protein tau (tau) is a major antigenic component of paired helical filaments in Alzheimer disease. Proc Natl Acad Sci (USA) 83 (1986) 4044- 40448.
238. Kosuge Y., Koen Y., Ishige K., Minami K., Urasawa H., Saito H., Ito Y., S-allyl-L-cysteine selectively protects cultured rat hippocampal neurons from amyloid beta-protein- and tunicamycin-induced neuronal death. Neuroscience. 122 (2003) 885-895.
239. Kosuge Y., Sakikubo T., Ishige K., Ito Y., Comparative study of endoplasmic reticulum stress-induced neuronal death in rat cultured hippocampal and cerebellar granule neurons. Neurochem Int. 49 (2006) 285-293.
240. Kowall N., McKee A., Yankner B., Beal M., In vivo neurotoxicity of β-amyloid [β (1–40)] and the β(25–35) fragment. Neurobiol Aging 13 (1992) 537–542.
241. Kowalska A., The beta-amyloid cascade hypothesis: a sequence leading to neurodegeneration in Alzheimer’s disease. Neurol Neurochir Pol. 38 (2004) 405-411.
242. Kozin S.A., Zirah S., Rebuffat S., Hoa G.H.B., Debey P., Zinc binding to Alzheimer’s Aβ (1-16) peptide results in stable soluble complex. Biochem. Biophys. Res. Comm. 285 (2001) 959-964.
243. Kril J.J., Hodges J., Halliday G., Relationship between hippocampal volume and CA1 neuron loss in brains of humans with and without Alzheimer's disease. Neurosci Lett. 361 (2004) 9-12.
244. Kubo T., Kumagae Y., Miller C.A., Kaneko I., Beta- amyloid racemizedat the Ser26 residue in the brains of patients with Alzheimer disease: Implications in the pathogenesis of Alzheimer disease. J. Neurpathol. Exp. Neurol. 62 (2003) 248-259.

245. Kubo T., Nishimura S., Kumagae Y., Kaneko I., Invivo conversion of racemized beta- amyloid ([D-Ser 26] A beta 1-40) to truncated and toxic fragments ([D-Ser 26] Abeta 25-35/40) and fragment presence in the brains of Alzheimer's patients. J. Neurosci. Res. 70 (2002) 474-483.

246. Kuo Y.M., Emmerling M.R., Vigo-Pelfrey C., Kasunic T.C., Kirkpatrick J.B., Murdoch G.H., Ball M.J., Roher A.E., Water-soluble Abeta (N-40, N-42) oligomers in normal and Alzheimer disease brains. J. Biol. Chem. 271 (1996) 4077-4081.

247. Kuroda Y., Kawahara M., Tohoku J., Aggregation of amyloid beta-protein and its neurotoxicity: enhancement by aluminum and other metals. Exp. Med. 174 (1994) 263-268.

248. Laemmli U.K., Cleavage of structural proteins during the assembly of head of bacteriophage T4. Nature 227 (1970) 680-685.

249. Lahiri D.K., Greig N.H., Lethal weapon: amyloid beta-peptide, role in the oxidative stress and neurodegeneration of Alzheimer's disease. Neurobiol Aging 25 (2004) 581-587.

250. Lamb B.T., Sisodia S.S., Lawler A.M., Slunt H.H., Kitt C.A., Kearns W.G., Pearson P.L., Price D.L., Gearhart J.D., Introduction and expression of the 400 kilobase amyloid precursor protein gene in transgenic mice Nat Genet 5 (1993) 22-30.

251. Lambert J.C., Amouyel P., Genetic heterogeneity of Alzheimer's disease: Complexity and advances. Psychoneuroendocrinol. 32S1 (2007) S62-S70.

252. Lambert J.C., Berr C., Pasquier F., Delacourte A., Frigard B., Cottel D., Perez-Tur J., Mouroux V., Mohr M., Cecyre D., Galasko D., Lendon C., Poirier J., Hardy J., Mann D., Amouyel P., Chartier-Harlin M.C., Pronounced impact of Th1/E47 cs mutation compare with-492 AT mutation on neural APOE gene expression and risk of developing Alzheimer's disease. Hum Mol Genet. 7 (1998) 1511-1516.

253. Lambert M.P., Barlow A.K., Chromy B.A., Edwards C., Freed R., Liosates M., Morgan T.E., Rozovsky I., Trommer B., Viola K.L., Wals P., Zhang C., Finch C.E., Krafft G.A., Klein W.L., Diffusible, nonfibrillar ligands derived from

Abeta1-42 are potent central nervous system neurotoxins. Proc. Natl. Acad. Sci. USA 95 (1998) 6448-6453.

254. Lambert M.P., Velasco P.T., Chang L., Viola K.L., Fernandez S., Lacor P.N., Khuon D., Gong Y., Bigio E.H., Shaw P., De Felice F.G., Krafft G.A., Klein W.L., Monoclonal antibodies that target pathological assemblies of Abeta. J. Neurochem. 100 (2007) 23-35.
255. Laskowski R.A., M.W. MacArthur, D.S. Moss and J.M. Thornton, PROCHECK: a program to check the stereo chemical quality of protein structures, J. Appl. Cryst. 26 (1993) 283-291.
256. Lassmann H., Bancher C., Breitschopf H., Wegiel J., Bobinski M., Jellinger K., Wisniewski H.M., Cell death in Alzheimer's disease evaluated by DNA fragmentation in situ. Acta Neuropathol. 89 (1995) 35-41.
257. Latha K.S., Anitha S., Rao K.S., Viswamitra M.A., Molecular understanding of aluminum-induced topological changes in (CCG)12 triplet repeats: relevance to neurological disorders. Biochim Biophys Acta. 1588 (2002) 56-64. Erratum in: Biochim Biophys Acta 1637 (2003) 182.
258. Lawson L.D., Bioactive organosulfur compounds of garlic and garlic products. American Chemical society, Washington, DC. In: ACS Symposium Series 534, human Medicinal Agents From Plants (Kinghorn, A.D. & Balandrin, M.F., eds.) (1993) 306-330.
259. LeBlanc A., Increased production of 4 kDa amyloid beta peptide in serum deprived human primary neuron cultures: possible involvement of apoptosis. J Neurosci. 15 (1995) 7837-7846.
260. Lee K.H., Shin B.H., Shin K.J., Kim D.J., Yu J., A hybrid molecule that prohibits amyloid fibrils and alleviates neuronal toxicity induced by beta-amyloid (1-42). Biochem Biophys Res Commun. 328 (2005) 816-823.
261. Lemaire H.G., Salbaum J.M., Multhaup G., Kang J., Bayney R.M., Unterbeck A., Beyreuther K., Muller-Hill B., The PreA4 (695) precursor protein of Alzheimer's disease A4 amyloid is encoded by 16 exons. Nucleic Acids Res. 17 (1989) 517-522.

262. Lendon C.L., Ashall F., Goate A.M., Exploring the etiology of Alzheimer's disease using molecular genetics. JAMA. 277 (1997) 825-831.

263. Lesne S., Koh M.T., Kotilinek L., Kayed R., Glabe C.G., Yang A., Gallagher M., Ashe K.H., A specific Amyloid beta protein assembly in the brain impairs memory. Nature. 440 (2006) 352-357.

264. LeVine H.I., Thioflavine T interaction with synthetic Alzheimer's disease beta-amyloid peptides: detection of amyloid aggregation in solution. Protein Sci. 2 (1993) 404-410.

265. Levy-Lahad E., Wasco W., Poorkaj P., Romano D.M., Oshima J., Pettingell W.H., Yu C.E., Jondro P.D., Schmidt S.D., Wang K., Candidate gene for the chromosome 1 familial Alzheimer's disease locus. Science. 269 (1995) 973-977.

266. Levy-Lahad E., Wijsman E.M., Nemens E., Anderson L., Goddard K.A., Weber J.L., Bird T.D., Schellenberg G.D., A familial Alzheimer's disease locus on chromosome 1. Science. 269 (1995) 970-973.

267. Lim G.P., Chu T., Yang F., Beech W., Frautschy S.A., Cole G.M., The curry spice curcumin reduces oxidative damage and amyloid pathology in an Alzheimer transgenic mouse. J Neurosci. 21 (2001) 8370-8377.

268. Lippa S.M., Lippa C.F., Mori H., Alpha-Synuclein aggregation in pathological aging and Alzheimer's disease: the impact of beta-amyloid plaque level. Other Demen. 20 (2005) 315-318.

269. Loo D.T., Copani A., Pike C.J., Whittemore E.R., Walencewicz A.J., Cotman C.W., Apoptosis is induced by beta-amyloid in cultured central nervous system neurons. Proc Natl Acad Sci. (U S A) 90 (1993) 7951-7955.

270. Loomis P.A., Howard T.H., Castleberry R.P., Binder L.I., Identification of nuclear tau isoforms in human neuroblastoma cells. Proc Natl Acad Sci. (USA) 87 (1990) 8422-8426.

271. Lorenzo A., Yankner B.A., Amyloid fibril toxicity in Alzheimer's disease and diabetes. Ann N Y Acad Sci. 777 (1996) 89–95.

272. Lott I.T., Head E., Alzheimer disease and Down syndrome: factors in pathogenesis. Neurobiol Aging. 26 (2005) 383-389.

273. Lovell M.A., Robertson J.D., Teesdale W.J., Campbell J.L., and Markesber W.R., Copper, iron and zinc in Alzheimer's disease senile plaques. J.Neurol. Sci. 158 (1998) 47-52.

274. Lowenson J.D., Clarke S., Roher A.E., Chemical modifications of deposited amyloid-beta peptides.Methods Enzymol. 309 (1999) 89-105.

275. Lu M., Kosik K.S., Competition for microtubule-binding with dual expression of tau missense and splice isoforms. Mol Biol Cell. 12 (2001) 171-184.

276. Lucassen P.J., Chung W.C., Kamphorst W., Swaab D.F., DNA damage distribution in the human brain as shown by in situ end labeling; area-specific differences in aging and Alzheimer disease in the absence of apoptotic morphology. J Neuropathol Exp Neurol. 56 (1997) 887-900.

277. Lue L.F., Kuo Y.M., Roher A.E., Brachova L., Shen Y., Sue L., Beach T., Kurth J.H., Rydel R.E., Rogers J., Soluble amyloid beta peptide concentration as a predictor of synaptic change in Alzheimer's disease. Am. J. Pathol. 155 (1999) 853-862.

278. Lukiw W.J., Krishna B., Wong L., Kruck T.P.A., Bergeson C., McLachlan D.R.C. Nuclear compartmentalization of aluminium in Alzheimer's disease. Neurolbiol Aging 13 (1991) 115-121.

279. Lyras L., Cairns N.J., Jenner A., Jenner P., Halliwell B., An assessment of oxidative damage to proteins, lipids, and DNA in brain from patients with Alzheimer's disease. J Neurochem. 68 (1997) 2061-2069.

280. Maccioni R.B., Cambiazo V., Role of microtubule-associated proteins in the control of microtubule assembly. Physiol Rev. 75 (1995) 835-864.

281. Mandavilli B.S., Rao K.S., Accumulation of DNA damage in aging neurons occurs through a mechanism other than apoptosis. J. Neurochem. 67 (1996) 1559-1565.

282. Mandavilli B.S., Rao K.S., Neurons in the cerebral cortex are most susceptible to DNA-damage in aging rat brain. Biochem Mol Biol Int. 40 (1996) 507-514.

283. Mann D., The pathological association between Down syndrome and Alzheimer's disease. Mech Age Dev. 43 (1985) 99-136.

284. Mann D., Yates P., Marcyniuk B., Ravindra C. The topography of plaques and tangles in Down's syndrome patients of different ages. J Neuropathol Appl Neurobiol. 12 (1986) 447-457.

285. Mann D.M., Iwatsubo T., Pickering-Brown S.M., Owen F., Saido T.C., Perry R.H., Preferential deposition of amyloid beta protein (Abeta) in the form Abeta40 in Alzheimer's disease is associated with a gene dosage effect of the apolipoprotein E E4 allele. Neurosci Lett. 221 (1997) 81-84.

286. Martin J.B., Molecular basis of the neurodegenerative disorders, N. Engl. J. Med. 340 (1999) 1970-1980.

287. Martin S.J., Green D.R., Cotter T.G., Dicing with death: dissecting the components of the apoptosis machinery. Trends Biochem Sci. 19 (1994) 26-30.

288. Martineau M., Baux G., Mothet J.P., Gliotransmission at central glutamatergic synapses: D- serine on stage. J. Physiol. –Paris 99 (2006) 103-110.

289. Martyn C.N., Barker D.J., Osmand C., Harris E.C., Edwardson J.A., Lacey R.F., Geographical relation between Alzheimer's disease and aluminum in drinking water. Lancet. 1 (1989) 59–62.

290. Masters C.L., Simms G., Weinman N.A., Multhaup G., McDonald B.L., Beyreuther K., Amyloid plaque core protein in Alzheimer disease and Down syndrome. Proc. Natl. Acad. Sci. (USA) 82 (1985) 4245-4249.

291. Mathura V.S., Paris D., Ait-Ghezala G., Quadros A., Patel N.S., Kolippakkam D.N., Volmar C.H., Mullan M.J., Model of Alzheimer's disease amyloid beta peptide based on a RNA binding protein. Biochem. Biophys, Res. Commun. 332 (2005) 585-592.

292. Matilla A., Koshy B.T., Cummings C.J., Isobe T., Orr H.T., Zoghbi H.Y., The cerebellar leucine-rich acidic nuclear protein interacts with ataxin-1. Nature. 389 (1997) 974-978.

293. Matsui T., Ingelsson M., Fukumoto H., Ramasamy K., Kowa H., Frosch M.P., Irizarry M.C., Hyman B.T., Expression of APP pathway mRNAs and proteins in Alzheimer's disease. Brain Res. 1161 (2007) 116-123.

294. Mattson M.P., Goodman Y., Different amyloidogenic peptides share a similar mechanism of neurotoxicity involving reactive oxygen species and calcium. Brain Res. 676 (1995) 219-224.

295. McKhann G., Drachman D., Folstein M., Katzman R., Price D., Stadlan E.M. Clinical diagnosis of Alzheimer's disease Report of the NINCDS-ADRDA work group under the auspices of Department of Health and Human Services Task Force on Alzheimer's disease. Neurology. 34 (1984) 939-944.

296. Mclean C.A., Cherny R.A., Fraser F.W., Fuller S.J., Smith M.J., Beyreuther K., Bush A.I., Masters C.L., Soluble pool of Abeta amyloid as a determinant of severity of neurodegeneration in Alzheimer's disease. Ann. Neurol. 46 (1999) 860-866.

297. Mecocci P., Beal M.F., Cecchetti R., Polidori M.C., Cherubini A., Chionne F., Avellini L., Ronano G., Senin U., Mitochondrial membrane fluidity and oxidative damage to mitochondrial DNA in aged and AD human brain. Mol Chem Neuropathol. 31 (1997) 53-64.

298. Mecocci P., MacGarvey U., Beal M.F., Oxidative damage to mitochondrial DNA is increased in Alzheimer's disease. Ann Neurol. 36 (1994) 747-751.

299. Meda L., Cassatella M.A., Szendrei G.I., Otvos Jr. L., Baron P., Villalba M., Ferrari D., Rossi F., Activation of microglial cells by beta-amyloid protein and interferon-gamma. Nature. 374 (1995) 647-650.

300. Mendoza-Ramirez J.L., Beltran-Parrazal L., Verdugo-Diaz L., Morgado- Valle C., Drucker-Colin R., Delay in manifestations of aging by grafting NGF cultured chromaffin cells in adulthood. Neurobiol Aging. 16 (1995) 907-916.

301. Minn A., Boise L., Thomson C., Bcl-x(s) antagonizes the protective effects of Bcl- x(L). J Biol Chem. 271 (1996) 6306-6312.

302. Minn A.J., Rudin C.M., Boise L.H., Thomson C.B., Expression of bcl-xL can confer a multidrug resistance phenotype. Blood. 86 (1995) 1903-1910.

303. Mirra S.S., Heyman A., McKeel D., Sumi S.M., Crain B.J., Brownlee L.M., Vogel F.S., Hughes J.P., Van Belle G., Berg L., Participating CERAD neuropathologists The Consortium to Establish a Registry for Alzheimer's disease

(CERAD). Part II. Standardization of the neuropathologic assessment of Alzheimer's disease. Neurology. 41 (1991) 479-486.

304. Monji A., Tashiro K., Yoshida I., Kaname H., Hayashi Y., Matsuda K., Tashiro N., Laminin inhibits both Abeta40 and Abeta42 fibril formation but does not affect Abeta40 or Abeta42-induced cytotoxicity in PC12 cells. Neurosci Lett. 266 (1999) 85-88.
305. Moreno H., Wu W.E., Lee T., Brickman A., Mayeux R., Brown T.R., Small S.A., Imaging the Abeta-related neurotoxicity of Alzheimer disease. Arch. Neurol. 64 (2007) 1467-1477.
306. Mori H., Ishii K., Tomiiyama T., Furiya Y., Sahara N., Asano S., Endo N., Shirasawa T., Takio K., Racemization: its biological significance on neuropathogenesis of Alzheimer's disease. Tohoku J. Exp. Med. 174 (1994) 251-262.
307. Moriguchi T., Matsuura H., Kodera Y., Itakura Y., Katsuki H., Saito H., Nishiyama N., Neurotrophic activity of organosulfur compounds having a thioallyl group on cultured rat hippocampal neurons. Neurochem Res. 22 (1997b) 1449-1452.
308. Moriguchi T., Saito H., Nishiyama N., Aged garlic extract prologs longevity and improves spatial memory deficit in senescence-accelerated mouse. Biol Pharm Bull. 19 (1996) 305-307.
309. Moriguchi T., Saito H., Nishiyama N., Anti-ageing effect of aged garlic extract in the inbred brain atrophy mouse model. Clin Exp Pharmacol Physiol. 24 (1997a) 235-242.
310. Morocz M., Kalam J., Juhasz A., Sinko I., McGlynn A.P., Downes C.S., Janka Z., Rasko I., Elevated levels of oxidative damage in lymphocytes from patients with Alzheimer's disease. Neurobiol Aging. 23 (2002) 47-53.
311. Mortimer J., Van Duijn C.M., Chandra V., Fratiglioni L., Graves A.B., Heyman A., Jorm A.F., Kokmen E., Kondo K., Rocca W.A., Shalat S.L., Soininen H., Hofman A., for the Eurodem risk factors research group: Head trauma as a risk factor for Alzheimer's disease: a collaborative re-analysis of case-control studies. Int J Epidemiol., 20 (Suppl 2) (1991) S28-S35.

312. Mosbaugh D.W., Linn S., Characterization of the action of Escherichia coli DNA polymerase I at incisions produced by repair endodeoxyribonucleases. J. Biol. Chem. 257 (1982) 575-583.

313. Mudher A., Lovestone S., Alzheimer's disease-do tauists and baptists finally shake hands? Trends Neurosci. 25 (2002) 22-26.

314. Mullaart E., Boerrigter M.E., Ravid R., Swaab D.F., Vijg J., Increased levels of DNA breaks in cerebral cortex of Alzheimer's disease patients. Neurobiol Aging. 11 (1989) 169-173.

315. Nagae S., Ushijima M., Hatono S., Imai J., Kasuga S., Matsuura H., Itakura Y., Higashi Y., Pharmacokinetics of the garlic compound S-allylcysteine. Planta Med. 60 (1994) 214-217.

316. Naiki H., Nakakuki K., First-order kinetic model of Alzheimer's beta-amyloid fibril extension in vitro. Lab Invest. 74 (1996) 374-383.

317. Nandi P.K., Interaction of prion peptide HuPrP106-126 with nucleic acid. Arch Virol. 142 (1997) 2537-2545.

318. Nandi P.K., Leclerc E., Nicole J.C., Takahashi M., DNA-induced partial unfolding of prion protein leads to its polymerisation to amyloid. J. Mol. Biol. 322 (2002) 153-161.

319. Nandi P.K., Nicole J.C., Nucleic acid and prion protein interaction produces spherical amyloids which can function in vivo as coats of spongiform encephalopathy agent. J. Mol. Biol. 344 (2004) 827-837.

320. Nandi P.K., Polymerization of human prion peptide HuPrP 106-126 to amyloid in nucleic acid solution. Arch Virol. 1431(1998) 1251-1263.

321. Nandi P.K., Sizaret P.Y., Murine recombinant prion protein induces ordered aggregation of linear nucleic acids to condensed globular structures. Arch Virol. 146 (2001) 327-345.

322. Necula M., Kayed R., Milton S., Glabe C.G., Small-molecule inhibitors of aggregation indicate that amyloid beta oligomerization and fibrillization pathways are independent and distinct. J. Biol. Chem. 282 (2007) 10311-10324.

323. Nelson D.L., Cox M.M., In Lehninger Principles of Biochemistry. Worth Publishers Inc 2000.

324. Nicholson D.W., Thornberry N.A., Caspases: killer proteases. Trends Biochem Sci. 22 (1997) 299-306.

325. Nishiyama N., Moriguchi T., Saito H., Beneficial effects of aged garlic extract on learning and memory impairment in the senescence-accelerated mouse. Exp Gerontol. 32 (1997) 149-160.

326. Nistor M., Don M., Parekh M., Sarsoza F., Goodus M., Lopez G.E., Kawas C., Leverenz J., Doran E., Lott I.T., Hill M., Head E., Alpha- and beta-secretase activity as a function of age and beta-amyloid in Down syndrome and normal brain. Neurobiol Aging. 28 (2006) 1493-1506.

327. Numagami Y., Ohnishi S.T., S-allylcysteine inhibits free radical production, lipid peroxidation and neuronal damage in rat brain ischemia. J Nutr. 131 (2001) 1100S-1105S.

328. Nunomura A., Perry G., Pappolla M.A, Wade R., Hirai K., Chiba S., Smith M.A., RNA oxidation is a prominent feature of vulnerable neurons in Alzheimer's disease. J Neurosci. 19 (1999) 1959–1964.

329. Nunomura A., Perry G., Pappolla M.A., Freidland R.P., Hirai K., Chiba S., Smith M.A., Neuronal oxidative stress precedes amyloid-beta deposition in Down syndrome, J Neuropathol Exp Neurol. 59 (2000) 1011-1017.

330. O'Donovan A., Wood R.D., Identical defects in DNA repair in xeroderma pigmentosum group G and rodent ERCC group 5. Nature 363 (1993) 185-188.

331. Ohnishi S., Takano K., Amyloid fibrils from the viewpoint of protein folding. Cell Mol Life Sci. 61 (2004) 511-524.

332. Ohyagi Y., Asahara H., Chui D.H., Tsuruta Y., Sakae N., Miyoshi K., Yamada T., Kikuchi H., Taniwaki T., Murai H., Ikezoe K., Furuya H., Kawarabayashi T., Shoji M., Checler F., Iwaki T., Makifuchi T., Takeda K., Kira J., Tabira T., Intracellular Abeta42 activates p53 promoter: a pathway to neurodegeneration in Alzheimer's disease. FASEB J. 19 (2005) 255-257.

333. Ohyagi Y., Yamada T., Asahara H., Taniwaki T., Nakebuppu Y., Younkin S., Kira J., Amyloid β protein (Aβ)42 forms a 24 kD complex in normal cells: its nuclear shift in response to differentiation and genotoxicity. Soc. Neurosci. Abstr. 25 (1999) 1805.

334. Okouchi M., Ekshyyan O., Maracine M., Aw T.Y., Neuronal apoptosis in neurodegeneration. Antioxid Redox Signal. 9 (2007) 1059-1096.

335. Olsson A., Vanderstichele H., Anderson N., De Meyer G., Wallin A., Holmberg B., Rosengreen L., Vanmechelen E., Blenow K., Simultaneous measurement of Abeta (1-42), total tau and phosphorylated tau (Thr 181) in cerebrospinal fluid by the xMAP technology. Clin Chem. 51 (2004) 336-345.

336. Oltvai Z.N., Milliman C.L., Korsmeyer S.J., Bcl-2 heterodimerizes in vivo with a conserved homolog, Bax, that accelerates programmed cell death. Cell. 74 (1993) 609-619.

337. Ono K, Hasegawa K, Yamada M, Naiki H. Nicotine breaks down preformed Alzheimer's beta-amyloid fibrils in vitro. Biol Psychiatry 52 (2002) 880-886.

338. Ono K., Hasegawa K., Naiki H., Yamada M., Curcumin has potent anti-amyloidogenic effects for Alzheimer's β-amyloid fibrils in vitro. J.Neurosci. Res. 75 (2004) 742-750.

339. Ono K., Hirohata M., Yamada M., Ferulic acid destabilizes preformed beta-amyloid fibrils in vitro. Biochem Biophys Res Commun. 336 (2005) 444-449.

340. Ono K., Yamada M., Antioxidant compounds have potent anti-fibrillogenic and fibril-destabilizing effects for alpha-synuclein fibrils in vitro. J Neurochem. 97 (2006) 105-115.

341. Ono M., Kung M.P., Hou C., Kung H.F., Benzofuran derivatives as Abeta-aggregate-specific imaging agents for Alzheimer's disease. Nucl Med Biol. 29 (2002) 633-642.

342. Pan M.H., Huang T.M., Lim J.K., Biotransformation of curcumin through reduction and glucuronidation in mice. Drug Metab and Disposition. 27 (1999) 486-494.

343. Pappolla M., Bozner P., Soto C., Shao H., Robakis N.K., Zagorski M., Frangione B., Ghiso J., Inhibition of Alzheimer beta-fibrillogenesis by melatonin. J Biol Chem. 273 (1998) 7185-7188.

344. Parihar M.S., Hemnani T., Experimental excitoxicity provokes oxidative damage in mice brain and attenuation by exatract of Asparagus racemosus. J Neural transm. 111 (2004) 1-12.

345. Parihar M.S., Hemnani T., Phenolic antioxidants attenuate hippocampal neuronal cell damage against kainic acid induced neurotoxicity. J Biosci. 28 (2003) 121-128.

346. Patro B.S., Rele S., Chintalwar G.J., Chattopadhyay S., Adhikari S., Mukherjee T., Protective Activities of Some Phenolic 1,3-Diketones against Lipid Peroxidation: Possible Involvement of the 1,3-Diketone Moiety. ChemBioChem. 3 (2002) 364–370.

347. Peng Q., Buz'Zard A.R., Lau B.H., Neuroprotective effect of garlic compounds in amyloid-beta peptide-induced apoptosis in vitro. Med Sci Monit. 8 (2002) BR328-BR337.

348. Pereira C., Santos M.S., Oliveira C., Mitochondrial function impairment induced by amyloid beta-peptide on PC12 cells. Neuroreport. 9 (1998) 1749-1755.

349. Perez-Severiano F., Salvatierra-Sanchez R., Rodriguez-Perez M., Cuevas-Martinez E.Y., Guevara J., Limon D., Maldonado P.D., Medina-Campos O.N., Pedraza-Chaverri J., Santamaria A., S-Allylcysteine prevents amyloid-beta peptide-induced oxidative stress in rat hippocampus and ameliorates learning deficits. Eur J Pharmacol. 489 (2004) 197-202.

350. Pericak-Vance M.A., Bebout J.L., Gaskell P.C., Yamaoka L.A., Hung W.Y., Alberts M.J., Walker A.P., Bartlett R.J., Haynes C.S., Welsh K.A., Earl N.L., Heyman A., Clark C.M., Roses A.D., Linkage studies in familial Alzheimer's disease: evidence for chromosome 19 linkage. Am J Hum Genet. 48 (1991) 1038-1050.

351. Perry G., Castellani R.J., Hirai K., Smith M.A., Reactive Oxygen Species Mediate Cellular Damage in Alzheimer Disease. J Alzheimers Dis. 1 (1998) 45-55.

352. Pert C.B., Pert A., Chang J.K., Fong B.T., (D-Ala2)-Met-enkephalinamide: a potent, long-lasting synthetic pentapeptide analgesic. Science. 194 (1976) 330-332.

353. Pierrot N., Ghisdal P., Caumont A.S., Octave J.N., Intraneuronal amyloid-beta1-42 production triggered by sustained increase of cytosolic calcium concentration induces neuronal death. J. Neurochem. 88 (2004) 1140–1150.

354. Pike C.J., Burdick D., Walencewicz A.J., Glabe C.G., Cotman C.W., Neurodegeneration induced by beta-amyloid peptides in vitro: the role of peptide assembly state. J Neurosci. 13 (1993) 1676-1687.

355. Pike C.J., Cummings B.J., Cotman C.W., β-amyloid induces neuritic dystrophy in vitro: similarities with Alzheimer pathology. Neuroreport. 3 (1992) 769–772.

356. Pike C.J., Walencewicz A.J., Glabe C.G., Cotman C.W., Aggregation-related toxicity of synthetic beta-amyloid protein in hippocampal cultures. Eur J Pharmacol. 207 (1991) 367–368.

357. Pike C.J., Walencewicz-Wasserman A.J., Kosmoski J., Cribbs D.H., Glabe C.G., Cotman C.W., Structure–activity analyses of beta-amyloid peptides: contributions of the beta 25–35 region to aggregation and neurotoxicity. J Neurochem. 64 (1995) 253–265.

358. Pollack S.J., Sadler I.I., Hawtin S.R., Tailor V.J., Shearman M.S., Sulfated glycosaminoglycans and dyes attenuate the neurotoxic effects of beta-amyloid in rat PC12 cells. Neurosci Lett. 184 (1995) 113-116.

359. Porat Y., Abramowitz A., Gazit E., Inhibition of amyloid fibril formation by polyphenols: structural similarity and aromatic interactions as a common inhibition mechanism. Chem Biol Drug Des. 67 (2006) 27-37.

360. Prasad K.N., Hovland A.R., Cole W.C., Prasad K.C., Naheini P., Edwards-Prasad J., Andretta C.P., Multiple antioxidants in the prevention and treatment of Alzheimer disease: analysis of biological rationale. Clin Neuropharmacol. 23 (2000) 2-13.

361. Pratico D., Uryu K., Leight S., Trojanoswdi J.Q., Lee V.M., Increased lipid peroxidation precedes amyloid plaque formation in an animal model of Alzheimer amyloidoses. J Neurosci. 21 (2001) 4183-4187.

362. Priller C., Bauer T., Mitteregger G., Krebs B., Kretzschmar H.A., Herms J., Synapse formation and function is modulated by the amyloid precursor protein. J Neurosci. 26 (2006) 7212-7221.

363. Rachakonda V., Pan T.H., Le W.D., Biomarkers of neurodegenerative disorders: how good are they? Cell Res. 14 (2004) 347-358.

364. Rajan M.T., Champion C.S.K.D., Vishnuvardhan D., Rao K.S.J., Viswamitra M.A., Interaction of Co, Mn, Mg and Al with d(GCGTACGC): a spectroscopic study. Mol Biol Rep. 22 (1996) 47–52.

365. Rao K.S.J., Rao R.V., Shanmugavelu P., Menon R.B., Trace elemental inter-relationships in Alzheimer's brain: A new hypothesis. Alz Rep. 2 (1999) 241-246.

366. Rapport S.I., Pettingrew K.D., Schapiro M.B., Discordance and condcordance of dementia of the Alzheimrer's type (DAT) in monozygotic twin indicate heritable and sporadic forms of Alzheimer's disease. Neurology. 41 (1991) 1549-1553.

367. Reed J.C., Bcl-2: Prevention of apoptosis as a mechanism of drug resistance. Hematol Oncol Clin North Am. 9 (1995) 451-473.

368. Reiter R.J. Oxidative process and antioxidation in Alzheimer's disease. Free Radic Biol Med. 9 (1997) 526-533.

369. Reynolds A., Laurie C., Lee Mosley R., Gendelman H.E., Oxidative stress and the pathogenesis of neurodegenerative disorders. Int Rev Neurobiol. 82 (2007) 297-325.

370. Rich A., DNA comes in many forms. Gene. 135 (1993) 99-109.

371. Riddell D.R., Christie G., Hussain I., Dingwall C., Compartmentalization of beta-secretase (Asp2) into low-buoyant density, noncaveolar lipid rafts. Curr Biol. 11 (2001) 1288-1293.

372. Ringman J.M., Frautschy S.A., Cole G.M., Masterman D.L., Cummings J.L., A potential role of the curry spice curcumin in Alzheimer's disease. Curr Alzheimer Res. 2 (2005) 131-136.

373. Rivière C., Richard T., Quentin L., Krisa S., Mérillon J.M., Monti J.P., Inhibitory activity of stilbenes on Alzheimer's beta-amyloid fibrils in vitro. Bioorg Med Chem. 15 (2007) 1160-1167.

374. Rivlin R.S., Is garlic alternative medicine? J Nutr.136 (2001) 713S-715S.

375. Rochet J.C. and Lansbury P.T., Amyloid fibrillogenesis: themes and variations. Curr. Opin. Struct. Biol. 10 (2000) 60-68.

376. Rodriguez W.G., Becerra M.S., Milton S., Glabe C.G., Soluble Amyloid Aβ (1-40) exists as a stable dimer at low concentrations. J. Biol. Chem. 272 (1997) 21037-21044.

377. Rogaev E.I., Sherrington R., Rogaeva E.A., Levesque G., Ikeda M., Liang Y., Chi H., Lin C., Holman K., Tsuda T., Familial Alzheimer's disease in kindreds with missense mutations in a gene on chromosome 1 related to the Alzheimer's disease type 3 gene. Nature. 376 (1995) 775-778.

378. Rogaev E.I., Sherrington R., Wu C., Levesque G., Liang Y., Rogaeva E.A., Analysis of the 5' sequence, genomic structure, and alternative splicing of the presenilin-1 gene (PSEN1) associated with early onset Alzheimer disease. Genomics. 40 (1997) 415-424.

379. Rogers J., Morrison J.H., Quantitative morphology and regional and laminar distributions of senile plaques in Alzheimer's disease. J Neurosci. 5 (1985) 2801-2808.

380. Roher A.E., Lowenson J.D., Clarke S., Wolkow C., Wang R., Cotter R.J., Reardon I.M., Zurcher-Neely H.A., Heinrikson R.L., Ball M.J., Structural alterations in the peptide backbone of beta-amyloid core protein may account for its deposition and stability in Alzheimer’s Disease. J. Biol. Chem. 268 (1993) 3072-3083.

381. Rottkamp C.A., Nunomura A., Hirai K., Sayre L.M., Perry G., Smith M.A., Will antioxidants fulfill their expectations for the treatment of Alzheimer disease? Mech Ageing Dev. 116 (2000) 169-179.

382. Safarian S., Moosavi-Movahedi A.A., Hosseinkhani S., Xia Z., Habibi-Rezaei M., Hosseini G., Sorenson C., Sheibani N., The structural and functional studies of His119 and His12 in RNase A via chemical modification. J Protein Chem. 22 (2003) 643-654.

383. Samudralwar D.L., Diprete C.C. and Ni B.F., Elemental imbalances in the olfactory pathway in Alzheimer’s disease. J. Neurol. Sci. 130 (1995) 139-145.

384. Sanderson K.L., Butler L., Ingram V.M., Aggregates of a beta-amyloid peptide are required to induce calcium currents in neuron-like human teratocarcinoma cells: relation to Alzheimer's disease. Brain Res. 744 (1997) 7-14.

385. Saunders A.M., Schmader K., Breitner J.C., Benson M.D., Brown W.T., Goldfarb L., Goldgaber D., Manwaring M.G., Szymanski M.H., McCown N., Dole K.C., Schmechel D.E., Strittmatter W.J., Pericak-Vance M.A., Roses A.D.,

Apolipoprotein E e4 allele distributions in late-onset Alzheimer`s disease and in other amyloid-forming diseases. Lancet. 342 (1993) 710-711.

386. Savory J., Rao K.S.J., Huang Y., Letada P.R., Herman M.M., Age related changes in Bcl-2 and Bax ratio, oxidative stress, redox active iron, and apoptosis associated with aluminium induced neurodegeneration: increased susceptibility with aging. Neurotoxicol. 20 (1999) 805–818.
387. Sayre L.M., Perry G., Harris P.L.R., Liu Y., Schubert K.A., Smith M.A., In situ oxidative catalysis by neurofibrillary tangles and senile plaques in Alzheimer's disease: a central role for bound transition metals. J Neurochem. 74 (2000) 270–279.
388. Scandalios J.G., Oxidative stress responses--what have genomescale studies taught us? Genome Biol, 3 (2002).
389. Scatchard G., The attraction of proteins for small molecules and ions. Ann. N. Y. Acad. Sci. (USA) 51 (1949) 660-672.
390. Schenk D., Amyloid-β immunotherapy for Alzheimer’s disease: The end of the beginning. Nature Rev Neurosci. 3 (2003) 824-828.
391. Schmitz C., Rutten B.P., Pielen A., Schafer S., Wirths O., Tremp G., Czech C., Blanchard V., Multhaup G., Rezaie P., Korr H., Steinbusch H.W., Pradier L., Bayer T.A., Hippocampal neuron loss exceeds amyloid plaque load in a transgenic mouse model of Alzheimer's disease. Am J Pathol. 164 (2004) 1495-1502.
392. Schroeter E.H., Kisslinger J.A., Kopan R., Notch-1 signalling requires ligand-induced proteolytic release of intracellular domain. Nature. 393 (1998) 382-386.
393. Schuurmans Stekhoven J.H., Renkawek K., Otte-Holler I., Stols A., Exogenous aluminum accumulates in the lysosomes of cultured rat cortical neurons. Neurosci Lett. 119 (1990) 71–74.
394. Selkoe D.J., Alzheimer’s disease: genes, proteins and therapy. Physiol Rev. 81 (2001) 741-766.
395. Selkoe D.J., Amyloid beta-protein and the genetics of Alzheimer’s disease. J Biol Chem. 271 (1996) 18295–18298.

396. Selkoe D.J., Biochemistry of altered brain proteins in Alzheimer's disease. Ann. Rev. Neurosci. 12 (1989) 463-490.

397. Selkoe D.J., Polisny M.B., Joachim C.L., Vickers E.A., Lee G., Fritz L.C., Oltersdorf T., β-amyloid precursor protein of Alzheimer disease occurs as 110- to 135-kilodalton membrane-associated proteins in neural and nonneural tissues. Proc Natl. Acad. Sci. (USA) 85 (1988) 7341-7345.

398. Selkoe D.J., Wolfe M.S., In search of gamma-secretase: presenilin at the cutting edge. Proc. Natl. Acad. Sci. USA. 97 (2000) 5690-5692.

399. Semisotnov G.V., Rodionova N.A., Kutyshenko V.P., Ebert B., Blanck J., Ptitsyn O.B., Sequential mechanism of refolding of carbonic anhydrase B. FEBS Lett. 224 (1987) 9-13.

400. Serban D., Benevides J.M., Jr. Thomas G.J., DNA secondary structure and Raman markers of supercoiling in Escherichia coli plasmid pUC19. Biochemistry 41 (2002) 847-853.

401. Shankar G.M., Bloodgood B.L., Townsend M., Walsh D.M., Selkoe D.J., Sabatini B.L., Natural oligomers of the Alzheimer amyloid β-protein induce reversible synapse loss by modulating an NMDAR-dependent signaling pathway. J. Neurosci. 27 (2007) 2866-2875.

402. Shapira R., Austin G.E. and Mirra S.S., Neuritic plaque amyloid in Alzheimer's disease is highly racemized. J. Neurochem. 50 (1988) 69-74.

403. Shapira R., Wilkinson K.D., Shapira G., Racemization of individual aspartate residues in human myelin basic protein. J. Neurochem. 50 (1988) 649-654.

404. Shcherbatykh I., Carpenter D.O., The role of metals in the etiology of Alzheimer's disease. J Alzheimers Dis. 11 (2007) 191-205.

405. Shen J., Bronson R.T., Chen D.F., Xia W., Selkoe D.J., Tonegawa S., Skeletal and CNS defects in presenilin-1 deficient mice, Cell. 89 (1997) 629–639.

406. Shen Z.X., Brain cholinesterases: II. The molecular and cellular basis of Alzheimer's disease. Med Hypotheses. 63 (2004) 308-321.

407. Sheng J.W., Hu Y.E. and Xia Z.Q., Structure and biological activity of beta amyloid protein precursor. Sheng. Li. Ke. Xue. Jin. Zhan. 3 (2000) 166-168.

408. Sherrington R., Rogaev E.I., Liang Y., Rogaeva E.A., Levesque G., Ikeda M., Chi H., Lin C., Li G., Holman K., Cloning of a gene bearing missense mutations in early-onset familial Alzheimer's disease. Nature. 375 (1995) 754-760.

409. Shimizu T., Matsuoka Y., Shirasawa T., Biological significance of isoaspartate and its repair system. Biol. Pharm. Bull. 28 (2005) 1590-1596.

410. Shimizu T., Watanabe A., Ogawara M., Mori H., Shirasawa T., Isoaspartate formation and neurodegeneration in Alzheimer's Disease. Arch. Biochem. Biophys. 381 (2000) 225-234.

411. Shin Y.A., Eichhorn G.L., Formation of psi(+) and psi (–) DNA. Biopolymers. 23 (1984) 325–335.

412. Shoji M., Golde T.E., Ghiso J., Cheung T.T., Estus S., Shaffer L.M., Cai X.-D., McKay D.M., Tinter R., Frangione B., Younkin S.G., Production of the Alzheimer amyloid beta protein by normal proteolytic processing. Science 258 (1992) 126-129.

413. Shoji M., Kawarabayashi T., Mastubara E., Ikeda M., Ishiguro K., Harigaya Y., Okamoto K., Distibution of amyloid beta protein precursor in Alzheimer's disease brain. Psychiatry Clin Neurosci. 54 (2000) 45-54.

414. Shukla P.K., Khanna V.K., Khan M.Y. and Srimal R.C., Protective effect of curcumin against lead neurotoxicity in rat. Hum. Exp. Toxicol. 22 (2003) 653–658.

415. Simmons L.K., May P.C., Tomaselli K.J., Rydel R.E., Fuson K.S., Brigham E.F., Wright S., Lieberburg I., Becker G.W., Brems D.N., Secondary structure of amyloid beta peptide correlates with neurotoxic activity in vitro. Mol. Pharmacol. 45 (1994) 373-379.

416. Sirover M.A., Loeb L.A., On the fidelity of DNA replication. Effect of metal activators during synthesis with avian myeloblastosis virus DNA polymerase. J. Biol. Chem. 252 (1977) 3605-3610.

417. Sisodia S.S., Secretion of the beta-amyloid precursor protein. Ann N Y Acad Sci. 674 (1992) 53–57.

418. Skinner P.J., Koshy B.T., Cummings C.J., Klement I.A., Helin K., Servadio A., Zoghbi H.Y., Orr H.T., Ataxin-1 with an expanded glutamine tract alters nuclear

matrix-associated structures. Nature. 389 (1997) 971-974. Erratum in: Nature. 391 (1997) 307.

419. Skovronsky D.M., Doms R.W. and Lee V.M., Detection of a novel intraneuronal pool of insoluble amyloid beta protein that accumulates with time in culture. J. Cell. Biol. 141 (1998) 1031-1039.
420. Smale G., Nichols N.R., Brady D.R., Finch C.E., Horton W.E., Evidence for apoptotic cell in Alzheimer's disease. Exp Neurol. 133 (1995) 225-230.
421. Smith M.A., Harris P.L.R., Sayre L.M., Perry G., Iron accumulation in Alzheimer disease is a source of redox-generated free radicals. Proc Natl Acad Sci. (USA) 94 (1997) 9866–9868.
422. Smith M.A., Perry G., Richey P.L., Sayre L.M., Anderson V.E., Beal M.F., Kowall N., Oxidative damage in Alzheimer's. Nature. 382 (1996) 120–121.
423. Smith M.A., Richey P.L., Taneda S., Kutty R.K., Sayre L.M., Monnier V.M., Perry G., Advanced Maillard re action products are associated with Alzheimer disease pathology. Proc Natl Acad Sci. (USA) 91 (1994) 5710–5714.
424. Smith M.A., Sayre L.M., Monnier V.M., Perry G., Radical ageing in Alzheimer's disease. Trends Neuosci. 18 (1995) 172-176.
425. Solomon A.R., Marcinowski K.J., Friedland R.P., Zagorski M.G., Nicotine inhibits amyloid formation by the beta-peptide. Biochemistry 35 (1996) 13568-13578.
426. Solomon B., Koppel R., Frankel D., Hanan-Aharon E., Disaggregation of Alzheimer beta-amyloid by site-directed mAb. Proc Natl Acad Sci. (USA) 94 (1997) 4109-4112.
427. Solomon B., Koppel R., Hanan E., Katzav T., Monoclonal antibodies inhibit in vitro fibrillar aggregation of the Alzheimer beta-amyloid peptide. Proc Natl Acad Sci. (USA) 93 (1996) 452-455.
428. Soto C., Kindy M.S., Baumann M, Frangione B., Inhibition of Alzheimer's amyloidosis by peptides that prevent beta-sheet conformation. Biochem Biophys Res Commun. 226 (1996) 672-680.

429. Spina M.B., Squinto S.P., Miller J., Lindsay R.M., Human BDNF protects dopaminergic neurons against 6-OH and MPP^+ toxicity: involvement of the glutathione syatem. J Neurochem. 59 (1992) 99-105.

430. Sreejayan N. and Rao M.N, Curcuminoids as Potent Inhibitors of Lipid Peroxidation. J Pharm Pharmacol. 46 (1994) 1013–1016.

431. Stadelmann C., Bruck W., Bancher C., Jellinger K., Lassmann H., Alzheimer's disease: DNA fragmentation indicates increased neuronal vulnerability, but not apoptosis. J Neuropathol Exp Neurol. 57 (1998) 456-464.

432. Steiner M. and Li W. Aged garlic extract, a modulator of cardiovascular risk factors. J. Nutr. 131 (2001) 980S-984S.

433. Stellar H., Mechanisms and genes of cellular suicide. Science 267 (1995) 1445-1449.

434. Stephenson R.C., Clarke S., Succinimide formation from aspartyl and asparaginyl peptides as a model for the spontaneous degradation of proteins. J Biol Chem. 264 (1989) 6164-6170.

435. Strittmatter W.J., Roses A.D., Apolipoprotein E and Alzheimer disease. Proc Natl Acad Sci. (USA) 92 (1995) 4725-4727.

436. Strittmatter W.J., Saunders A.M., Schmechel D., Pericak-Vance M., Enghild J., Salvesen G.S., Roses A.D., Apolipoprotein E: high avidity binding to b-amyloid and increased frequency of type 4 allele in late-onset familial Alzheimer's disease. Proc Natl Acad Sci. (USA) 90 (1993) 1977-1981.

437. Su J., Satou T., Anderson A., Cotman C., Upregulation of Bcl-2 is associated with neuronal damage. Neuroreport 7 (1996) 437-440.

438. Su J.H., Anderson A,J., Cummings B.J. and Cotman C.W., Immunohistochemical evidence for apoptosis in Alzheimer's disease. Neuroreport. 5 (1994) 2529-2533.

439. Su J.H., Cummings B.J., Cotman C.W., Early phosphorylation of tau in Alzheimer's disease occurs at Ser 202 and is preferentially located within neurites. Neuroreport. 84 (1994) 265-272.

440. Sugaya K., Reeves M., McKinney M., Topographic association between DNA fragmentation and Alzheimer's disease neuropathology in the hippocampus. Neurochem Int. 31 (1997) 275-281.

441. Suh Y.H., An etiological role of amyloidogenic carboxyl- terminal fragments of the beta- amyloid precursor protein in Alzheimer's disease. J. Neurochem. 68 (1997) 1781-1791.

442. Sun X., Shimizu H., Yamamoto K., Identification of a novel p53 promoter element involved in genotoxic stress-inducible p53 gene expression. Mol Cell Biol. 15 (1995) 4489-4496.

443. Suram A., Hegde M.L., Rao K.S.J., A new evidence for DNA nicking property of amyloid β-peptide (1-42): Relevance to Alzheimer's disease. Arch. Biochem. Biophys. 463 (2007) 245-252.

444. Suram A., Rao K.S., Latha K.S., Viswamitra M.A., First evidence to show the topological change of DNA from B-dNA to Z-DNA conformation in the hippocampus of Alzheimer's brain. Neuromolecular Med. 2 (2002) 289-297.

445. Syme C.D., Nada R.C., rigby S.E.J., Viles J.H., Copper binding to the Amyloidβ-peptide associated with Alzheimer's disease. J. Biol. Chem. 279 (2004) 18169-18177.

446. Takahashi R.H., Milner T.A., Li F., Nam E.E., Edgar M.A., Yamaguchi H. et al., Intraneuronal Alzheimer A{beta } 42 accumulates in multivesicular bodies and is associated with synaptic pathology. Am. J. Pathol. 161 (2002) 1869-1879.

447. Takahashi R.H., Nam E.E., Edgar M., Gouras G.K., Histol. Alzheimer β-amyloid peptides: normal and abnormal localization. Histopathol. 1 (2002) 239-246.

448. Takeuchi H., Hanamura N., Harada I., Structural specificity of peptides in Z-DNA formation and energetics of the peptide-induced B-Z transition of poly(dG-m5dC). J. Mol. Biol. 236 (1994) 610-617.

449. Takuma K., Yan S.S., Stern D.M., Yamada K., Mitochondrial dysfunction, Endoplasmic reticulum stress, and apoptosis in Alzheimer's Disease. J. Pharmacol. Sci. 97 (2005) 312-316.

450. Tamagno E., Bardini P., Guglielmotto M., Danni O., Tabaton M., The various aggregation states of beta-amyloid 1-42 mediate different effects on oxidative stress, neurodegeneration, and BACE-1 expression. Free Radic. Biol. Med. 41 (2006) 202-212.

451. Tanzi R.E., Kovacs D.M., Kim T.W., Moir R.D., Guentte S.Y., Wasco W., The gene defects responsible for familial Alzheimer's disease. Neurobiol Dis. 3 (1996) 159-168.

452. Teller J.K, Russo C, DeBusk L.M, Angelini G, Zaccheo D, Dagna-Bricarelli F, Scartezzini P, Bertolini S, Mann DM, Jabaton M, Gambettip P., Presence of soluble amyloid-beta peptide precedes amyloid plaque formation in Down's syndrome. Nat Med. 1 (1996) 93–95.

453. Terry R.D., Katzman R., Senile dementia of the Alzheimer type. Ann Neurol. 14 (1983) 497-506.

454. Terry R.D., Neuropathological changes in Alzheimer disease. Prog Brain Res. 101 (1994) 383-390.

455. Thomas T.J., Bloomfield V.A., Quasielastic laser light scattering and electron microscopy studies of the conformational transitions and condensation of poly (dA-dT).poly(dA-dT). Biopolymers. 24 (1985) 2185-2194.

456. Thomas T.J., Thomas T., Direct evidence for the presence of left handed conformation in a supramolecular assembly of polypeptide. Nucleic Acids Res. 17 (1989) 3795-3810.

457. Thompson M.A., Argus Lab 4.0.1 Planaria Software LLC, Seattle, WA.

458. Thomson M., Ali M., Garlic [Allium sativum]: a review of its potential use as an anti-cancer agent. Curr Cancer Drug Targets. 3 (2003) 67-81. Review.

459. Tienari P.J., Ida N., Ikonen E., Simons M., Weidemann A., Multhaup G., Masters C.L., Dotti C.G., Beyreuther K., Intracellular and secreted Alzheimer beta-amyloid species are generated by distinct mechanisms in cultured hippocampal neurons. Proc. Natl. Acad. Sci. (USA) 94 (1997) 4125-4130.

460. Tjernberg L.O., Tjernberg A., Bark N., Shi Y., Ruzsicska B.P., Bu Z., Thyberg J., Callaway D.J., Assembling amyloid fibrils from designed structures containing a significant amyloid beta-peptide fragment. Biochem J. 366 (2002) 343-351.

461. Tomaselli S., Esposito V., Vangone P., Van Nuland N.A., Bonvin A.M., Guerrini R., Tancredi T., Temussi P.A., Picone D., The alpha-to-beta Conformational Transition of Alzheimer's Abeta-(1-42) Peptide in Aqueous Media is Reversible:

A Step by Step Conformational Analysis Suggests the Location of beta Conformation Seeding. Chembiochem. 7 (2006) 257-267.

462. Tomiyama T., Asano S., Furiya Y., Shirasawa T., Endo N., Mori H., Racemization of Asp23 residue affects the aggregation properties of Alzheimer amyloid beta protein analogues. J. Biol. Chem. 269 (1994) 10205-10208.
463. Tomiyama T., Shoji A., Kataoka K., Suwa Y., Asano S., Kaneko H., Endo N., Inhibition of amyloid beta protein aggregation and neurotoxicity by rifampicin. Its possible function as a hydroxyl radical scavenger. J. Biol. Chem. 271(1996) 6839-6844.
464. Trumbore C.N., Myers Y.N., Hyde C.K., Hudson R.D., Rhodes C.N., Masselink J.K., Metal-ion assisted, radiation-induced conversion of B- to Z-DNA in poly (dGdC) and two natural DNAs. Int. J. Radiat. Biol. 66 (1994) 479-483.
465. Turnbull S., Tabner B.J., El-Agnaf O.M.A., Twyman L.J., Allsop D., New evidence that the Alzheimer β-amyloid peptide does not spontaneously form free radicals: an ESR study using a series of spin-traps. Free Radic Biol Med. 30 (2001) 1154–1162.
466. Turner P.R., O'Connor K., Tate W.P., Abraham W.C., Roles of amyloid precursor protein and its fragments in regulating neural activity, plasticity and memory. Prog Neurobiol. 70 (2003) 1-32.
467. Tyler-Cross R., Schirch V., Effects of amino acid sequence, buffers, and ionic strength on the rate and mechanism of deamidation of asparagine residues in small peptides. J. Biol. Chem. 266 (1991) 22549-22556.
468. Ugolini G., Cattaneo A., Novak M., Co-localization of truncated tau and DNA fragmentation in Alzheimer's disease neurones. Neuroreport. 8 (1997) 3709-3712.
469. Van Aalten D.M., Bywater R., Findlay J.B., Hendlich M., Hooft R.W., Vriend G.J., PRODRG, a program for generating molecular topologies and unique molecular descriptors from coordinates of small molecules, Comput. Aided. Mol. Des. 10 (1996) 255-262.
470. Van Duijn C.M., Clayton D., Chandra V., Fratiglioni L., Graves A.B., Heyman A., Jorm A.F., Kokmen E., Kondo K., Mortimer J., Rocca W.A., Shalat S.l., Soininen H., Hofman A., for the Eurodem risk factors research group: Familial

aggregation of Alzheimer's disease and related disorders: a collaborative re-analysis of case-control studies. Int J Epidemiol. 20(Suppl 2) (1991) S13-S20.

471. Van Leeuwen F.W., Burbach J.P., Hol E.M., RNA: a first example of molecular misreading in Alzheimer's disease. Trends Neurosci. 21 (1998) 331-335.
472. Vetrivel K.S., Cheng H., Lin W., Sakurai T., Li T., Nukina N., Wong P.C., Xu H., Thinakaran G., Association of gamma-secretase with lipid rafts in post-Golgi and endosome membranes. J Biol Chem. 279 (2004) 44945-44954.
473. Vetrivel K.S., Zhang Y.W., Xu H., Thinakaran G., Pathological and physiological functions of presenilins. Mol Neurodegener. 1 (2006) 4.
474. Vitek M.P., Bhattacharya K., Glendening J.M., Stopa E., Vlassara H., Bucala R., Manogue K., Cerami A., Advanced glycation end products contribute to amyloidosis in Alzheimer's disease. Proc Natl Acad Sci. (USA) 91 (1994) 4766-4770.
475. Vriend G., WHAT IF: a molecular modeling and drug design program. J Mol Graph. 8 (1990) 52-56.
476. Waldemar G., Dubois B., Emre M., Georges J., McKeith I.G., Rossor M., Scheltens P., Tariska P., Winblad B., Recommendations for the diagnosis and management of Alzheimer's disease and other disorders associated with dementia: EFNS guideline. Eur J Neurol. 14 (2007) e1–e26.
477. Walsh D.M., Klyubin I., Fadeeva J.V., Rowan M.J., Selkoe D.J., Amyloid β oligomers: their production, toxicity and therapeutic inhibition. Biochem. Soc. Trans. 30 (2002) 552-557.
478. Walsh D.M., Selkoe D.J., Oligomers on the brain: the emerging role of soluble protein aggregates in neurodegeneration. Protein Pept. Lett. 11 (2004) 213-228.
479. Walsh D.M., Tseng B.P., Rydel R.E., Podlisny M.B., Selkoe D.J., The oligomerization of amyloid beta-protein begins intracellularly in cells derived from human brain. Biochemistry 39 (2000) 10831-10839.
480. Wang Y.J., Pan M.H., Cheng A.L., Lin L.I., Ho Y.S., Hsieh C.Y. and Lin J.K. Stability of curcumin in buffer solutions and characterization of its degradation products. J Pharm Biomed Anal. 15 (1997) 1867-1876.

481. Wei G., Shea J.E., Effects of solvent on the structure of the Alzheimer amyloid-beta(25-35) peptide. Biophys J. 91 (2006) 1638-1647.

482. Weisgraber K.H., Mahley R.W., Human apolipoprotein E: the Alzheimer's disease connection. FASEB J. 10 (1996) 1485-1494.

483. Welsh K.M., Lu A.L., Clark S., Modrich P., Isolation and characterization of the Escherichia coli mutH gene product. J. Biol. Chem. 262 (1987) 15624-15629.

484. Wenk G.L., Neuropathologic changes in Alzheimer's disease. J Clin Psychiatry. 9 (2003) 7-10.

485. Werner P., Knowledge about symptoms of Alzheimer's disease: correlates and relationship to help-seeking behavior. Int J Geriatr Psychiatry. 18 (2003) 1029-1036.

486. Wertkin A.M., Turner R.S., Pleasure S.J., Golde T.E., Younkin S.G., Trojanowski J.Q. et al., Human neurons derived from a teratocarcinoma cell line express solely the 695-amino acid amyloid precursor protein and produce intracellular beta-amyloid or A4 peptides. Proc. Natl. Acad. Sci. (USA) 90 (1993) 9513-9517.

487. Westermark P., Classification of amyloid fibril proteins and their precursors: an ongoing discussion. Amyloid. 4 (1997) 216-218.

488. Williams D.R., Tauopathies: classification and clinical update on neurodegenerative diseases associated with microtubule-associated protein tau. Intern Med J. 36 (2006) 652-660.

489. Wisniewski K., Wisniewski H., Wen G., Occurrence of neuropathological changes and dementia of Alzheimer’s disease in Down’s syndrome. Ann Neurol. 17 (1985) 277-282.

490. Wolfe M.S., Presenilin and gamma-secretase: structure meets function. J Neurochem. 76 (2001) 1615-1620.

491. Wolfe M.S., Therapeutic strategies for Alzheimer’s disease. Nature Rev Drug Discov. 1 (2002) 859-866.

492. Wu C., Heat shock transcription factors: structure and regulation. Annu. Rev.Cell Dev.Biol. 11 (1995) 441-469.

493. Xu H., Gouras G.K., Greenfield J.P., Vincent B., Naslund J., Mazzarelli L., Fried G., Jonanvic J.N., Seeger M., Relkin N.R., Liao F., Checler F., Barbaum J.D.,

Chait B.T., Thinakaran G., Sisodia S.S., Greengard P., Gandy S., Estrogen reduces neuronal generation of Alzheimer's β-amyloid peptides. Nature Med. 4 (1998) 447-451.

494. Xu H., Sweeney D., Wang R., Thinakaran G., Lo A.C., Sisodia S.S. et al., Generation of Alzheimer beta-amyloid protein in the trans-Golgi network in the apparent absence of vesicle formation. Proc. Natl. Acad. Sci. (USA) 94 (1997) 3748-3752.
495. Xu Y.X., Pindolia K.R., Janakiraman N., Chapman R.A., Gautam S.C., Curcumin inhibits IL1 alpha and TNF-alpha induction of AP-1 and NF-kB DNA-binding activity in bone marrow stromal cells. Hematopathol. Mol. Hematol. 11 (1997-1998) 49-62.
496. Yamasaki T., Lau B.H., Garlic compounds protect vascular endothelial cells from oxidant injury Nippon Yakurigaku Zasshi. 110 (1997) 138P-141P.
497. Yang A.J., Chandswangbhuvana D., Margol L. and Glabe C.G., Loss of endosomal/lysosomal membrane impermeability is an early event in amyloid Abeta1-42 pathogenesis. J. Neurosci. Res. 52 (1998) 691-698.
498. Yang A.J., Knauer M., Burdick D.A. and Glabe C., Intracellular A beta 1-42 aggregates stimulate the accumulation of stable, insoluble amyloidogenic fragments of the amyloid precursor protein in transfected cells. J. Biol. Chem. 270 (1995) 14786-14792.
499. Yang F., Lim G.P., Begum A.N., Ubeda O.J., Simmons M.R., Ambegaokar S.S., Chen P.P., Kayed R., Glabe C.G., Frautschy S.A., Cole G.M., Curcumin inhibits formation of amyloid beta oligomers and fibrils, binds plaques, and reduces amyloid in vivo. J Biol Chem. 280 (2005) 5892-5901.
500. Yankner B.A. New clues to Alzheimer's disease: unraveling the roles of amyloid and tau. Nat Med. 2 (1996) 850-852.
501. Yankner B.A., Duffey L.K., Kirshner D.A., Neurotrphic and neurotoxic effects of amyloid β protein: reversal by tachykinin neuropeptides. Science 250 (1990), 279-282.

502. Ye H., Cande C., Stephanou N.C., Jiang S., Gurbuxani S., Larochette N., Daugas E., Garrido C., Kroemer G., Wu H., DNA binding is required for the apoptogenic action of apoptosis inducing factor. Nat. Struct. Biol. 9 (2002) 680-684.
503. Yeh Y.Y., Liu L., Cholesterol-lowering effect of garlic extracts and organosulfur compounds: human and animal studies. J Nutr. 131 (2001) 989S-993S.
504. Yoshiike Y., Tanemura K. and Murayama O. et al., New Insights on How Metals Disrupt Amyloid β-Aggregation and Their Effects on Amyloid-β Cytotoxicity, J Biol Chem. 276 (2001) 32293–32299.
505. Yoshikai S., Sasaki H., Doh-ura K., Furuya H., Sakaki Y., Genomic organization of the human amyloid beta-protein precursor gene Gene 87 (1990) 257-263.
506. Zhao B.L., Li X.J., He R.G., Cheng S.J., Xin W.J., Scavenging effect of extracts of green tea and natural antioxidants on active oxygen radicals. Cell Biophys. 14 (1989) 175-185.
507. Zheng H., Koo E.H., The amyloid precursor protein: beyond amyloid. Mol. Neurodegener. 3 (2006) 5.
508. Zhu M., Rajamani S., Kaylor J., Han S., Zhou F., Fink A.L., The flavonoid baicalein inhibits fibrillation of alpha-synuclein and disaggregates existing fibrils. J Biol Chem. 279 (2004) 26846-26857.

CHAPTER 6

Bibliography

List of publications

1.Veer Bala Gupta, Anitha S, Hegde ML**,** Zecca L, Garruto RM, Ravid R, Stein R, Shankar S, Shanmugavelu P, Rao KSJ (2005) Aluminum in Alzheimer's disease: Are we at a cross roads. *Cell. Mo.l Life Sci.*, 62, 143-158.

2. Hegde ML, **Veer Bala Gupta**, Anitha M, Harikrishna T, Shankar SK, Muthane U, Subba Rao K, Rao KSJ (2006) Studies on brain DNA topology and stability in brain regions of Parkinson's Disease. *Archive. Biochem. Biophys., 449*, 143-156.

3. Veer Bala Gupta, Hegde ML, Rao KSJ (2006) The role of protein conformational dynamics and DNA integrity in neuronal Cell death in neurodegeneration. *Curr. Alz. Res., 3*, 297-309.

4. Veer Bala Gupta, Rao KSJ (2007) Anti-amyloidogenic activity of S-allyl L-cysteine and its activity to destabilize Aβ fibrils *invitro*. *Neurosci. Letts. 429*, 75-80.

5. Veer Bala Gupta, Indi SS, Rao KSJ (2007) Role of amino acid stereospecificity on Amyloid beta aggregation. *J. Mol. Neurosci.* (in press).