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Ph.D. Dissertation

Exploration of cell death signal transduction pathway induced by extracellular Aβ

Graduate School of Chosun University

Department of Biomedical Sciences

Tahmina Bilkis

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세포밖에 위치하는 Amyloid-β에 의해 유도되는 세포사 신호전달체계

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세포밖에 위치하는 Amyloid-β에 의해 유도되는 세포사 신호전달체계

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노인성 플라크의 주성분인 β-아밀로이드(Aβ)는 알츠하이머병에서 세포 내 및 세포 외 과정에 의해 세포 사멸을 유도합니다. 이전에 우리는 세포 내 Aβ 펩타이드가 세포 사멸 카스파 제 활성화보다 일찍 발생하는 펩타이드 특이적 라민 단백질 단편화를 유도한다는 것을 발견했습니다. 현재 연구에서 우리는 세포 외 Aβ가 동일한 유형의 라민 단편화를 유도하고 단편화로 이어지는 세포 사멸 과정을 조사했습니다. 세포외에 위치한 Aβ는 세포내 Aβ와 다른 세포사멸 경로를 유도하므로 상이할 것으로 예상하였다. 이전에 우리와 다른 사람들은 세포 외로 투여된 Aβ가 세포에 들어가 펩티드 특이적 사멸 과정을 유도할 수 있다고 보고했습니다. 따라서 연구를 위해서는 세포 불투과성 Aβ 펩티드가

유용해야 합니다. 테스트된 Aβ 펩타이드 구성물 중에서 FLAG 태그가 지정된 42개 아미노산 Aβ42(fAβ42)가 세포 불투과성 및 세포독성인 것으로 밝혀졌습니다. 공초점 현미경에 의해 밝혀진 세포 불투과성 펩타이드 FLAG 태그 42-아미노산 Aβ42(fAβ42)가 사용되었으며, 라민 단백질 절단 및 카스파제 활성화를 유도합니다. 세포 생존율은 야생형 Aβ42 및 Tat-Aβ42보다 fAβ42로 처리된 세포에서 최대입니다. fAβ42는 세포 불투과성이지만 고독성 Tat-Aβ42와 같은 단량체 및 올리고머 조건 모두에서 LA/LC 및 LB를 절단합니다. 더욱이, fAβ42에 의해 유도된 라미네이트 단편화의 절단된 생성물은 STS 처리된 세포에서 인지되는 라민 단편화와 유사하다. 반면에, 이러한 LA/LC 및 LB의 단편화는 야생형 Aβ42 및 Tat-Aβ42 처리된 세포와 완전히 달랐다. 우리는 이전에 카스파제 의존적 세포자멸사와 비-세포자멸사 세포 사멸이 모두 Aβ에 의해 유도되었음을 관찰했습니다. 이것이 웨스턴 블롯팅 및 효소 분석을 사용하여 세포 독성, 카스파제 및 이들의 기질 처리를 분석한 이유입니다. 우리의 결과는 caspase 활성화 후 fAβ42로 처리된 세포에서 lamin A/C와 lamin B 단편화가 발생함을 보여주었습니다. 28-kDa 및 46-kDa에서 LA/LC 및 LB의 STS 및 fAβ42 유도 절단은 20μM z-VAD-FMK 및 20μM z-DEVD-FMK로 처리한 후 유의하게 억제되었습니다. 카스파제는 STS 및 fAβ42 처리된 세포에서 LA/LC 및 LB의 단편화에

관여했으며, 이는 해당 세포 사멸 경로에서 야생형 Aβ42와 라민 단편화의 다른 특성을 확인시켜줍니다. 이 메커니즘은 아직 명확하게 이해되지 않습니다. fAβ42 독성과 라민 단편화 사이의 상관관계는 카스파제 활성화의 억제가 알츠하이머병의 병리학적 과정을 조절하는 효과적인 방법이 될 수 있음을 시사합니다.

핵심어: β-아밀로이드; 알츠하이머병; 세포 불투과성; 라민 절단; 플래그-Aβ

ABSTRACT

Exploration of cell death signal transduction pathway induced by extracellular Aβ

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β-Amyloid (Aβ), a major constituent of senile plaques, induces cell death by intracellular and extracellular processes in Alzheimer's disease. Previously, we found the intracellular Aβ peptide induces the peptide-specific lamin protein fragmentation which occurs earlier than apoptotic caspase activation. In the current study, we examined extracellular Aβ also induces the same type of lamin fragmentation and the cell death process leading to the fragmentation. Because Aβ located extracellularly induced the cell death pathway different from that of intracellular Aβ, it was expected that they are different. Previously, we and others reported that Aβ administered extracellularly can enter the cells to induce the peptide-specific death processes.Thus, for the study cell-impermeable Aβ peptide should be useful. Among tested Aβ peptide constructs, FLAG-tagged 42-amino-acids Aβ42 (fAβ42) was found to be cell-impermeable and cytotoxic. Cell-impermeable peptide FLAG- tagged 42-amino-acids Aβ42 (fAβ42) revealed by confocal microscopy was used, induces lamin protein cleavage and caspase activation. Cell survival rate is maximum with the cells treated by fAβ42 rather than wild type Aβ42 and Tat-Aβ42. Though fAβ42 is cell-impermeable, it cleaves

the LA/LC and LB in both monomeric and oligomeric condition like highly toxic Tat-Aβ42. Moreover, the cleaved product of lamin fragmentation induced by fAβ42 is similar to lamin fragmentation perceived in STS-treated cells. On the other hand, this fragmentation of LA/LC and LB was totally different from wild type Aβ42 and Tat-Aβ42 treated cells. We previously observed that both caspase dependent apoptosis and non-apoptotic cell death was induced by Aβ. That's why cytotoxicity, caspases and their substrates processing were analyzed using western blotting and enzyme assays. Our results showed that lamin A/C and lamin B fragmentation occur in cells treated by fAβ42 after caspase activation. The STS and fAβ42-induced cleavage of LA/LC and LB at 28-kDa and 46-kDa were significantly inhibited after treatment with 20μM z-VAD-FMK and 20μM z-DEVD-FMK. Caspases were involved in fragmentation of LA/LC and LB in STS and fAβ42 treated cells, which confirmed the differing nature of lamin fragmentation that wild type Aβ42 in that cell death pathway. This mechanism is not clearly understood yet. A correlation between fAβ42 toxicity and lamin fragmentation suggests that inhibition of caspase activation could be an effective way of regulating the pathological process of AD.

Key words: β-amyloid; Alzheimer's disease; cell-impermeability; lamin cleavage; Flag-Aβ

1. INTRODUCTION

I-1. The Aβ peptide linked with Alzheimer's disease

Amyloid precursor protein (APP) was found for the first time in the brains and cerebrovasculature of Alzheimer's disease patients, and it is now considered to be the primary component of amyloid plaques. APP is thought to be the primary source of amyloid beta (Aβ), a 4.5 kDa peptide with 39-43 amino acid residues [1-4]. Amyloid beta is formed as a consequence of APP cleavage and secretase [1-2], and this generation and accumulation of Aβ are connected to the pathophysiology of AD [2]. The most common kind of dementia is Alzheimer's disease, and it is distinguished by the slow degradation of the central nervous system, resulting in memory loss and diminished cognitive function severe enough to impede everyday life [4].

The presence of Aβ-composed senile plaques and neurofibrillary tangles in the brain is thought to be the primary neuropathological characteristic of Alzheimer's disease [6-8]. The heart, kidneys, lungs, spleen, gut, and brain all contain APP, a large type 1 transmembrane protein [6]. The majority of APP is broken down in the endoplasmic reticulum, with only a small amount going through the secretase cleavage pathway [1-8]. The enzymatic pathway that produces Aβ from APP is now well understood. APP use proteolytic cleavage to release some of its derivatives into vesicle lumens and the extracellular environment via the secretory route. A large soluble ectodomain fragment (CTF) is released when APP is cut at the end of its NH2-terminus [1, 9-11]. This CTF is kept in the membrane.

β-secretase cleavage of an APP's ectodomain leads in the discarding of APPsα- and APPsβ. The γ-secretase's final cleavage of α-CTF (APP carboxy-terminal fragments) results in the formation of p3 and Aβ peptides [8-9]. Differential cleavage by γ-secretase results in the distinct C-termini of Aβ. The two most prevalent versions are Aβ-40, which ends at Val at position 40 and Aβ42, which ends at Ala position 42. Because Aβ42 is more neurotoxic and hydrophobic than Aβ40, it has a larger tendency to oligomerize and aggregate, and it is the major element of senile plaques [12-13].

The equilibration of neuronally produced various forms of Aβ inside the brain's intestinal fluid is predicted. Any element that induces an imbalance in Aβ production and Aβ clearance hastens the development of amyloid plaques. Extracellular or intracellular Aβ deposition initiates a series of processes that lead to neurodegeneration: synaptic and neuritic injury, oxidative damage, microglial and astrocytic activation, disrupted neuronal ionic homeostasis, caspase/protease activation, and eventually cell death [11-15]. Because Aβ42 plays a significant role in Alzheimer's disease, the mechanism of its cell cytotoxicity has received a lot of studies.

To date, the precise mechanism of Aβ toxicity has not been thoroughly established. Although the senile plaque is largely extracellular, the significance of this extracellular Aβ in cytotoxicity is also unclear at this time, even though various ideas have been offered. The expression of Aβ inside cells helps to highlight the role of intracellular Aβ accumulation, while the repercussions of significant extracellular Aβ deposition remain unknown.

Furthermore, transfection of Aβ plasmids may affect the membrane characteristics of the cells, interfering with Aβ's cytotoxicity. These concerns led us to utilize TAT-Aβ42, FLAG-Aβ42 to treat the cells, as stated in the materials and methods portion. The percentage of cells killed by these three Aβ42s varies widely. The cells treated with FLAG-Aβ42 had the highest cell survival rate rather than TAT-Aβ42 and wild type Aβ42. The variation in the amount of cytotoxicity generated by the three Aβ42 groups can be explained by the fact that entrance in to the cell may be particularly important for the Aβ42 driven cell death pathway. Flag-Aβ42, a cell-impermeable protein, cleaves the lamin. This study also tried to figure out whether cytotoxocity is caused by deposits inside cells or outside cells.

I-2. Multimeric conformation of Aβ and cytotoxicity

According to the amyloid cascade theory, Aβ aggregation is a key cause of AD [8]. Low and high molecular weight oligomers, protofibrils, and fibrils have all been identified as pathogenic Aβ species responsible for the formation of senile plaques in AD brains. The fibrillar form of Aβ is the key ingredient of the senile plaque in the Alzheimer's disease patient's brain, which is characterized as a polymer of Aβ peptide with a cross-sheet structure [12]. Fibrillar Aβ causes several degenerative disorders, including tau hyperphosphorylation, which leads to neuronal malfunction and degeneration [4]. Aggregation is a sophisticated process that consists of several phases; nevertheless, the aggregation mechanism is not fully described [16-17]. According to several studies, nucleation and extension are two phases in the process of creating insoluble fibers from soluble monomers. As more monomers are added to the existing nucleus, the fibrils

grow quickly [10]. In nucleation, the formation of a nucleus can be seen as a sequence of monomeric peptides being accumulated [10].

During Aβ fibrillization, several conformational changes occur, including trimer, pentamer, high molecular weight Aβ derived diffusible ligands [15], oligomers comprising 15-20 monomers [16], protofibrils [17], and dodecameric oligomers Aβ [18]. These intermediates seen in the cerebral fluid of Alzheimer's disease patients [19] are referred to as "soluble Aβ" [20]. Initially, Aβ40 and Aβ42 aggregation mechanisms are distinct. The earliest aggregation step of Aβ42 is the formation of pentamer or hexamer units known as paranuclei [13, 16]. A greater amount of oligomerization of Aβ42 paranuclei results in the production of larger forms such as large oligomers, protofibrils, and fibrils, among other things [20]. Aβ42 comprises Ile and Ala at positions 41 and 42, which may play a major role in Aβ42's increased toxicity and quicker aggregation qualities when compared to Aβ40 [24-26]. Previously, many investigations demonstrated that extracellular fibrillar Aβ deposition is responsible for its toxicity [24-25]. It has recently been suggested Aβ oligomers, rather than fibrillar Aβ, have a role in Alzheimer's disease pathology [23], which is strongly supported by evidence of soluble Aβ assembly in the brains of Alzheimer's patients [19]. According to recent research, soluble, oligomeric, non-fibrillar forms of Aβ are more harmful than insoluble, fibrillar forms of Aβ, and higher levels of soluble Aβ enhance the degree of synapse loss as well as the severity of cognitive deficits [24-25]. Furthermore, oligomers can migrate across extracellurar areas and are present in both intracellular and extracellular regions. The monomer and insoluble plaque are described as nontoxic Aβ species [26]. It’s still unclear how Aβ causes toxicity [18, 28-31]. An investigation into

how soluble extracellular fAβ42 induces cytotoxicity was the focus of this work.

I-3. Intracellular and extracellular Aβ-induced cell death pathway mechanism (s)

The mechanism of Aβ42-mediated toxicity is unclear, even though it is widely believed to play an important role in the development of Alzheimer's disease. The cellular mechanisms that occur between Aβ production and neuronal loss are yet unknown. Several hypotheses about the relationship between Aβ production and neuronal degeneration and toxicity have been offered. The existence of intracellular Aβ is undeniably there, but the role of this intracellular Aβ in illness progression is debatable. The burning question is whether the accumulated Aβ inside the neuronal cells is the product of direct deposition of intracellularly produced Aβ or absorption of extracellularly produced Aβ. In addition to the plasma membrane, APP has been discovered in mitochondrial membranes, the trans Golgi network, the endoplasmic reticulum (ER), endosomes, autophagosomes, and lysosomes [3]. These subcellular compartments have been identified to contain β- and γ-secretases. As a result, intracellular accumulation may develop in favorable conditions due to intracellular Aβ production.

However, no doubt cleaving the APP and resulting in extracellular Aβ deposition creates a significant quantity of Aβ in the plasma membrane. According to reports, this extracellular Aβ can be re-up taken by cells via membrane micro domains known as lipid rafts and contribute to intracellular deposition [28-33]. A plasma membrane binding and peptide cytotoxicity

have been found to be linked in several studies [34-35]. The N-methyl-D-aspartic acid (NMDA) receptor [32], the low density receptor associated protein-1 [33], the α7 nicotinic acetylcholine receptor [34], the p75 neurotrophin receptor [34], and the receptor for advanced glycation products [35] have all been identified to bind to Aβ [35]. Membrane binding of Aβ has been described as a crucial step in peptide toxicity [36], whereas interaction of Aβ with receptor-associated protein (RAP) has been discovered to increase peptide cellular internalization [37].

A novel interaction of the Aβ peptide with lipoprotein lipase (LPL) has been revealed in recent research, which increased membrane attachment and cellular absorption of the peptide [38]. The first order interaction of Aβ with the cell membrane and receptors, on the other hand, promotes membrane permeability and peptide internalization. An aggregation happens when the anionic lipid comes into contact with the peptide. This makes it easier for the peptide to go from random coils to a β-sheet structure [39]. Along with increased membrane permeability, this increase in structural conversion results in the establishment of the "channel hypothesis", which is based on evidence of Aβ peptides generating ion channels [40], with at least eight distinct ion channels found [41]. According to one study, when Aβs interacts with the membrane, it forms small oligomeric complexes that create multimeric channels with a central pore-like structure [42].

I-4. Intracellular and extracellular Aβ are connected

Additional significant query concerning the relevance of intracellular Aβ is whether the intracellular and extracellular pools of Aβ are separate or

connected. Human studies have yielded inconclusive outcomes. Using immunohistochemistry and digital image analysis, it was discovered that brain locations with significant intracellular Aβ accumulation displayed signs of neuronal lysis, resulting in intracellular Aβ dispersion in the surrounding extracellular space [47-48]. In addition, it was found that Down syndrome brains had an inverse relationship between the amount of Aβ inside and outside the brain [23]. These findings indicate that extracellular Aβ is at least partially dependent on intraneuronal Aβ accumulation. However, they observed that intracellular Aβ deposition is not a predictor of extracellular Aβ deposition [45]. Even if the experiment is expanded to include different intracellular Aβ assembly states, it is unclear if the same conclusion would be reached.

Aβ immunotherapy is being used in a new study to see if the intracellular and extracellular Aβ pools are linked. Aβ immunotherapy has been used in several mouse models and has resulted in the quick and effective removal of extracellular plaque load [46] as well as improved cognition [47]. Extracellular Aβ plaques are removed in the 3x-AD model first, followed by intraneuronal Aβ clearance [48]. The existence of intraneuronal Aβ is noteworthy since it appears first, followed by the formation of extracellular plaques [49]. These date show that clearing extracellular Aβ with immunotherapy resulted in a decrease in intraneuronal Aβ accumulation, which would be an indirect impact. There is a dynamic balance between the intraneuronal and extracellular pool that is sequestered from the cell when extracellular pools are eliminated, suggesting that extracellular Aβ is obtained from intraneuronal pools, according to this research.

I-5. Extracellular and intracellular uptake of Aβ peptide by cells

We don't know how they harm synapses and cause neurodegeneration, but they are considered to have a role in Alzheimer's disease progression. The cellular processes that cause Aβ synthesis, accumulation, and neuronal death have yet to be identified. Several hypotheses are being investigated in order to establish a link between Aβ production and neuronal degeneration and toxicity. They may bind to a broad variety of biomolecules because of their unique structural features. The quantity of Aβ attached to the plasma membrane is strongly linked to the amount of cytotoxicity that Aβ produces in several investigations [34-35].

Aβ has been shown to interact with a number of receptors or receptor-related proteins, including low-density receptor-associated protein-1 [33]. 7-nicotinic acetylcholine receptor [34], p75 neurotrophin receptor [50], and receptor for advanced glycation end-products [35]. Aβ inhibits calcineurin and calmodulin-dependent protein kinase II, resulting in diminished N-methyl-D-aspartate (NMDA) receptor. Another research discovered that when Aβ oligomer interacts with Frizzle receptors, it suppresses Wnt signaling, which is needed for axon guidance and synapse formation. Cellular prion was recently discovered to be an Aβ oligomer receptor [49, 53-57].

In contrast, several studies have found that rather than interacting with particular receptors, Aβ prefers to attach to membrane lipids [51]. The interaction of Aβ with a receptor-related protein (RAP) has been shown to

help the peptide get inside cells, which is key step in making the peptide toxic [40-41]. It has been found that the interaction between Aβ peptide and lipoprotein lipase (LPL) enhances membrane association and cellular absorption of the peptide [38]. The first-order interaction of Aβ with the cell membrane and receptors increase the peptide's membrane permeability and internalization. There's also a good reason why anionic lipoprotein helps the peptide move faster from random coils to a β–sheet structure, which makes it easier to connect Aβ to membranes [39]. The "Channel hypothesis" is supported by the quick conformational shift and enhanced membrane permeability observed. Based on the evidence of Aβ-peptide-induced ion channel formation [40], the "Channel hypothesis" has been proposed for over a decade, and around eight unique ion channels have been discovered during that period [41].

When Aβs interact with the membrane, tiny oligomeric complexes form, which has been reported to produce multimeric channels with a core pore-like shape [42]. It is critical to keep the plasma membrane intact in order to keep the cell alive. Increased membrane permeability produced by Aβ creates channels that disrupt calcium homeostasis. The same number of monomers or fibrils can't produce the same amount of Ca2 + as oligomers can. The development of Aβ channels affects ion homeostasis, particularly Ca2+, which facilitates degenerative processes such as tua phosphorylation, mitochondrial malfunction, free radical production, glutamate receptor alterations, excitotoxocity, and circuitry hyper excitability [58-65].

The source of Aβ deposition inside neuronal cells, whether it is mediated by intracellularly derived Aβ or assimilation of extracellularly produced Aβ, is a major topic. The trans-Golgi network includes the endoplasmic reticulum

(ER), endosomes, autophagosomes, and lysosmes [3]. Furthermore, these subcellular compartments include β- and γ- secretases, which cause the successive proteolytic cleavage of APP to create Aβ, supporting the concept that intracellular Aβ production may cause intracellular accumulation. Exogenous Aβ buildup in neurons has been found in several investigations [61, 66]. According to an in vitro study [56], a dynamin-dependent and RhoA-mediated mechanism regulates Aβ endocytosis [67-68].

In contrast, an in-vivo study found that PC12 cells, cortical and hippocampal neurons, and other cells build up Aβ in a way that isn't saturable, energy-dependent, or endocytic. It's know that Aβ can interact with many molecules after it enters cells, including Aβ-related death-inducing protein (ABDIP), which can cause and speed up cell death when it binds to Aβ. A study in human neuroblastoma cells found that Aβ interacts directly with mitochondria, which makes it easier for peptides to get into the cells through the outer membrane translocase (TOM40) and the inner membrane translocase (TIM22) [64-65]. The interaction of the Aβ peptides with several mitochondrial proteins produces mitochondrial malfunciton, which results in Aβ related cytotoxicity [55]. Amyloid peptide binding protein (ERAB) is one of the proteins in this group (ABAD). It is also referred to by the name of β-amyloid binding alcohol dehydrogenase [69-71]. It is hypothesized to detoxify aldehydes while also preserving mitochondria [57]. When ABAD and Aβ interact, ROS are made because ABAD's detoxifying abilities are cut back. This could be the reason for the mitochondrial malfunction [62], GSK3 and CDK5 are all signaling proteins that have been connected to the neurodegenerative course of Alzheimer's disease. These proteins are called Fyn Kinase, GSK3, and CDK5.

Alzheimer's disease progresses through a variety of signaling pathways, including those of the MAPK family, including ERK and JNK, as well as additional pathways, such as p21-activated kinase. Synaptic failure and altered neurogenesis may happen if signaling pathways aren't working properly. Tau phosphorylation and aggregation, cytoskeletal abnormalities, activation of pro-apototic pathways, and calcium and calpain-dependent proteolysis all play a role [72-76].

I-6. Proteins that inhibit Aβ-associated cytotoxicity

Despite numerous studies, the mechanism of Aβ-associated cytotoxicity remains a mystery, as it involves a variety of pathways, including ion dyshomeostasis [42], apoptosis in combination with other pathways [44], toxic radical formation [46], and complement formation [47]. In the pathophysiology of Aβ, it is believed that the transformation of Aβ monomer conformation into hazardous Aβ oligomers, protofibrillar, and fibrillar species is the most significant stage. To prevent the conversion of monomers into toxic species, a variety of molecules that interact with Aβ were used in the therapeutic strategy [48]. The aggregation and toxicity of Aβ can be controlled by several chaperons, both intracellular and extracellular, which have been discovered. The identification and utilization of proteins that bind to Aβ and prevent aggregation may be relevant in the development of treatment solutions of Alzheimer’s disease in the future.

Cellular functions such as protein folding, degradation, and subcellular trafficking are dependent on molecular chaperones [65]. There are several indications, such as the inhibition of Aβ amyloidogenesis by

small heat shock protein (sHsp) [30, 64, 77], the presence of Aβ binding chaperons in cerebrospinal fluid [63], the suppression of in vivo Aβ toxicity by overexpression of small chaperone [68], and the interaction of Aβ peptide with several chaperone proteins and the subsequent suppression of cellular toxicity in transgenic *Caenorhabdities legans* [69] Apolipoprotein J (ApoJ), also known as "clusterin" or "extracellular chaperone," increases the formation of Aβ fibrils. However, the anti-aggregation protein sHsp αB-crystallin (sHsp αB-crystallin) inhibits the formation of Aβ fibril and at higher concentrations, prevents the accumulation of toxic oligomers [61]. It has also been found that Hsp90 and Hsp70 hinder Aβ assembly during the early stages of fibrillogenesis [70]. The examples provided suggest that chaperones may counteract Aβ's biological function by controlling its aggregation. Even though several proteins have been discovered as inhibitors of Aβ accumulation, the mechanism by which Aβ causes cytotoxicity is currently being explored. Overexpression of various proteins has also been shown to have an inhibitory impact on in vivo cytotoxicity [76, 78], but it has also been shown in multiple in vitro experiments that αB-crystalline [72], ApoE [73], and α-anticymotrypsin [74] have an additive effect on cytotoxicity.

In the case of chaperones, the effect of concentration is very important. For example, at a low concentration, sHsp20 can have an inhibitory effect, but at a high concentration, it can't [67]. While it has been said that the opposite is true for ApoJ [75] According to the researchers, maltose-binding protein (MBP) from *Escherichia coli* is also said to decrease the toxicity of Aβ by prolonging the time it takes for Aβ to polymerize,

which in turn slows down the pace at which fibrils form. This is because the Aβ-MBP complex, which is made up of Aβ and MBP, is sequestered.

I-7. Chemical compounds that inhibit the cytotoxicity associated with Aβ

Therapeutic chemical compounds are categorized based on their ability to block one or more phases of the Aβ monomer >oligomer> fibril production pathway [76], As a result of its direct interaction with small Aβ species, curcumin inhibits the formation of aggregation and fibril formation, as well as the ability to disaggregate mature fibrils [77], making it the most important molecule to be considered as a therapeutic agent for Alzheimer's disease pathogenesis. Using both in vivo and in vitro circumstances, curcumin has been found to suppress the development of aggregates and fibrils, as well as the capacity to disaggregate mature fibrils, among other things. More than a dozen research papers have studied whether thioflavin (ThT) [78] and the sulfonate dye Congo red and its derivatives [79] bind to Aβ and stain it in a tissue slice. These two dyes are commonly utilized in the study of Aβ-mediated fibrillization to assess its properties.

The fascinating thing about multiple studies is that the greatest reported Aβ-peptide inhibitors share certain structural similarities with Congo red and ThT; they are planar and aromatic, respectively. Aromatic stacking is a phenomenon that occurs in both chemistry and biology and is vital in the self-assembly process [80]. Furthermore, in the case of Aβ-related proteins and peptides, an abundant number of aromatic residues are

present; indicating that aromatic stacking may be involved in the aggregation process of Aβ pathogenesis [81- 82]. As evidenced by the protein folding process, which indicates that aromatic stacking has a major influence on the amyloidogenic propensity of proteins, the validity of this theory has been established [83]. According to the findings of a mechanistic investigation into the structural similarities of Aβ assembly inhibitors, the majority of Aβ inhibitors include a minimum of two phenolic rings, two to six atom linkers, and at least three OH groups in their structure.

Flavonoids [91-94] are compounds with anti-allergic, anti-inflammatory, anti-cancer, antioxidant, and antimicrobial properties that are found in abundance in plants. Flavonoids have been shown to have anti-cancer, anti-allergic, anti-inflammatory, antioxidant, and antimicrobial properties [84- 94]. Flavonoids have also been shown to lower the chance of developing Alzheimer's disease [85]. Many studies on *Ginkgo biloba* extracts have shown that flavonoid molecules are necessary for anti-amyloidogenic and anti-apoptotic activity in brain cells, and these studies have used the HE208 and EGb761 chemicals [95-96]. Because fibrillogenesis inhibitor can only save a small percentage of cells exposed to Aβ, they are not always cytoprotective [76, 77]. For example, different identified flavonoids such as myricetin, querecetin, kaempferol, morin, catechin, and baicaleinn act as anti-fibrillogenic and fibril destabilizing agents, but only a few of them have been reported to improve cytotoxicity [97-99], making the correlation of anti-amyloidogenic and anti-cytotoxic effects of flavonoids more difficult.

Aside from that, the antifibrillogenic feature may potentially augment Aβ associated cytotoxicity by limiting the aggregation of poisonous Aβ species to form mature fibrils, with clusterin serving as an example [79]. The

flavonoids present in plants are classified into two types: monoflavonoids and biofavonoids. Monofavonoids are flavonoids that are isolated from plants. Monoflavonoids with two terminal phenyl rings, such as myricetin, morin, querecetin, kaempferol, epicatechin, and apigenin, are planar flavonoids with two terminal phenyl rings. Myricetin, morin, quercetin, kaempferol, epicatechin, and apigenin are examples of monoflavonoids the two terminal phenyl rings, whereas bioflavonoids are composed of two interlinked monoflavonoids, such as quercetin and epicatechin. When compared to monoflavonoids such as apigenin, flavones such as taiwaniaflavone (TF), amentoflavone, and sumaflavone have been shown to suppress Aβ cytotoxicity more effectively [89]. This is attributed to their ability to inhibit Aβ fibrillogenesis.

I-8. Cell-penetrating peptides

Cell-penetrating peptides (CPPs) are units of 5-30 amino acid residues that may pass through the tissue or cell membrane without engaging with specific receptors in an energy-dependent or energy-independent manner [90]. CPPs are also known as peptidic delivery factors because they may transport physiologically active conjugates such as peptides, proteins, DNAs, siRNAs, and tiny medicines into cells [91].Two independent groups created the first CPP in 1988. They discovered that cells from the surrounding media could take up HIV-1's trans-acting activator of transcription (TAT) protein [102,103]. In terms of their physical and chemical characteristics, CPPs may be divided into three groups: cationic peptides, amphipathic peptides, and hydrophobic peptides. Because of the inclusion of arginines and lysines in their structures, cationic CPPs retain strongly positive net charges at physiological pH when compared to other

proteins. The cationic group consists of TAT-derived peptides, penetration, polyarginine [104-108], and Diatos peptide vector 1047 (DPV1047]. Similarly, truncated analogs of arginine (R9), which supports the hypothesis that the transduction ability of CPPs may be dependent on the number and order of amino acids, particularly arginines, contained within the peptide [106-107]. Also reported is that arginines, as opposed to lysines, contribute more to cellular uptake [106-107] because arginine contains a guanidine head group, which can form bidentate hydrogen bonds with negatively charged groups of cell membrane constituents, resulting in increased cellular internalization [108-109].

Amphipathic CPPs are amino acid mixtures that include both hydrophilic and hydrophobic amino acids. Hydrophobic residues including valine, leucine, isoleucine, and alanine are found in amphipathic CPPs, in addition to lysine and arginine. According to the literature [99], amphipathic CPPs are classified into three groups: primary secondary, and proline-rich. Among the many examples of primary, secondary, and proline-rich peptides are MPG and Pep-1 [100], model amphipathic peptide (MAP) [101], transportation [102], and synthetic fragments of Bac7 fragments [103] are all examples. Hydrophobic CPPs vary from other CPPs in that they can translocate across membranes without using energy [104]. The hydrophobic CPPs include the C105Y peptide and Pep-7 [116-117].

The precise mechanism by which CPPs regulate cellular internalization is unknown [118-119]. Multiple studies have shown that CPPs are not carried by passive diffusion or a receptor and transporter-dependent process [94]. A single CPP can enter the cell by a variety of different entrance pathways, which can be generally grouped into two

categories: direct and indirect entry. The first is energy-independent direct plasma membrane penetration, while the second is energy-dependent endocytosis. It was recently postulated that the transport mechanism of CPPs might be an artifact of chemical fixation prior to microscope viewing [81]. Despite disagreements and doubts about the process of translocation, their cell-penetrating characteristics make them an appealing carrier for carrying genes, proteins, medicines, and imaging reagents [107].

I-9. Apoptosis and the Role of Aβ

Apoptosis also known and regarded as a unique and significant method of programmed cell death, entails a sequence of biochemical reactions that remove genetically determined cells. Caspases, a series of cysteine proteases activated in response to an initial stimulus, are generally required in this highly coordinated and energy-dependent process, which links the stimulus to the final death of cells [120-121]. The apoptotic process is an essential part of cellular homeostasis throughout growth and aging, as well as a protective reaction when cells are damaged by pathogens or other potentially dangerous substances. There are two primary apoptotic mechanisms, according to scientific studies: the extrinsic or death receptor route and the intrinsic or mitochondrial system. During intrinsic mitochondria-dependent apoptosis, when Cyto-c is released from the mitochondria, apoptosomes are made that contain Apaf-1 and Cyto-c, as well as dATP and procaspase-9, which are part of the apoptosomes [111]. This protein complex is required for intrinsic apoptosis and is carefully controlled in healthy cells when Cyto-c is stored in mitochondria and the intracellular concentration of K+ is high enough (150mM) to block its formation [80]. Heat shock protein 70, which binds to Apaf-1 and inhibits its development, is

another protein that regulates the multiprotein complex [112]. The creation of the apoptosome causes caspase-9 to be activated, which then proteolytically cleaves caspase-3 for activation [113]. TNF, CD95, and Trail create the death-inducing signaling complex (DISC) by binding to death receptors and activating caspase-8 through the receptor-mediated extrinsic apoptotic pathway [114]. Execution is the last phase of both intrinsic and extrinsic apoptosis. These effector caspases are processed here, including caspase-3, -6, and -7. Most of the apoptosis's aftereffects are mediated by these caspases [126-127].

In vitro and in vivo apoptotic processes have been stated to be linked to Aβ, according to several researches. The mechanism of Aβ-induced apoptosis can be explained by a number of different possibilities (programmed cell death) [128, 81]. There is evidence that mitochondrial apoptosis-inducing factor (AIF) is produced from mitochondria in cultured neurons, which has been linked to DNA breakage and chromatin condensation, which leads to caspase-independent cell death [125-126,129]. Extrinsic pathway activation can be triggered by Aβ binding to the p75 neurotrophin receptor, whereas intrinsic pathway activation can be triggered by mitochondrial malfunction induced by the peptide. In addition to the unfolded protein response signaling pathway, caspase-4-mediated apoptosis can also triggered by this pathway. This signaling pathway is linked to neurodegenerative diseases like Alzheimer's [119]. A third signaling pathway, called the unfolded protein response, is also thought to play a role in Alzheimer's.

1-9. Outline of the thesis

The thesis focused on the exploration of the cell death signal transduction pathway induced by extracellular Aβ. Previously, we found the intracellular Aβ peptide induces the peptide-specific lamin protein fragmentation, which occurs earlier than apoptotic caspase activation. The first part described how extracellular Aβ also induces the same type of lamin fragmentation and how the cell death process leads to the fragmentation. The latter portion investigated the importance of the cellular entrance of Aβ to induce its effect. The rationale for this thesis is summarized below.

1. The mechanism and role of the apoptotic pathway and lamin fragmentation induced by extracellular Flag-Aβ42 have been elucidated.
2. To better understand the role of Aβ42 in cytotoxicity and other events that occur when cells are treated with Aβ42, the physicochemical properties of Aβ42 fibrils, Flag-Aβ42, and Tat-Aβ42 were investigated.
3. Previously described Aβ42 inhibitors were employed to demonstrate the correlation between cellular internalization and cellular processes that happened during Aβ42 treatment.

II. MATERIALS AND METHODS

II-1. Materials

The fetal bovine serum (FBS) was procured from Life Technology Inc. (Grand Island, USA). Dulbecco's modified Eagles medium (high glucose) and penicillin/Streptomycin were provided by Welgene (Daegu, South Korea). The urea and phosphate buffer saline (PBS) were supplied by Georgia Chem (Georgia, USA) and Ameresco (Solon, USA), respectively. OCI Company, Ltd. (Seoul, South Korea) provided methanol and ethanol, while Bioneer (Daegu, South Korea) provided isopropy-1-D1-thiogalactopyranoside (IPTG).

Unless otherwise specified, dithiotheritol (DTT), 1,1,1,3,3,3-hexafluoro-2-propanol (HFIP), thioflavin T (ThT), ammonium hydroxide (ND4OH), 3-(4,5-dimethylthiazol-2y1)-2,5-diphenyl tetrazolium bromide (MTT), N, N-dimethylformamide (DMF), and all other compounds were bought from Sigma (St, Louis, USA).

Caspase-9, Caspase-8, Caspase-6, and Caspase-3 substrates were provided by Alexis (Lausen, Switzerland), which also supplied N-acetyl Leu-Glu-His-Asp-amino methyl coumarin (Ac-LEHD-AMC), Ac-IETD-AMC, and Ac-DEVD-AMC. iNtron Biotechnology provided the Western blot detection kit (WEST-B) (Gyeonggi-do, Korea). Sigma was the source of all extra compounds unless otherwise stated (St. Louis, USA).

II-2. Cell Culture and Cytotoxicity Assay

The Dulbecco's modified Eagle's medium (high glucose) was used to culture the human epithelial HeLa cells and was supplemented with 10% FBS and 1% penicillin/streptomycin. The SH-SY5Y cells were cultured in Opti MEM medium and were supplemented with 10% FBS and 1% penicillin/streptomycin. Cytotoxicity assays were performed using 15,000-cell/well-seeded cells in 96-well plates (Nunc, Roskilde, Denmark) and grown for 24 hours before being serum-deprived for a further 12 hours before being treated with flag-Aβ42 and fibrils Aβ42 as instructed. All of the wells were filled with 20μl of a solution of 3-(4, 5-dimethylthiazole-2-yl)-2, 5-diphenyltetrazolium bromide (MTT) in PBS, and the cytotoxicity assay was used to look at the results.

After a 2-hour incubation period, the solubilization buffer [20% sodium dodecyl sulfate (SDS) solution in 50% (v/v) DMF (pH 4.7)] was mixed and incubated for 16 hours. A microplate reader (the Kisan Bio plate reader) was used to measure absorbance at 570 nm (Kisan Bio, Seoul, South Korea).

The Alamar Blue Assay was also used for the cytotoxicity assay, with 10μl of Alamar Blue (Life Technologies, Carlsbad, CA, USA) directly added to each well after treatment and incubated for 16 hours. A microplate spectrofluorometer, Gemini- XS, respectively (Molecular Devices, Sunnyvale, CA, USA) was used to measure absorbance.

II-3. Assessment of Caspase Activity

In a 96-well plate cells (2×10^4) were seeded, cultured at 37^0 C for 24 hours, and then serum-starved for another 12 hours, following the treatment plan. The cells were subjected to several treatments. After that, the cells were washed twice with ice-cold PBS to remove any remaining treatment residue.

In order to lyse the cells, 40µl of lysis buffer (20mM HEPES-NaOH, pH 7.0, 1mM ethylene diamine tetra acetic acid (EDTA), 1mM EGTA, 20mM NaCl, 0.25 percent Trition X-100, 1mM dithiotheritol, 1mM PMSF, 10µg/ml leupeptin, 5µg/ml pepstain A, 2µg/ml aprotinin, and 25µg/mlN-acetyl-Leu-Leu-Norleu-al) were applied to each well in each plate.

During the next 20 minutes, the mix was kept on ice. During that time, the caspase assay buffer was made with 20mM HEPES-NaOH, pH 7.0, 20mM NaCl, 1.5mM $MgCl_2$, 1mM EDTA, 1mM EGTA, and 10mM DTT. After that, the caspase assay buffer and the 10µM Ac-DEVD-AMC for DEVDase activity were added in the presence or absence of monomers and oligomers of Flag-Aβ42 and fibrils Aβ42.

Caspase-9, Caspase-8, and Caspase-6 activity were evaluated using 50µM Ac-LEHD-AMC, 50µM Ac-IETD-AMC, 50µM Ac-VEID-AMC in the presence or absence of monomers and oligomers of Flag-Aβ42 and fibrils Aβ42. At 5-minute intervals, the release of aminomethylcoumarin (AMC) was measured using a microplates spectrofluouometric Gemini-XS at excitation and emission wavelengths of 360 nm and 480 nm (Molecular Devices). The data was shown using the slope of total readings vs. time. A.

G. Scientific Inc. provided the caspase substrates (DEVD-AMC, LEHD-AMC, IETD-AMC, and VEID-AMC) (USA).

II-4. Preparation of Flag-Aβ42 solution, Aβ42 oligomers, Aβ42 fibrils and TAT-Aβ42

All of the purified peptides that had been monomerized by 1,1,1,3,3,3,- hexafluroro-2-propanol (HFIP) and dried under nitrogen flow for 30 minutes. They were then speed vacuumed and stored at -20^{0}C. The monomerized Aβ42 and Flag-Aβ42 were dissolved in 0.1% NH_4OH at a concentration of 2mg/ml. After that, bath sonication was done for 10 minutes, and then used. Peptides were diluted in a cell culture medium at a (1M) concentration, vortexed for 30 seconds, and then incubated at 4^0 C for 24 hours to produce Aβ42 oligomers. The TAT-Aβ42 peptide was dissolved in o.1% HCl prior to use.

For 15 minutes, the reaction mixture was centrifuged at 16000× g. After centrifugation, there was no visible particulate. Fibrils were created using a high peptide concentration. Aβ42 (100 M) was incubated in PBS containing 0.02 percent sodium azide for 4 days at 37^{0}C. For 30 minutes, the samples were centrifuged at 16000× g. PBS was used to wash the pellet fraction (fibrils) three times. Fibrils were sonicated for 10 minutes, measured with the Bradford method, and used right away or kept at -80^{0}C.

II-5. SDS-PAGE gel Analysis

The SDS-PAGE gel analysis was carried out in accordance with the prior publication's protocol. Protein samples were mixed in 6X SDS-PAGE gel loading buffer and heated at 100^0C for 5 minutes before being loaded at a concentration of 10-15 percent onto a polyacrylamide gel. After electrophoresis, the gel was stained with Coomassie brilliant blue to show the protein bands.

II-6. Preparation of cell extract

To make the cell extract, cells were rinsed in ice-cold PBS before being suspended in buffer A (20mM HEPES-KOH, pH 7.5, 0.5mM EDTA, 0.5mM EGTA, 10mM KCl, 1mM mercaptoethanol, 0.1mM PMSF, 10 mg/ml leupeptin, 5mg/ml pepstatin A, 2mg/ml aprotinin, and 25mg/ml ALLN). After centrifugation at 17,000 x g for one hour, the supernatant was collected and stored at -80^0C. The samples were then subjected to western blotting.

II-7. Western blot analysis

The extract was obtained by extracting cells, washing them in ice-cold PBS, and resuspending them for 20 minutes on ice in buffer B (50mM Tris-HCl, pH 8.0, 150mM NaCl, 1% Triton X-100, 5mM EDTA, 5mM EGTA. 1mM PMSF, 10mg/ml leupeptin, 2mg/ml pepstatin A, 2mg/ml aprotinin, and 25mg/ml ALLN). The Bradford assay was used to assess protein concentration. On a 10-15% SDS-PAGE, equal amounts of proteins

were separated and transferred to a PVDF membrane. To probe the membrane, primary antibodies, and horseradish peroxidase-conjugated secondary antibodies were utilized. The blots were visualized using the West-Zol plus reagent (iNtron Biotechnology, Inc., Seoul, Korea). The precipitates generated after microfuge centrifugation were also investigated, but no expected proteins were found. Antibodies for lamin A/lamin C (LA/LC), lamin B (LB), β-actin, and anti-mouse and anti-goat immunoglobulin were purchased from Santa Cruz Biotechnology (USA).

II-8. Circular Dichroism (CD) Spectroscopy

Peptide solutions of 20μM were prepared in PBS, and spectra were taken immediately or after 24 hours of incubation at 37^0C. A Jasco J-810 Spectropholarimeter (Jasco CO., Tokyo, Japan) was used to capture far UV circular dichroism (CD) spectra using a 1mm path length cuvette at 0.5nm intervals between 190 and 250nm at 25^0 C. With 0.1nm resolution, a 0.5-s response time, and a scan speed of 50nm/min, five accumulative measurements were averaged and obtained.

II-9. Fibrillogenesis Study

Peptide solution (20μM) was made in PBS and incubated at 37^0C in a final volume of 300 μl, 20 μl of which was withdrawn and mixed with 80 μl of newly prepared 5M ThT in PBS. It was done with a Gemini-XS microplate spectrofluormeter from Molecular Devices in Sunnyvale, Califormia. It had an excitation wavelength of 445 nm and emission wavelength of 490 nm.

II-10. Immunocytochemistry

HeLa cells and SH-SY5Y cells were seeded at 70% confluency on a 12 well plate, cultured for 24 hours, then incubated for another 12 hours in serum-free medium before being treated with 20μM Aβ42, fibrils Aβ42, and Flag- Aβ42 for varying periods of time. Cells were collected and permeabilized with 0.3% Triton X-100 after being fixed in methanol. Mouse anti-amyloid-beta (6E10) and rabbit anti-caspase-9 antibodies (dilution, 1:100) were added after blocking with 0.1% BSA, and incubated overnight at 4^0C. The Alexa-Fluor-488-conjugated goat anti-mouse IgG and Alexa-Flour-543-conjugated goat anti-rabbit IgG antibodies (dilution, 1:200) were incubated for 2 hours at room temperature after washing with PBS. A Carl Zeiss LSM 510 microscope (Jena, Germany) and software called LSM510 were used to take confocal images of the nuclei. The nuclei were stained with the nucleic acid fluorochrome Hoechst (blue). Alexa-Fluor-488-conjugated goat anti-mouse IgG and Alexa-Flour-543-conjugated goat anti-rabbit IgG antibodies were procured from Jackson Immunoresearch Laboratories (West grove, PA).

III. RESULTS AND DISCUSSION

III-1. Lamin cleavage induced by Flag-Aβ42

III-1-1. Monitoring entry of mAβ42, oAβ42, Aβ42 fibrils, mfAβ42 and ofAβ42 into the HeLa cells

At first we examined the entry of each Aβ into the cells by monitoring confocal image of each sample. For 24-h treatment samples with mAβ42, and oAβ42 at 20μM,~17% and~28% cells have the peptide inside respectively (Fig 1B, C).

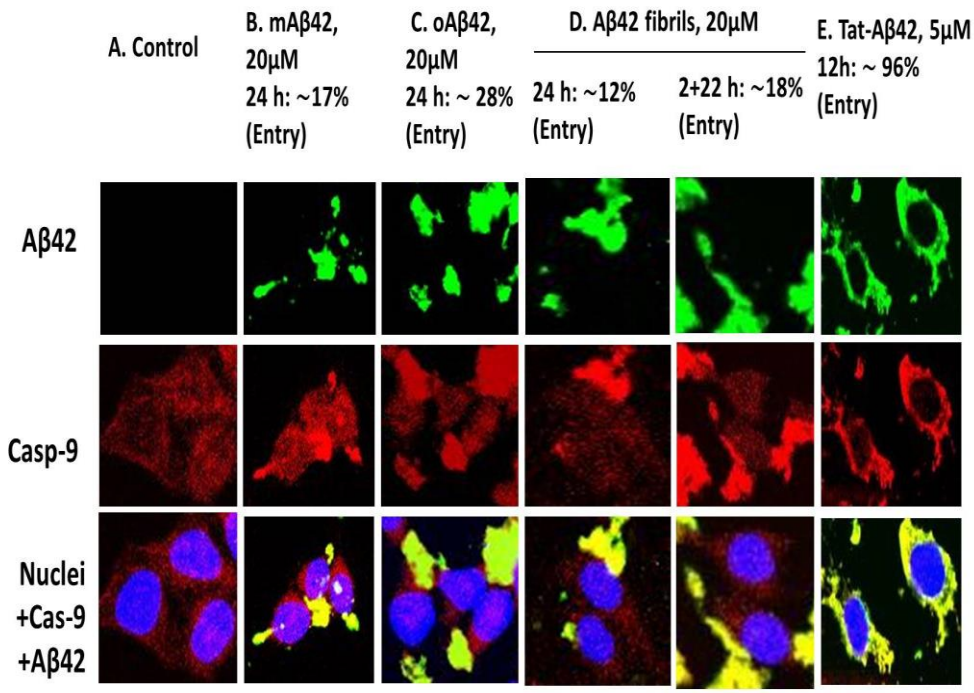

Fig 1: Extent of Cellular Internalization of mAβ42, oAβ42 and Aβ42 fibrils in HeLa cell. HeLa cells were treated with monomeric Aβ42 **(B)**,

oligomeric Aβ42 **(C)** and fibril Aβ42 **(D)** for specified time at indicated concentrations. In confocal microscope images of Aβ and caspase-9 in HeLa cells, mouse anti-Aβ(6E10) and rabbit anti-caspase-9 (p10) antibodies were used to identify Aβ (green) and caspase-9 (red) which were visualized by using secondary anti-mouse IgG-FITC and anti-rabbit IgG-rhodamine antibodies, respectively. Nuclei were stained with the nucleic acid flurochrome Hoechst (blue). Red color denotes the cytoplasm and yellow spots indicate the interaction of procaspase-9 and Aβ. Control is cells incubated without Aβ42 peptide **(A)**. Only representative images of cells are presented. Percentage indicates the entrance of peptide in to the cells. Monomeric Aβ42, oligomericAβ42, fibril Aβ42, Tat-Aβ42 are represented as mAβ42, oAβ42, Aβ42 fibrils and tAβ42 respectively. Images were using a confocal microscope (LSM 510, Carl Zeiss).

Samples incubated for longer time were not monitored, because the images of those samples were not clear, probably due to the debris of cells. The samples treated with Aβ42 fibrils for 24h which showed entry of peptide, (~12% and~18%) (Fig 1D). Moreover, when cells were treated with 5μM freshly prepared Tat- Aβ42 for 12 h,~96% peptide inside the cell (Fig 1E).

On the other hand, the cells were treated with mfAβ42, for 24h, 2+22h, 36h and 2+34h resulted less cells conveying the peptide that Aβ42 (~8%,~12%, ~17%, and~22%) (Fig 2A). In case of ofAβ42 the peptide entered into the cells~10% and~15%, in 24h and 2+22h treatment respectively (Fig 2B). The entry of fAβ42 is less than Aβ42 and Aβ42 fibrils. Though fAβ42 is cell impermeable rather than Aβ42 and tAβ42, it has cytotoxic effects and it cleaves lamin A/C and lamin B.

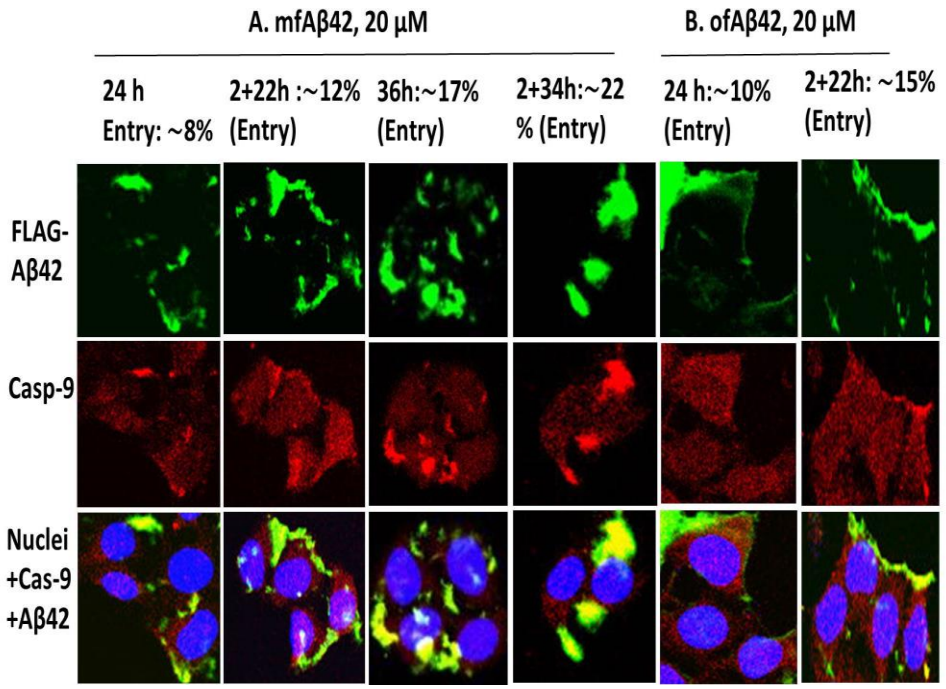

Fig 2: Extent of Cellular Internalization of mfAβ42 and ofAβ42 in HeLa cell. HeLa cells were treated with monomeric flag Aβ42 **(E)**, oligomeric flag Aβ42 **(F)** for specified time at indicated concentrations. Mouse anti-Aβ(6E10) and rabbit anti-caspase-9 (p10) antibodies were used to identify Aβ (green) and caspase-9 (red) which were visualized by using secondary anti-mouse IgG-FITC and anti-rabbit IgG-rhodamine antibodies, respectively in confocal microscope images of Aβ and caspase-9 in HeLa cells. Nuclei were stained with the nucleic acid flurochrome Hoechst (blue). Red color denotes the cytoplasm and yellow spots indicate the interaction of procaspase-9 and fAβ. Only representative images of cells are presented. Percentage indicates the entrance of peptide in to the cells. Monomeric flag Aβ42 and oligomeric flag Aβ42 are represented as mfAβ42 and ofAβ42 respectively. Images were visualized using a confocal microscope (LSM 510, Carl Zeiss).

We also observed the ratio of cells with intracellular Aβ (%) and cell death (%) from the cell death assay and confocal microscopy. In every cases cell death was not depended on entry of peptide into the cell except tAβ42. In case of fAβ42, the entry of peptide into the cell was very less than Aβ42 and tAβ42. The highest entry of cell was observed in case of tAβ42 at 12 h cell treatment.

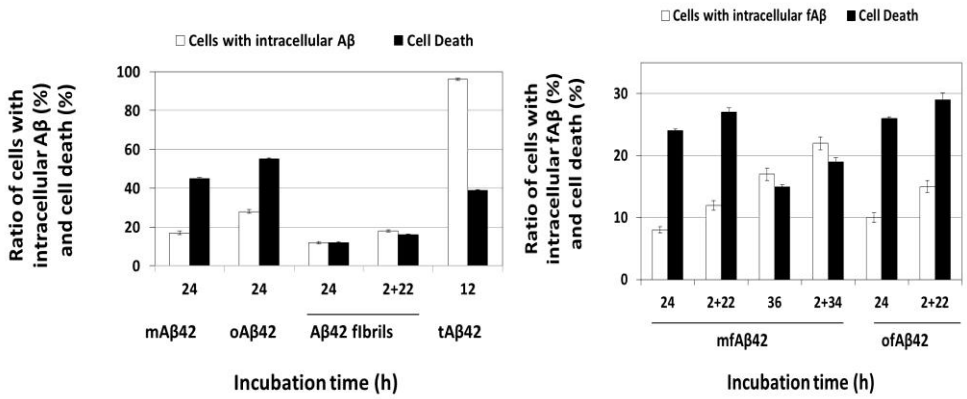

Fig 3: Ratio of cells with intracellular Aβ (%), Aβ42 fibrils (%), fAβ42 (%) and cell death (%) in HeLa cell. Summary of the comparison of the number of cells with intracellular Aβ42 and fAβ42 and cell death is presented. The number of cells with intracellular and extracellular Aβ was calculated using the results shown in Fig 1, Fig 2 and the data for cell death were obtained from Fig 3. Data are presented as the mean ± standard deviation of values from three independent experiments.

III-1-2. Cytotoxicity of fAβ42, Aβ42 fibrils and tAβ42 into Hela cells

We explored the cytotoxic effect of each preparation of fAβ42, Aβ42 fibrils and tAβ42. The cell death was monitored using MTT assay, results of which were confirmed by alamar blue assay throughout the current study. Freshly prepared fAβ42 (monomeric preparation, mfAβ42) ant up to 30μM induced less than 10% cell deat for 12h incubation (Fig 4A, B). Further incubation of the cells with the peptide for 24h increased the cell death up to 30% (Fig 4C,D). The oligmeric preparation of fAβ42 (ofAβ42) was not so cytotoxic for 12h incubation (Fig 4A, B) either and the toxicity increased when the cells were further incubated for 24h (Fig 4B, E). Both monomeric and oligomeric condition of fAβ42 induced less than 25% cell death in case of 36h treatment (Fig 4E, F).

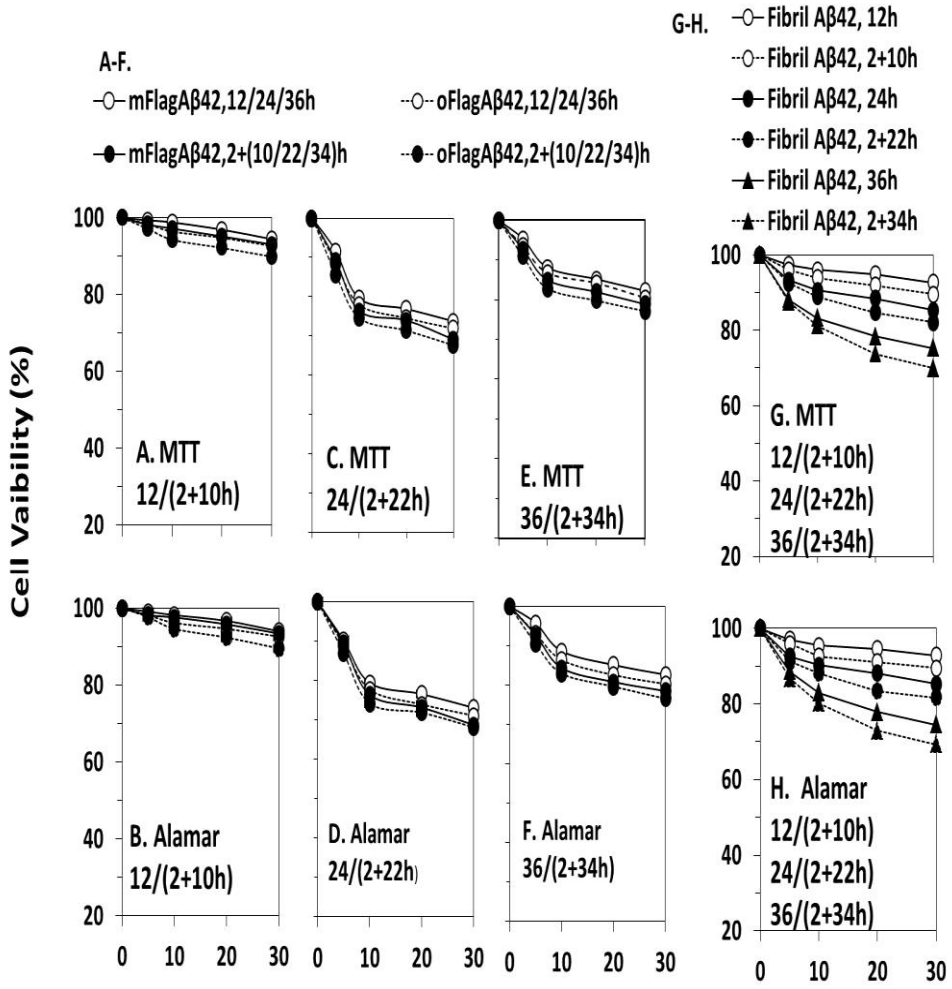

Fig 4: Cell Cytotoxicity of fAβ42 and Aβ42 fibrils by MTT reduction Assay and Alamar Blue Assay. (A-H) HeLa cells (1.5×10^{4}) were treated at different concentrations for the indicated time with monomeric and oligomeric flag-Aβ42, and Aβ42 fibrils. **(A-F)** After treatment, cell viabilities were assessed with MTT reduction assay and Alamar Blue assay. Effect of monomeric and oligomeric form of flag-Aβ42, and Aβ42 fibrils when cells are treated at different concentrations **(A-B)** for 12h or (2+10h) and **(C-D)** for 24h or (2+22h). **(E-F)** Effect of longer period of treatment for

36h or (2+34h) at different concentrations on cytotoxicity induced by monomeric and oligomeric form of flag-Aβ42. **(G-H)** cells were treated with Aβ42 fibrils at different concentrations for 12h or (2+10h), 24h or (2+22h) and 36h or (2+34h) respectively. Control is cells incubated without peptides. The results are expressed as mean±standard deviation of 3 independent experiments. Here, mfAβ42, ofAβ42 and Fibril Aβ42 represent monomeric flag Aβ42, oligomeric flag Aβ42 and Aβ42 fibrils.

When compared to wild type Aβ42, oAβ42 was more cytotoxic than mAβ42. According to one study, oligomerization of the peptide increased cytotoxicity by up to tenfold [120]. The difference was smaller than anticipated. This study found that mfAβ42 and ofAβ42 were less cytotoxic than wild type Aβ42.

After that wild-type Aβ42, Aβ42 fibrils and fAβ42 preparations were less cytotoxic than tAβ42 in the 12, 24 and 36 h incubation samples (Fig 4A-F); treatment of freshly prepared tAβ42 (mtAβ42) at 1-20 μM to cells for 24 h resulted in ~ 40% viability in the both assay (Fig 5B, C), while >20μM of wild-type oAβ42 necessary to induce those levels of viability (Fig.5). When compared to wild type Aβ42 was more cytotoxic than mAβ42. The difference was smaller than anticipated. This study found that mAβ42 and ofmAβ42 were less cytotoxic than wild type Aβ42. We also wonder if the double treatment of cells with Aβ peptide increases the peptide's entry into the cells because double treatment has been shown to increase cell death [121,122], and we found that apoptotic caspase activation and Aβ- specific lamin cleavage were only seen in the doubly treated cells [121], though the underlying mechanisms are still unknown.

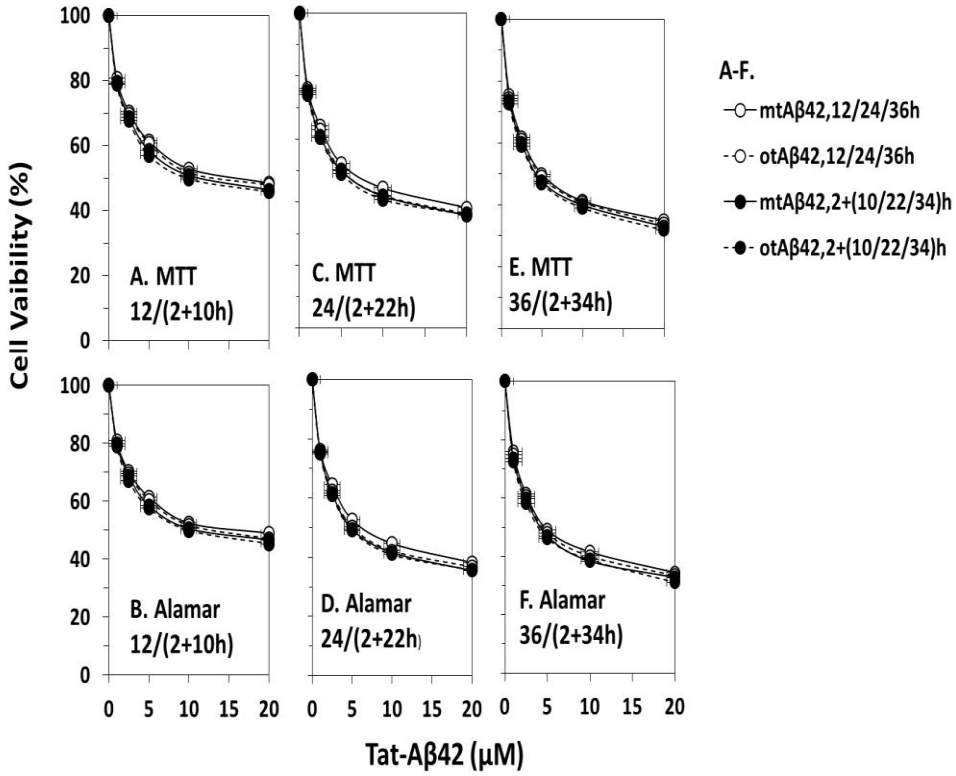

Fig 5: Cell Cytotoxicity of mtAβ42 and otAβ42 by MTT reduction Assay and Alamar Blue Assay in HeLa cell. (A-F) HeLa cells (1.5×10^{4}) were treated at different concentrations for the indicated time with monomeric and oligomeric tAβ42. **(A-F)** After treatment, cell viabilities were assessed with MTT reduction assay and Alamar Blue assay. Effect of monomeric and oligomeric form of tAβ42, when cells are treated at different concentrations **(A-B)** for 12h or (2+10h) and **(C-D)** for 24h or (2+22h). **(E-F)** Effect of longer period of treatment for 36h or (2+34h) at different concentrations on cytotoxicity induced by monomeric and oligomeric form of tAβ42. Control is cells incubated without peptides. The results are expressed as mean±standard deviation of 3 independent experiments. Here, mtAβ42 and otAβ42 represent monomeric tAβ42 and oligomeric tAβ42.

No prominent increase of the cytotoxicity by the double treatment was detected in the 12h, 24h and 36 h equivalent 2+(10,22 and 34) h samples with mfAβ42 (Fig 4A-F). There is no significant increase of cytotoxicity by the double treatment was observed in 2+10h samples with mfAβ42 (fig 4A-F). There is the significant increase of cytotoxicity by the double treatment was observed in 2+ 10h samples with of Aβ42 (Fig 4A, B), while the increase was also not so prominent in further incubated (2+22h, 2+34h) samples (Fig 4C, F).

Next we examined cytotoxicity with Aβ42 fibrils for 12h, 24h and 36h. There is increased cell death while the treatment time increased form less than 10% to 30 %. The cytotoxicity was also less than any other preparation of wild type Aβ42 (Fig 4G, H). For the this reason, in addition to the cell death tests, the Alamar Blue assay was also carried out [74]. A dose-dependent reduction in MTT formazan was seen i samples incubated for 24 and 36 hours, respectively, in the presence of f Aβ42 (Fig 5). Although the levels of Ala mar Blue decreased were lower than those seen in the MTT test, a consistent decline was detected in the 36-h samples (Fig5).

Cell death was seen in both the 24h and 36h samples, according to these findings. As a result, we found that the fAβ42 employed in this study was less cytotoxic than Aβ42. On the other hand, whether cell death period 12h samples with a drop in MTT formazan but no fAβ42-dose dependence are debatable. Furthermore, the levels of cell death in the sample were little decreased in the Alamar Blue assay . It has previously been demonstrated that Aβ42 fibrils cause extrinsic apoptosis, which activates caspase-8 and caspase-3 [123]. The acivity of caspase-3-like DEVDase was not found in

fibrillar Aβ42 –treated cells in this investigation (Fig. lA). MTT formazan levels were lowered in cells treated with the fibrillar form of Aβ42, but the reduction was not dose-dependent (Fig 14). According to the data, it appears that a non-specific decrease of MTT formazan may have been the dose-dependent same samples. An Alamar Blue test revealed a dose-dependent decrease in Aβ, indicating cell death caused by Aβ42. Overall, our findings show that fibrillary Aβ42 causes cell death independent of caspase-dependent apoptosis.

This was used as a baseline to see how the fAβ42 peptide formulation affects the cells. The levels of cell death determined by MTT formazan reduction were often higher than those determined by the Alamar Blue test (2+22 h and 2+34 h samples of MTT vs those of Alamar Blue), as was the case in the single treatment samples (2+22 h and 2+34 h samples of MTT vs those of Alamar Blue) (Fig 5). After two and a half days of treatment, the values were reduced by a somewhat greater amount than in the corresponding single treatment sample (i.e. 2+22 h as opposed to 24 h). In general, no significant changes in cell death triggering were seen between the single and double treatment samples.

III-1-3. Differential Fragmentation of Lamin A/C and Lamin B by Aβ42 oligomers, Aβ42fibrils, STS and Flag-Aβ42

Following that, we sought to detect LA and LB cleaved products. LA and LC each have 664 and 572 amino acids, respectively, whereas LB has 586 amino acids [124]. When the cells with treated with oAβ42 (2+22h) LA and LB can be cleaved at Try376 and Tyr377, respectively, to produce ~46- and ~21-kDa fragments that can be detected western blotting (Fig. 6A) [58,

125]. But, in case of Aβ42 fibrils (2+22htreatment), there was found no cleaved product of LA/LC and LB (Fig. 6A).

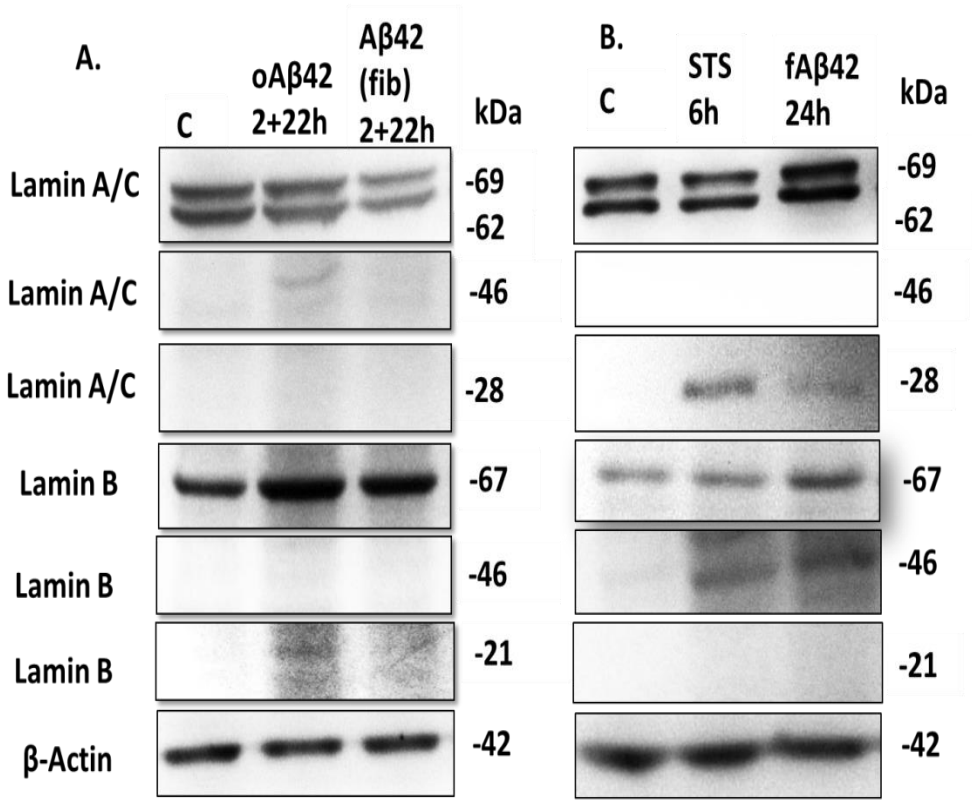

Fig 6: Differential Fragmentation of Lamin A/C and Lamin B by Aβ42 oligomers, fibrils and Flag-Aβ42. (A) HeLa cells (4×10^{4}) were treated with 20μM oligomeric Aβ42 and Aβ42 fibrils (20μM) for 2+22h. **(B)** HeLa cells were treated with STS (0.5μM) for 6h and fAβ42 (20μM) for 24h. Cell lysates were prepared and cleavage of Lamin A/C or Lamin B was evaluated by performing western blotting. As a loading control, β-Actin was used. Relative molecular weights (in kDa) are denoted at the top.

Caspase-6, on the other hand, cleaves the C-termini of LA/LC and LB to form 28- and 46-kDa fragments that can be identified using antibodies

specific to the N-terminus of LA/LC and the C-terminus of LB [62,125,126]. The fragments found in STS-treated cells matched to caspase-6 products (Fig. 6B), indicating that caspase-6 is involved in the STS-induced fragmentation of LA/LC and LB (Fig. 6B) [124]. When cells were treated with mfA42 and ofA42 for 24 hours, LA/LC and LB produced 28- and 46-kDa fragments, which were identified using the same antibodies as above (Fig. 6B). This outcome differed from wild type Aβ42 but was comparable to STS treated cells. The fragments found in STS-treated cells matched to caspase-6 products, confirming caspase-6's participation in the STS-induced fragmentation of LA/LC and LB [124].

III-1-4. LA/LC and LB Fragmentation by fAβ42 in different concentration in HeLa cell

The HeLa cells were treated with different concentration (5μM, 10μM, 20μM) of fAβ42 for 24 hrs. Results showed that fAβ42 cleaves LA/LC and LB at different concentration (Fig 8). Even it can cleave at very low concentration (5μM).One the other hand, wild type Aβ42 required 20μM for 2+22hrs treatment. Though fAβ42 was less cytotoxic than wild type Aβ42, it cleaved LA/LC and LB in very small concentration. The cleaved product was also different from wild type Aβ42 followed by STS treated cells.

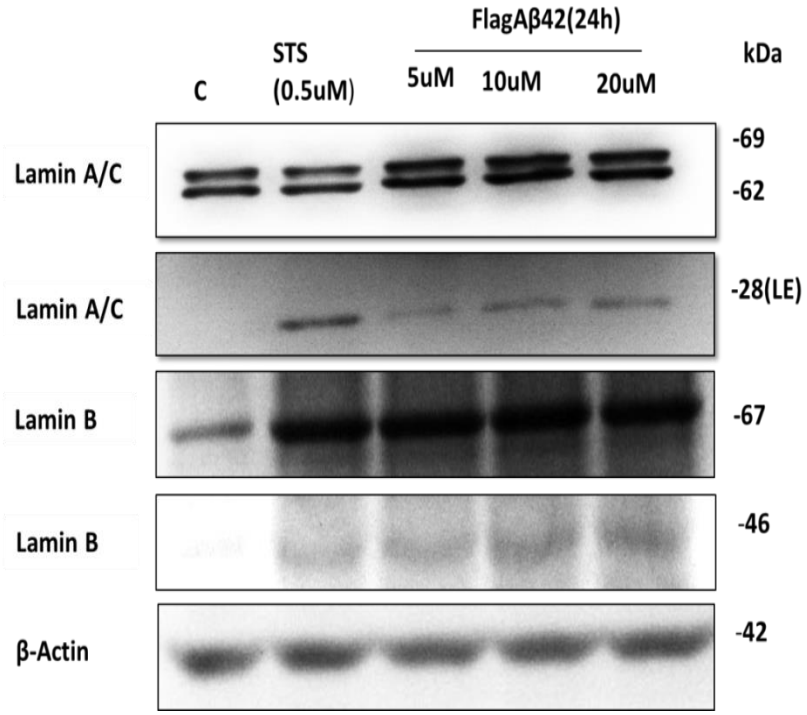

Fig 7: Concentration dependent cleavage of Lamin A/C and Lamin B by fAβ42. HeLa cells (4×10^{5}) were treated with 20μM fAβ42 for the indicated concentrations, total cell lysate were prepared and analysed for the processing of Lamin A/C and B by Western blotting. Actin was employed as a loading control. The relative molecular weights (in kDa) were indicated at the right.

III-1-5. Lamin A/C and Lamin B Fragmentation by fAβ42 in different time period in HeLa cell

Cells were treated with 20μM fAβ42 for different time period (12h, 2+10h, 24h, 2+22h, 36h, 2+34h) (Fig 9). In every stage we found cleaved product of LA/ LC and LB by fAβ42.

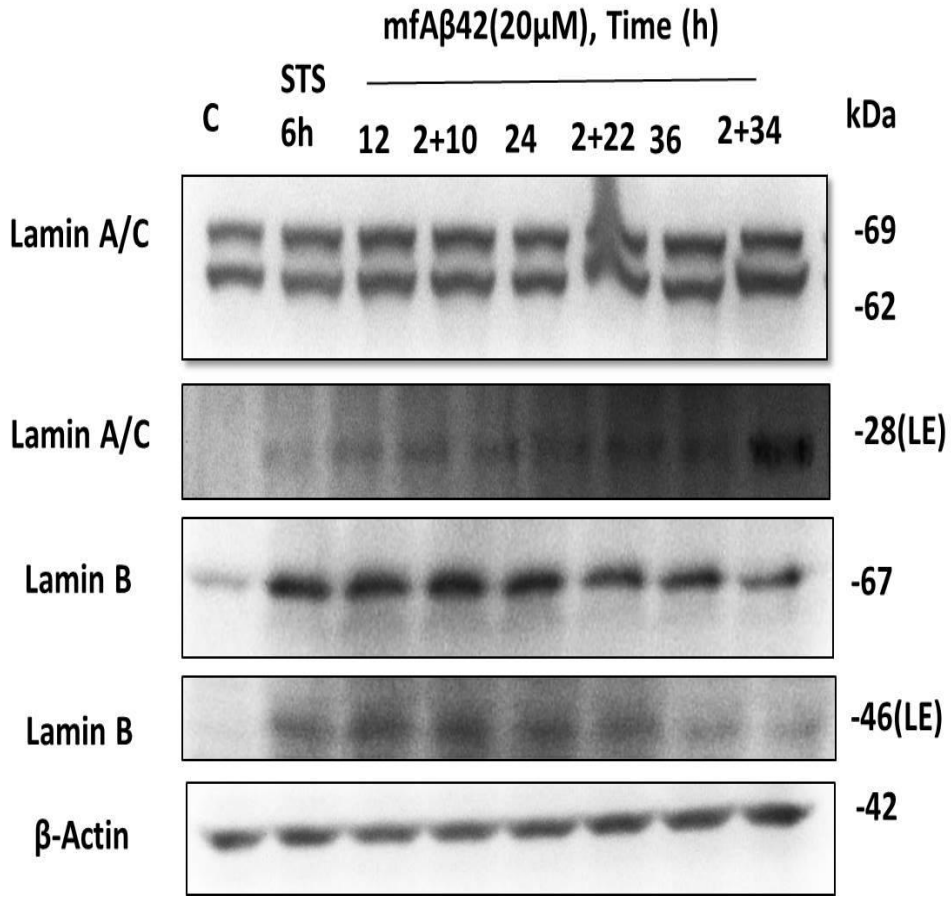

Fig 8: Time dependent cleavage of Lamin A/C and Lamin B by fAβ42. HeLa cells (4×10^{5}) were treated with 20μM fAβ42 for the indicated time periods, total cell lysate were prepared and analyzed for the processing of Lamin A/C and B by Western blotting. As a loading control, Actin was employed. The relative molecular weights (in kDa) were indicated at the right.

III-1-6. fAβ42 induces nuclear deformation which is suppressed by caspase inhibitor

Variation in stages of LA/LC and LB proteins likely causes nuclear abnormalities. Thus, we subsequently analyzed nuclear integrity of the 2+22 h sample with 20 μM Aβ42, 6h with 0.5μM STS and 24h with 20μM fAβ42 using Hoechst staining and immunofluorescence microscopy for LA/LC and LB (Fig 9, 10)

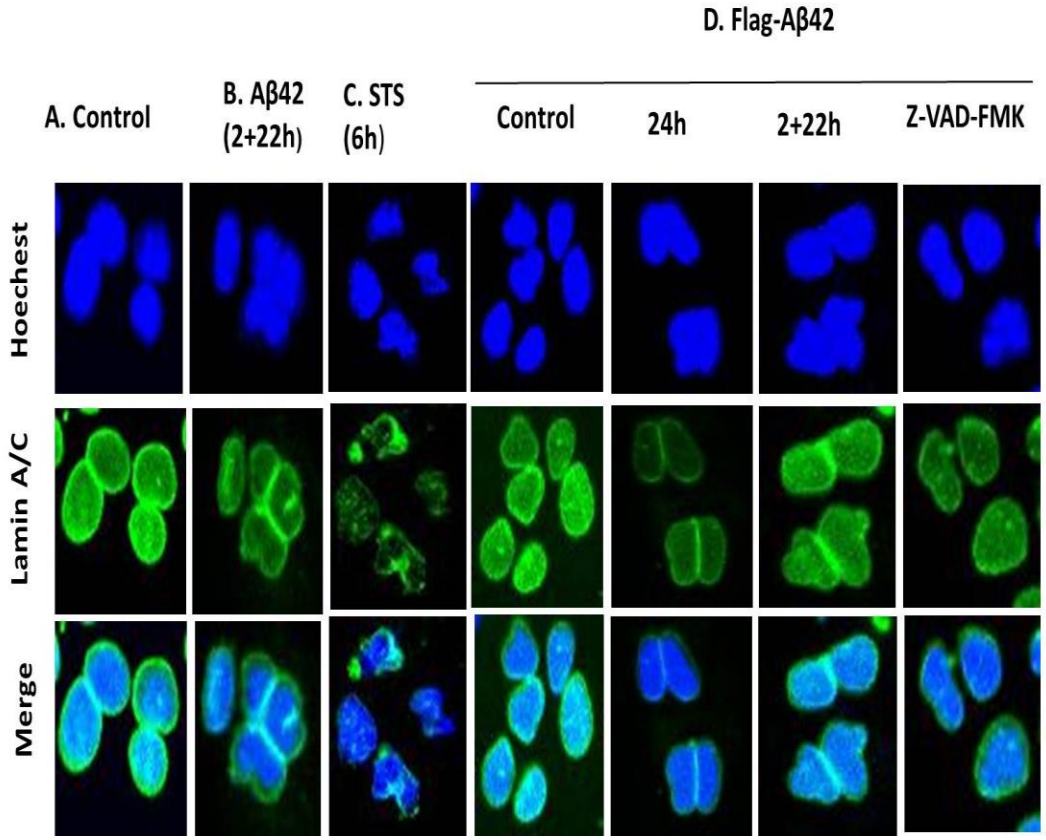

Fig 9: Flag-Aβ42 Induces nuclear deformation and LA/LC checking. (A) Confocal image analysis of HeLa cells treated without peptide, **(B)**

oligomeric Aβ42, **(C)** STS (0.5μM) **(D)** monomeric and oligomeric Flag-Aβ42 in the presence or absence of caspase inhibitor (z-VAD-FMK). Primary goat anti-lamin A/C in addition to secondary anti-goat IgG-FITC was used to detect Lamin A/C (green). Nuclei were seen with the Hoechst staining. Images were visualized using a confocal microscope (LSM 510, Carl Zeiss).

The sample had a significant frequency of nuclear deformation, as demonstrated in the images of representative cells (Hoechst stained samples in Fig 9 and 10). Changes in the lamin epitope identified by lamin A/C and lamin B antibodies in response to nuclear deformation were also detected (Fig 9 and 10). The distortion is comparable to that seen in primary fibroblasts with a lamin A/C mutation [58] or in Simian virus 40-infected cells [127]. The nuclear envelope dispersion, which has been seen before in Aβ-treated HT22 cells [128], was not seen in this sample of cells. Nuclear deformation and variations in the levels of the lamin A/C and B epitopes were greatly decreased in samples prepared with z-VAD-FMK at 20μM, which was very successful in suppressing the changes in the nucleus. These findings suggest that the nuclear deformations caused by fAβ42 are the consequence of a caspase-dependent mechanism (Fig 9 and 10).

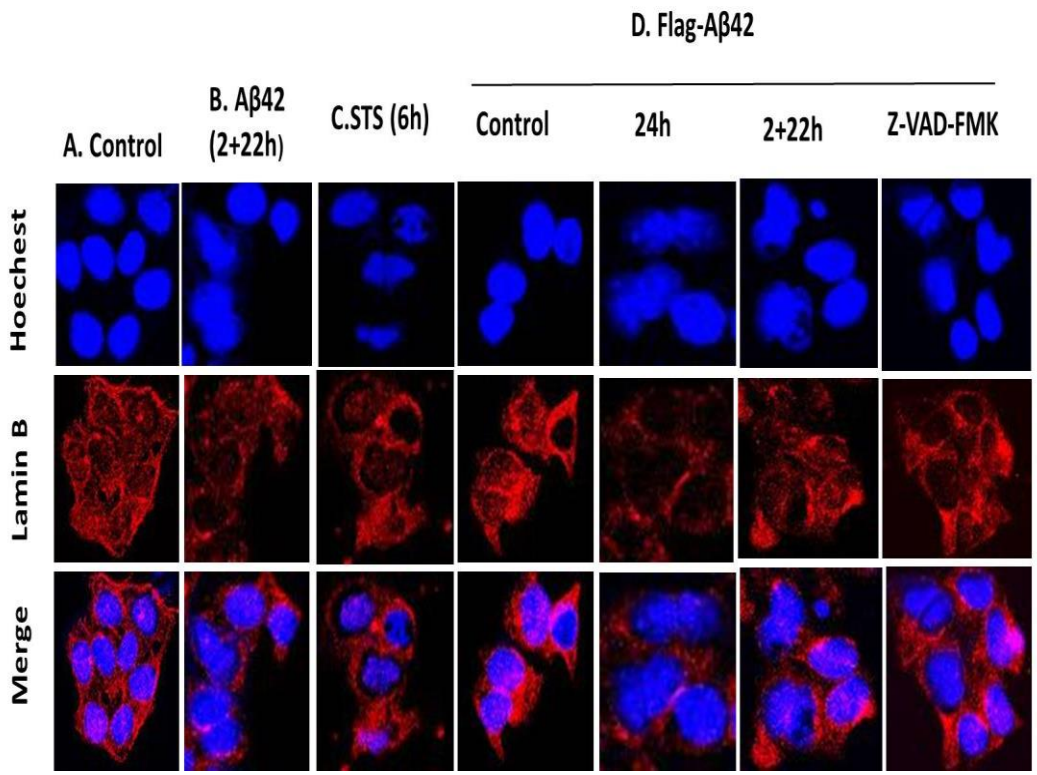

Fig 10: Flag-Aβ42 Induces nuclear deformation and LB checking. (A) Confocal image analysis of HeLa cells treated without peptide, **(B)** oligomeric Aβ42, **(C)** STS (0.5μM) **(D)** monomeric and oligomeric Flag-Aβ42 in the presence or absence of caspase inhibitor (z-VAD-FMK). Primary goat anti-lamin B in addition to secondary anti-goat IgG-FITC was used to detect Lamin B (red). Nuclei were seen with the Hoechst staining. Images were visualized using a confocal microscope (LSM 510, Carl Zeiss).

III-1-7. Differential processing of Caspases and their substrates in Aβ42, STS- and fAβ42-treated cells

Caspases are active in cells that have been treated with STS [129]. In the next set of experiments, immunoblot assays were used to compare the processing of lamin proteins in cells that had been treated with Aβ42, STS, or fAβ42. Lamin A and C have 664 and 572 amino acids, respectively, whereas lamin B1 has 586 amino acids. Caspase-6 cleaves the lamin at the C-terminus of Asp230 in lamin A/C and D231 in lamin B1. As an alternative, lamin A/C and lamin B1 are cleaved by NS protease at Tyr376 [130]. A product's size provides a good indicator of which enzyme was used to break it down. Caspase-9, -8. -3, -6, DFF40/45 (substrate of caspase-3) and lamin A/C were cleaved in STS-treated cells, as well as lamin B (Fig11). Previous investigations have shown that caspase dependent intrinsic apoptosis is activated in this study. However, procaspase-3, a substrate of active caspase-9, and DFF40/45. Which are substrates of active caspase-3, were only marginally altered in the experiments (Fig 11). Aa result, it is debatable whether the activity was caused by caspase-9. In the 2+22 h samples treated with high doses (20μM) of Aβ42, the levels of nuclear lamin A and B were significantly decreased (Fig 6). fAβ42 samples collected during the STS and those collected after 24 hours were found to differ significantly. STS treatment lowered the levels of both lamin A and lamin C in cells (Fig 6).

Furthermore, STS-and fAβ42-treated cells revealed the presence of a novel protein (46kDa), which seems to be a cleaved product of lamin B (ref) (Fig 6). Stem cell-treated cells were found to have protein bands of 28 kDa (Fig 6). Antibodies against the N-terminus of lamin A/C and the C-terminus of lamin B1 identified peptides of 28 kDa and 46 kDa in the STS-and fAβ42-treated cells in immunoblot analysis (Fig 6). These peptides correlate to caspase-6 products (ref), demonstrating that caspase-6 is involved in lamin cleavage triggered by STS and fAβ42. One the other hand, the decrease of lamin A in Aβ42-treated cells (Fig 6) appears to be attributable to the catalytic of NS protease, since the peptide formed by cleavage of lamin A is identified in the immunoblot analysis using an antibody specific for the N-terminus of lamin A/C (Fig 6). However, the Aβ42-treated cells slightly decreased the amount of lamin C, which is a lamin A homolog (Fig 6).

Lamin A was shown to preferentially cleave into lamin C, we couldn't locate anything similar in other articles that would explain this. This observation is still open to further investigation. Even after long exposure of the immunoblot to the detection equipment, we could not identify a 21-kDa-peptide [58] generated by NS protease cleavage of lamin B1 in Aβ42-treated cells. In order to explain the decline in lamin B1, we hypothesize that the cleavage by NS protease was responsible. In this investigation, lamin A/C and B fragmentation were shown to be identical in the STS- and fAβ42-induced cell death pathway, which were completely distinct from the Aβ42-induced cell death route. fAβ42 treated cells showed strong caspase-3,-6, and-9 activity with three distinct substrates in the 24 h samples as compared to untreated cells (Fig 11).

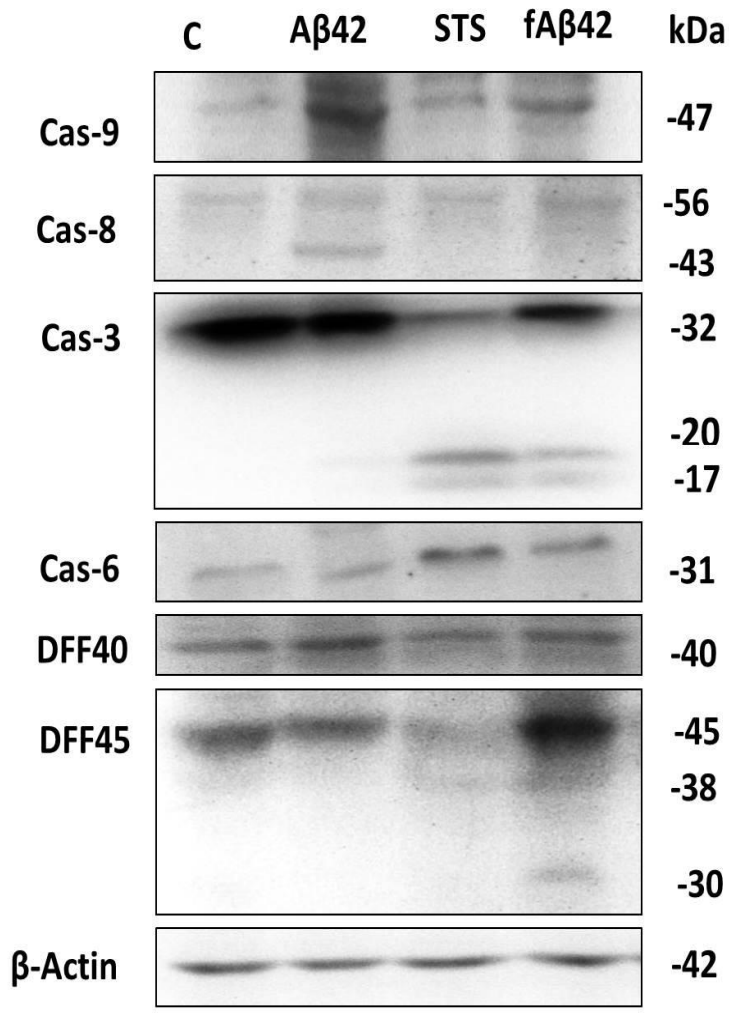

Fig 11: Differential Processing of Caspases and their substrates in HeLa cells treated with Aβ42, STS and fAβ42. (A) Western blot analysis of caspase and its substrates. HeLa cells were incubated with Aβ42 and fAβ42 at the indicated concentrations and time points or with 0.5μM STS for 6h. Total cell extracts were prepared and assessed for processing of Caspase-3,-6, -8, -9 and its substrates DFF40, DFF45, Lamin A/C and Lamin B by western blotting. Actin was employed as a loading control. The relative molecular weights (in kDa) were indicated at the right.

As a result, the immunoblot test revealed that the sample included caspases in the process of being processed (Fig 11). There was no evidence of Caspase-9 processing. The presence of cleaved caspase-8 in Aβ42-treated cells was also observed (Fig 11). After being exposed to larger concentrations (20μM) of Aβ42, procaspase-3 and its substrate, DFF40/45, were partly digested in the same sample as before (Fig 9F). Although the lamin proteins in the Aβ42 samples were reduced in the same way as in the STS and fAβ42 treated cells, the pattern of decrease in the 2+22 h samples of Aβ42 was different.

Following that, we looked for settings in which caspases are potently activated, and then investigated the apoptotic pathways associated with caspase activation in fAβ42-treated cells. Based on a study that found nucleation-dependent polymerization is needed for Aβ-mediated cell death, we used a “double treatment” method in which the fAβ42 peptide was given to cells two times in a row. This method was called “double treatment” [60,131]. This led us to believe that the polymerization process may provide a differential or greater signal that would activate caspase. The cells were then incubated for 10 (2+10 h), 22 (2+22 h), and 34 (2+34) hours with a fresh preparation of oligomeric fAβ42 peptide at the same concentration. Incubation samples with fAβ42 for 2+22 h and longer revealed DEVDase activity similar to that of caspase-3 in fAβ42-treated cells (Fig 13). A ~20 kDa processed caspase-3 fragment was discovered in the samples treated with high doses of fAβ42 for 24 hours, while a completely processed ~17 kDa caspase-3 fragment was also detected in the samples treated with low concentrations of fAβ42 for 24 hours (Fig 11). Although the intensity of the

processed fragment and the extra~17 kDa was present in the 24 h samples (Fig 11), the DEVDase activities of those samples were identical to those of the 24 h samples (Fig 12), demonstration that there is no apparent association between the activity and the processed fragments.

DFF45, a caspase-3 substrate, was found to be reduced (Fig. 11), indicating that caspase-3 was catalytically activated. DFF45 product fragment was also observed (Fig. 11). The activity of caspase-9-like LEHDase was highest in the 24 h sample, which also demonstrated fAβ42-dose dependence, and it was lower in the longer incubation samples, indicating that the enzyme was deactivated (Fig 12). Also shown in Fig. 11 is the processing of procaspase-3 which is a substrate of caspse-9, which results in the catalytic activation of caspase-9. Consequently, it appears that the catalytic activation of caspase was indeterminate in samples studied, despite the fact that a portion of it was processed into two distinct types of fragments. In the double treatment samples cell death was measured. This was used as a baseline to see how the Aβ42 peptide formulation affects the cells. A reduction in DFF45, a substrate of caspase-3 was observed (Fig 11), suggesting the catalytic activation of caspase-3. The product fragment of DFF45 was also seen (Fig 11). The activity of caspase-9-like LEHDase was highest in the 24 h sample, which also demonstrated fAβ42-dose dependence, and it was lower in the longer incubation samples, indication that the enzyme was deactivated (Fig 12). Also shown in Fig. 12 is the processing of procaspase-3, which is a substrate of casoase-9, which results in the catalytic activation of caspase-9. Consequently, it appears that the catalytic activation of caspase was indeterminate in the samples studied, even though a portion of them was processed into two distinct types of fragments. In the double treatment samples, cell death was measured.

III-1-8. Activation of caspase-3, -6, -8, and -9 by fAβ42 treated cells

Despite the fact that several studies, including ours, have shown that apoptosis and related caspase activation play a role in Aβ-induced cell death [89,123,132-134], additional study is required. Caspase-3, -6,-8, and -9, which are involved in the intrinsic and extrinsic pathways, the two principal caspase-dependent apoptotic processes, were shown to be active in the research.

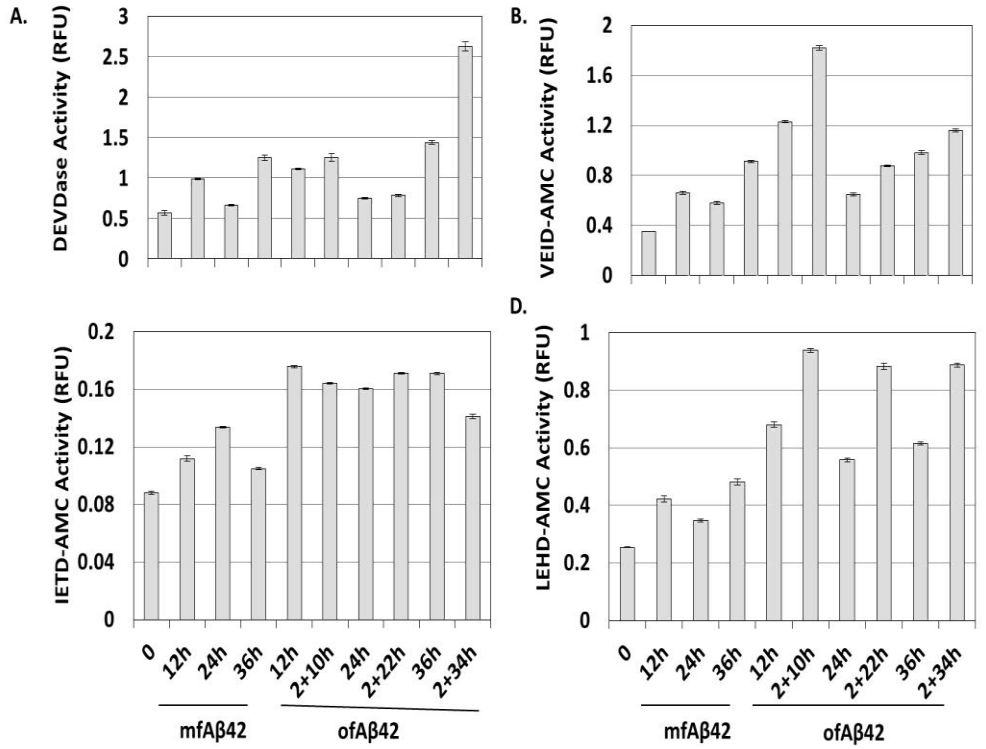

Fig 12: Effects of flag-Aβ42 (20μM) on activation of caspases and cell death in HeLa cells. (A-D) Cells (1.5×10^{4}) were treated with monomeric and oligomeric form of flag-Aβ42 at the indicated concentrations and time.

Then, the activities of Caspases-3, -6, -8, -9 of cell extracts were measured with their respective synthetic substrates (10μM DEVD-AMC for caspase-3, 50μM VEID-AMC for caspase-6, 50μM IETD-AMC for caspase-8, 50μM LEHD-AMC for caspase-9.

For each caspase, activity was evaluated using four synthetic substrates (DEVE-AMC, VEID-AMC, IETD-AMC, and LEHD-AMC). The cells were exposed to up to 20μM of fAβ42 for up to 36 hours (Fig 13). The monomeric fAβ42 preparation was employed in the tests rather than oligomeric preparations unless otherwise stated in the literature. Oligomeric Aβ42 is better than monomer or fibrillar Aβ42 at causing caspase activity and cell death [6,16] than monomer or fibrillar Aβ42. This is based on previous research and our lab.

The presence of caspase activities with the synthetic substrates was found in fAβ42-treated cells, particularly in samples collected after 36 hours of incubation (Fig 13A-13D). However, we are not confident that this was caused by Aβ42-induced damage because no concentration-dependent activity of fAβ42 was seen, and the activity may be attributable to background activities that were boosted by cell division during the treatment period, as previously reported. It is also likely that serum deprivation of the cells prior to Aβ42 treatment will result in an increase in background activity in the treated cells. As a result, determining whether the activity is produced by fAβ42 or by other causes is hard.

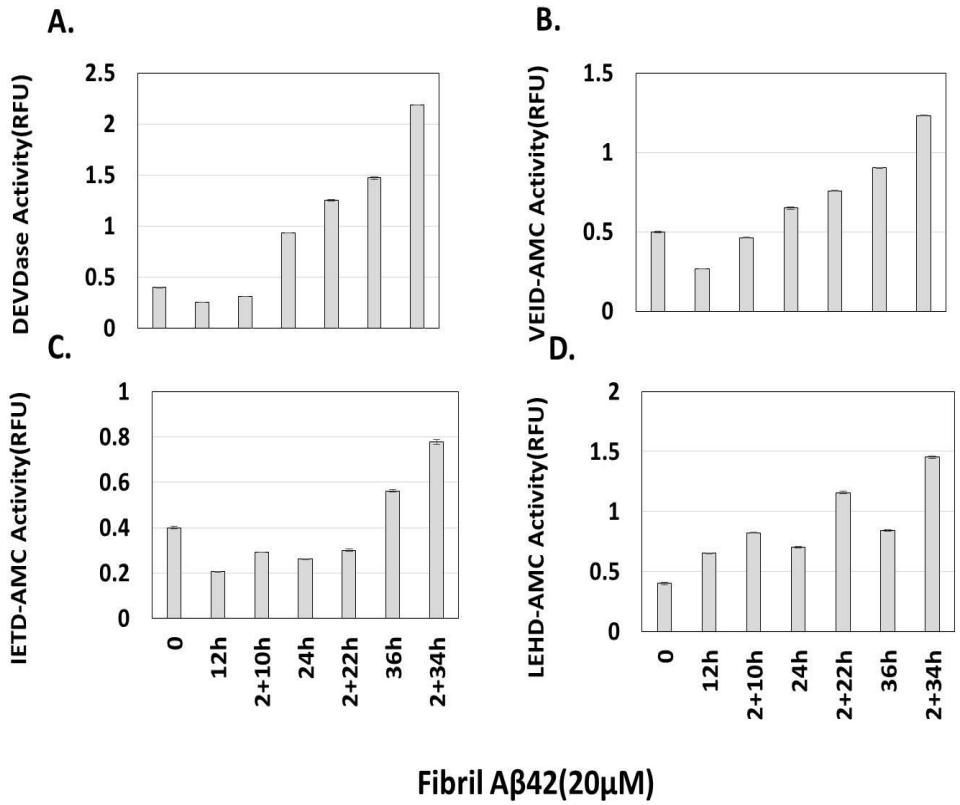

Fig 13: Effects of Aβ42 fibrils (20μM) on activation of caspases and cell death in HeLa cells. (A-D) Cells (1.5×10^{4}) were treated with Aβ42fibrils at the indicated concentrations and time. Then, the activities of Caspases-3, -6, -8, -9 of cell extracts were measured with their respective synthetic substrates (10μM DEVD-AMC for caspase-3, 50μM VEID-AMC for caspase-6, 50μM IETD-AMC for caspase-8, 50μM LEHD-AMC for caspase-9.

Furthermore, the activity levels were lower by a factor of many times compared to those caused by other harmful agents such as strontium [89]. As result, it is difficult to determine whether the activity is caused by fAβ42 or by other different factors. The immunoblot test was used to investigate the

activation of caspases in the cells that had been exposed to fAβ42. Each caspase was predicted to produce cleaved fragments (see Fig 12) if it was activated, and this was confirmed. All in all, caspase-3 was activated in the cells that were treated with fAβ42 in the way that was described in this report. fAβ42 cytotoxicity was investigated to ensure that the degree of caspase activation was not caused by a flaw in the peptide production. MTT was used primarily to measure cell death [134]. When cells are treated for longer period of time; soluble fAβ42 can cause a drop in MTT formazan synthesis even in the absence of overt cell death due to the presence of soluble fAβ42.

lII-1-9. Caspase inhibitor reduces lamin A and lamin B cleavage induced by Aβ42

The decrease of lamin A/C produced by STS and fAβ42, as well as the generation of a 46 kDa product from lamin B, were significantly reduced by 20 M z -VAD-FMK (Figure 15).

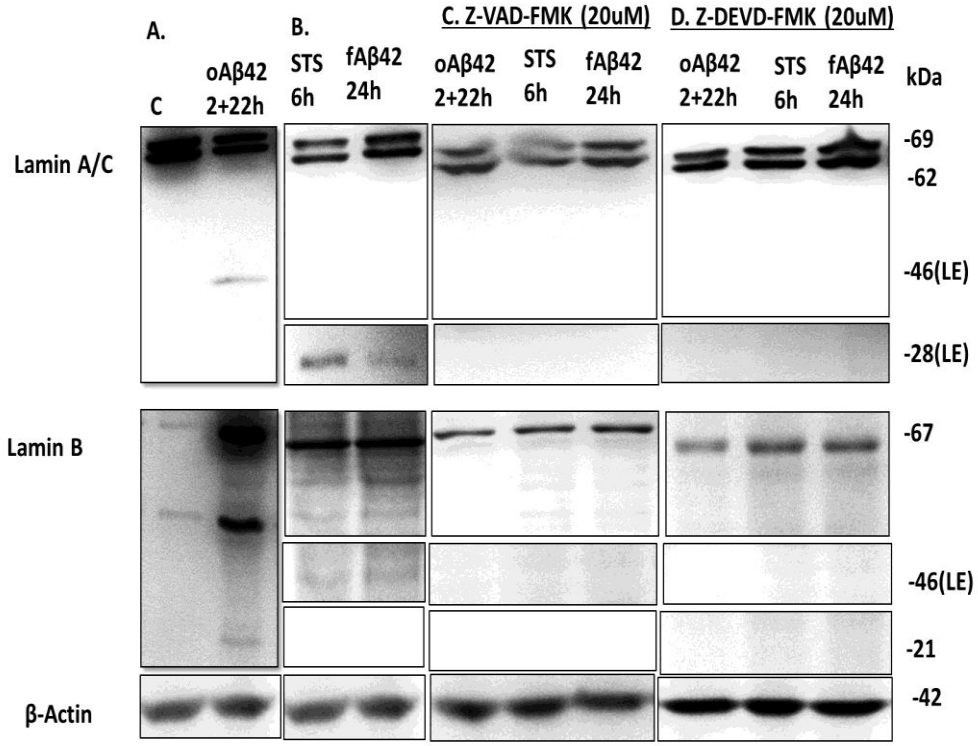

Fig 14: Caspase inhibitor reduces Aβ42, STS and fAβ42 induced Lamin A/C and Lamin B. (A-D) HeLa cells were exposed to indicated concentrations of Z-VAD-FMK and Z-DEVD-FMK for 1h prior to Aβ42 treatment and incubated with 20μM Aβ42 and fAβ42 or 0.5μM STS in the presence or absence of Z-VAD-FMK or Z-DEVD-FMK for 2+22h, 24h and 6h respectively. Cell extracts were prepared and analyzed for Lamin A/C and Lamin B processing. Actin was employed as a loading control. The relative molecular weights (in kDa) were indicated at the right.

III-1-10. Biophysical activity checking of fAβ42 in HeLa cell

Fibrillogenesis kinetics was analyzed by ThT assay (Fig 16 A) and β -sheet formation was assessed by CD spectroscopy (Fig 16B) of Aβ42 and fAβ42.

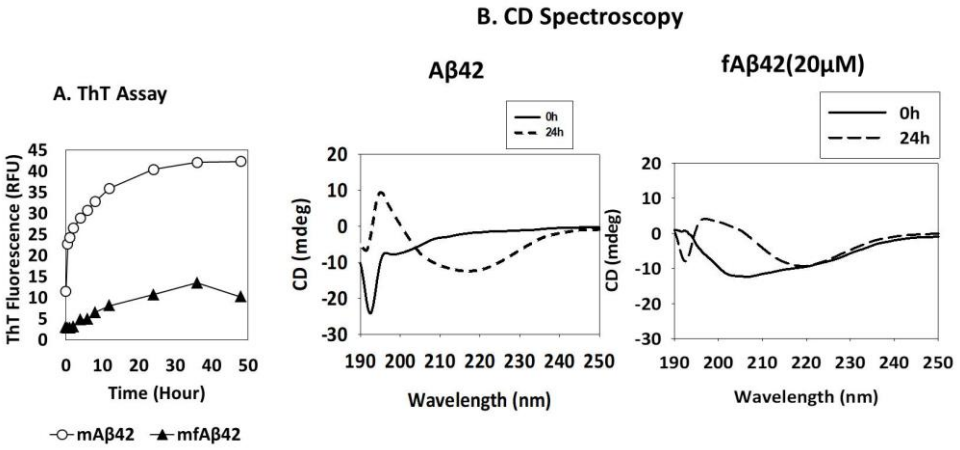

Fig 15: Physical characterization of monomeric Aβ42 and fAβ42. (A) Time dependent ThT-fluorescence assay of mAβ42 and mfAβ42. RFU represents relative fluorescence unit. (B) CD spectra recorded of freshly prepared (solid line), 24 h (broken line) incubated samples of mAβ42 and mfAβ42.

III-1-11. Cell death pathway induced by fAβ42

Here, we have shown that the cell death pathway in HeLa cells is induced by fAβ42. From this diagram, we can say that cell death happened after caspase activation, and after that, lamin A/C and lamin B cleavage occurred (Fig 16).

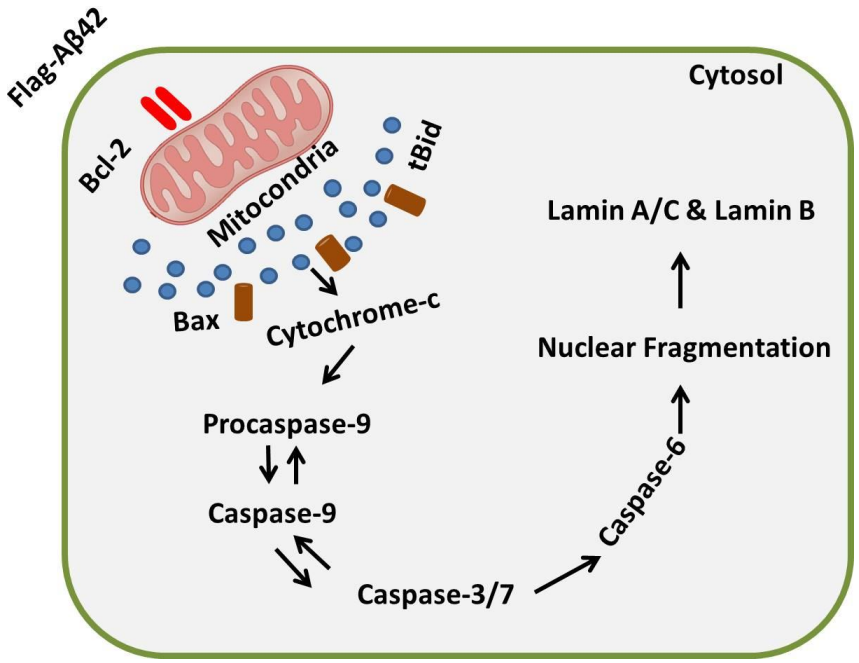

Fig 16: Graphical representation of cell death pathway induced by fAβ42

III-1-11. DISCUSSION

Nuclear lamina, the fundamental structural components of the nuclear scaffold, is formed by the polymerization of intermediated filament proteins, nuclear lamin A and B. Many nuclear processes, such as chromation architecture, DNA replication, transcription, and DNA repair, are also dependent on lamins. Nuclear lamin protein modifications are a typical mechanism in dying cells. As a result of the change, nuclear deformation, dispersion, and fragmentation of the nuclear envelop have been seen in cells [127, 128]. One of the changes that occurred in Aβ-treated cells was CDK5-mediated phosphorylation of lamin A and B, which led to nuclear dispersion [128]. In the current investigation, however, the decrease of lamin A and B (Fig 7) and distortion of the nuclear lamina (Fig 10, 11) were seen in the fAβ42-treated cells rather than nuclear dispersion.

Even though peptides have the same size, various conformations (such as oligomers vs. fibrils) have diverse biological effects (Fig 7). Additionally, cells were treated twice with Aβ42 in the current investigation to achieve the condition depicted in figure 7. Figure 7 shows the differing effects of STS therapy on cells treated with the same fAβ42 preparation alone. Another significant alteration that contributes to nuclear deformation and fragmentation is the proteolytic degradation of lamins. At least two different enzymes can cleave lamins. During apoptosis, caspase-6, and executioner caspase, is triggered by upstream caspases such as caspase-3 [131].

Although caspase-6 cleavage of the lamin is a late process that occurs after activation of the apoptotic cell death pathway, NS protease cleavage can occur before other cell death processes, as demonstrated in glutamate-induced cerebellar granule cell apoptosis, where lamin cleavage and dissolution of the microtubule network occurred before chromation fragmentation [135-137]. It's also possible to start apoptosis before the cell dies. There's been a link between CDK5 phosphorylation of lamin A and B and early cell death in cells that have been treated with Aβ, which causes nuclear dispersion.

In the current research, we further show that after caspase activation, fAβ42 causes nuclear deformation and a decrease in lamin proteins. z-VAD-FMK and z-DEVD-FMK dramatically decreased the cell death generated by fAβ42, indication the relevance of caspase-dependent apoptosis in cell death. Caspases, which were activated in the A-treated 24 h sample, cleaved the lamins, which was surprising (Fig 12). Although caspase-6-like VEIDase activity was found in the cells (Fig 12), casapase-6 activation was not (Fig 13B). It needs to be seen if a caspase-6 activity is down or if other unknown factors are to blame for caspase-dependent lamin cleavage.

Finally, we show that after caspase activation, cells treated with fAβ42 experience a decrease in lamin A and B, as well as a morphological alteration in the nucleus. Our findings suggest that the loss of lamin proteins might be attributable to caspase activation. The inhibitors z-VAD-FMK and z-DEVD-FMK substantially prevented the cell death mediated by fAβ42 by inhibiting caspase activation. In this study, it was found that blocking the

activation of caspases may be a good way to manage the process of Alzheimer's disease degeneration.

Confocal imaging analysis of fAβ42-treated cells to investigate the fAβ42-caspase relationship. Using confocal microscope image analysis, we investigated the interaction of fAβ42 with the chosen caspases. Procaspase-9 has previously been shown to interact with the peptides. Procaspase-9 has previously been shown to interact with the peptides. The current research examined Caspase-9. We chose it because it is known to play a significant part in the intrinsic apoptosis pathway, and one of our study objectives was to assess the potential interaction with the apoptosis process. Previously, it was discovered that caspase-3 does not interact with Aβ42 [134].

The images of cells treated with fAβ42 and caspase-9 were evaluated. By displaying the yellow-colored spots in the merged images, the interaction was determined. In caspase-9 samples, there were prominent yellow dots (Fig. 1, 3). In the current study, we focused on exploration of caspase-9 and its potential interaction with fAβ42. Fibrillogenesis of fAβ42 was assessed by ThT binding assay and it did not follow the saturation kinetics even after 48-h incubation in ThT binding assay like wild type Aβ42, although it appeared to form β sheet structure (Fig 16A). Formation of secondary structure was analyzed by CD spectroscopy and it was found that fAβ42 did not form β-sheet structure (Fig 16B) like Aβ42.

III-2. Cellular Internalization of mAβ42, oAβ42, tAβ42 and Effect of Aβ42 toxicity inhibitor on the entry of Aβ42 into SH-SY5Y cells

III-2-1: Monitoring entry of Aβ42 into the cells

The entrance of each Aβ42 into the SH-SY5Y cells was next investigated by observing the confocal image of each sample. ~95% percent of cells in 12 h treatment samples with mAβ42 and oAβ42 at 20 μM did not contain the peptide (Fig 17B and 17E). Longer incubation of the cells with the peptide for 24 hours resulted in ~30% and ~54% more cells transmitting the peptide for mAβ42 and oAβ42, respectively (Fig 17C and 17F). In comparison to mAβ42, samples treated twice with oAβ42 for 2+10 h showed higher lelvels of peptide entry (~38%). On the other hand, the peptide was found in ~97% of cells treated with tAβ42 for 12 hours (Fig 17H). In summary, the confocal microscope monitoring of A42 peptide entrance was similar with the levels of cytotoxicity revealed in Fig 19.

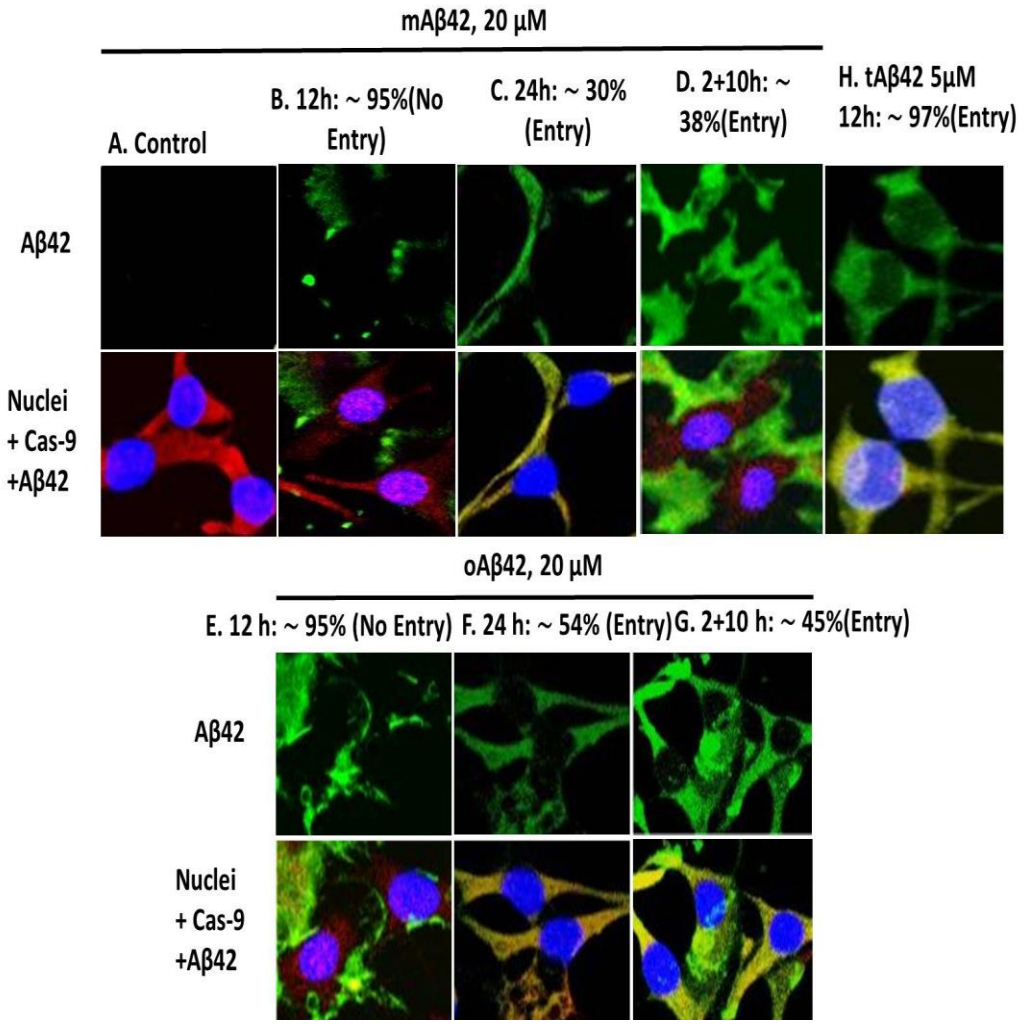

Fig 17: Extent of Cellular Internalization of Aβ42 in SH-SY5Y cell. (A-H) SH-SY5Y cells were treated with mAβ42 **(A-D)**, oAβ42 **(E-G)** and tAβ42 **(H)** for specified time at indicated concentrations. Mouse anti-Aβ (6E10) and rabbit anti-caspase-9 (p10) antibodies were used to identify Aβ (green) and caspase-9 (red) which were visualized by using secondary anti-mouse IgG-FITC and anti-rabbit IgG-rhodamine antibodies, respectively in confocal microscope images of Aβ and caspase-9 in SH-SY5Y cells. Nuclei were stained with the nucleic acid flurochrome Hoechst (blue). Red color denotes the cytoplasm and yellow spots indicate the interaction of

procaspase-9 and Aβ. Only representative images of cells are presented. Monomeric Aβ42, oligomeric Aβ42, monomeric tat-Aβ42 are represented as mAβ42, oAβ42, tAβ42 respectively.

III-2-2: Effect of Aβ42-toxicity inhibitor (AF and MBP) on the entry of Aβ42 into SH-SY5Y cells

Using confocal microscope, the effect of amentoflavone (AF) and MBP on the entry of oAβ42 and tAβ42 were monitored. Double treatment of oAβ42 was not used, because in the inhibitor-containing assay it often caused highly viscous samples which disturbed the clarity of the confocal images. Fewer cells were found to have the intracellular oAβ42 and tAβ42 in the presence of amentoflavone (~15% and ~15% of Fig 18A and 18D). MBP reduced the entry of oAβ42 only when the protein was pre-incubated with the Aβ42 (~14% of Fig 14C, compared to 41% in Fig 18B), while it could not for tAβ42 peptide (~86% of Fig 18).

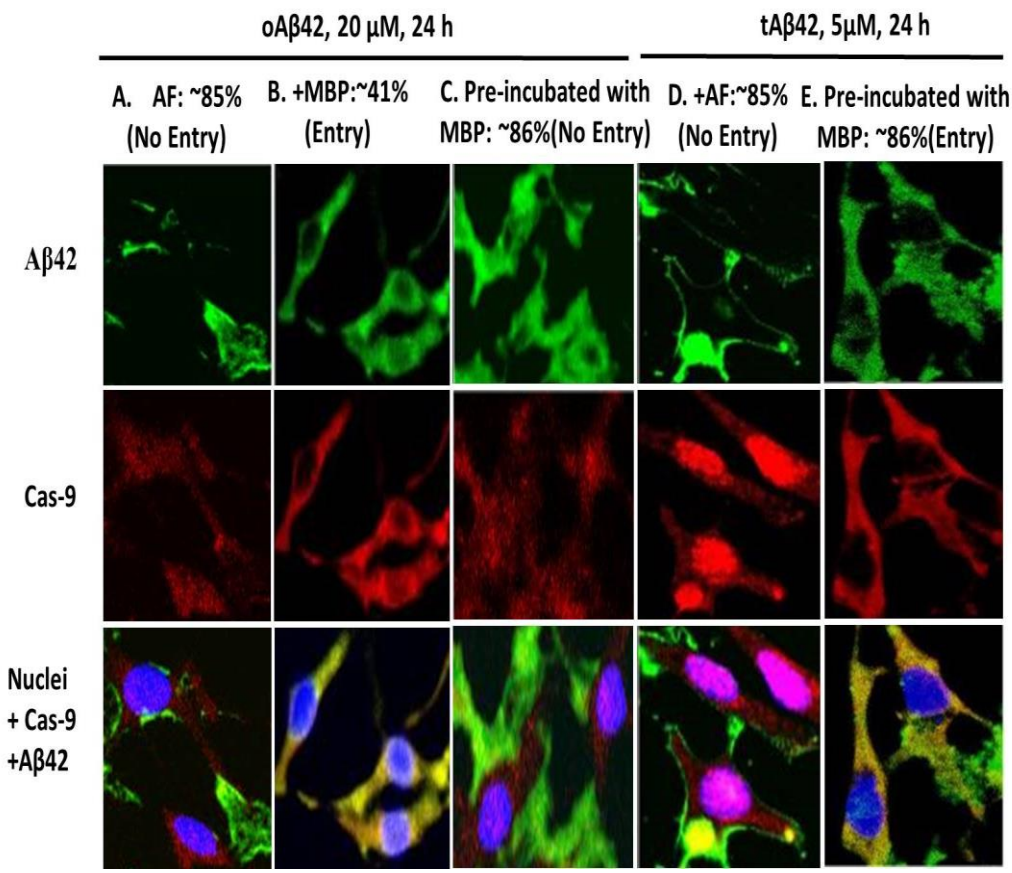

Fig 18: Effect of amentoflavone (AF) and maltose binding protein (MBP) on cellular internalization of Aβ42. (A-E) SH-SY5Y Cells were tre ated with 20 µM oAβ42 or 5 µM tAβ42 for 24h in presence of either 10 µM or 0.4 µM MBP. Freshly prepared mixture of peptide and MBP with or witho ut pre-incubation at 37^0C for 24 h were used. Confocal microscopic images were visualized by the same way discussed earlier in Fig 14. Incubation at least three independent experiments were carried out and only representative images of cells are shown here.

III-2-3: DnaK inhibits the entry of Aβ42 into the cells

DnaK was pre-incubated with Aβ42 and it prevents the entry of peptide into the cells. When the molar ratio of DnaK/Aβ42 was 0.5, the cells ~65% with peptide were found (Fig 19C). Furthermore, ~77% cells with peptide were found in case of DnaK/Aβ42 molar ratio was 1.0 (Fig 19D).

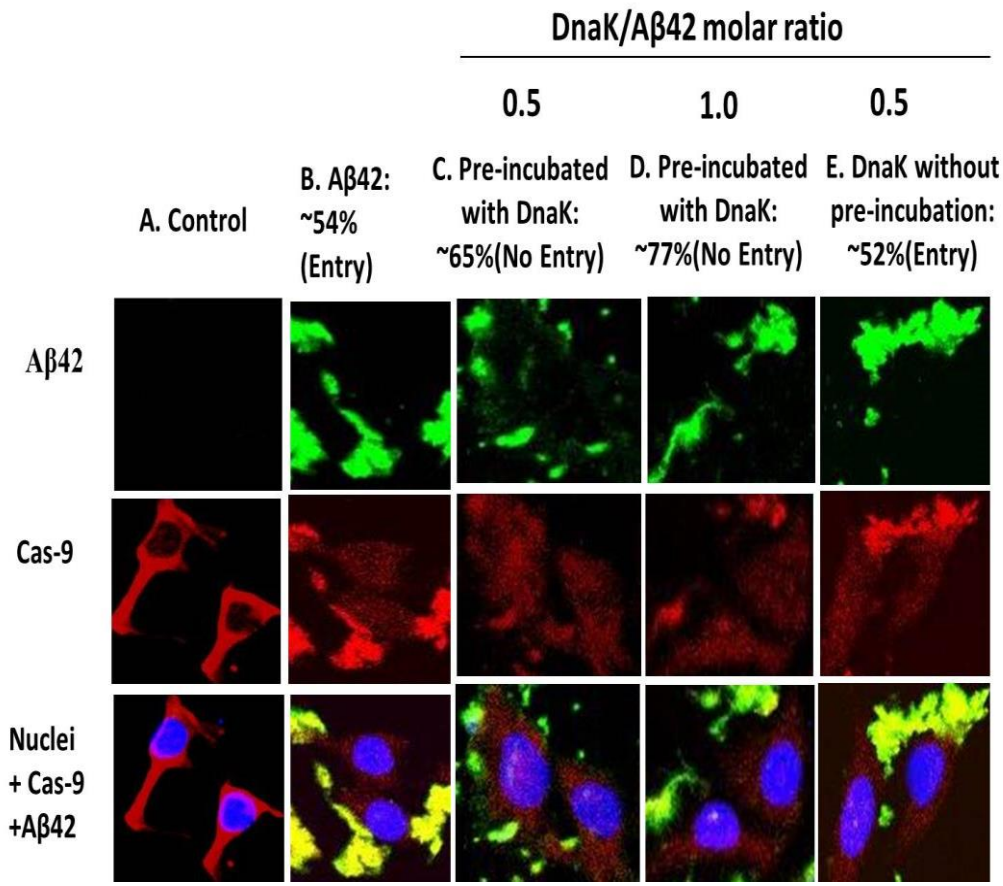

Fig 19: Effect of DnaK on cellular internalization of Aβ42. (A-E) SH-SY5 Y Cells were treated with 20 μM oAβ42 for 24h in presence of either 10μM or 20μM DnaK. Freshly prepared mixture of peptide and DnaK with or witho

ut pre-incubation at 37^0C for 24 h were used. Confocal microscopic images were visualized by the same way discussed earlier in Fig 14. Incubation at least three independent experiments were carried out and only representative images of cells are shown here.

III-2-4: Cell Cytotoxicity of Aβ42 and tAβ42 by MTT reduction Assay and Alamar Blue Assay in SH-SY5Y Cell

Next we explored the cytotoxic effect of each preparation of mAβ42, oAβ42, mtAβ42 and otAβ42. The cell death was monitored using MTT assay, results of which were confirmed by Alamar Blue assay throughout the current study. Freshly prepared Aβ42 (monomeric preparation, mAβ42) at up to 20μM induced less than 10% cell death for 12h incubation (Fig 20A, B). Further incubation of the cells with the peptide for 24h increased the cell death up to 30% (Fig 20 C, D). The oligmeric preparation of Aβ42 (oAβ42) was cytotoxic for 12h incubation (Fig 20A, B) and the toxicity increased when the cells were further incubated for 24h (Fig 20B, E). Both monomeric and oligomeric condition of Aβ42 induced cell death.

After that mAβ42, oAβ42 preparations were less cytotoxic than tAβ42 in the 12, 24 and 36 h incubation samples (Fig 20 A-F); treatment of freshly prepared tAβ42 (mtAβ42) at 1-20 μM to cells for 24 h resulted in~ 40% viability~40% viability in the both assay (Fig 20 B, C). When incubation increased up to 48h the cell viability~ 40% was found similar to 24 h treatment in both case (oAβ42, and tAβ42).

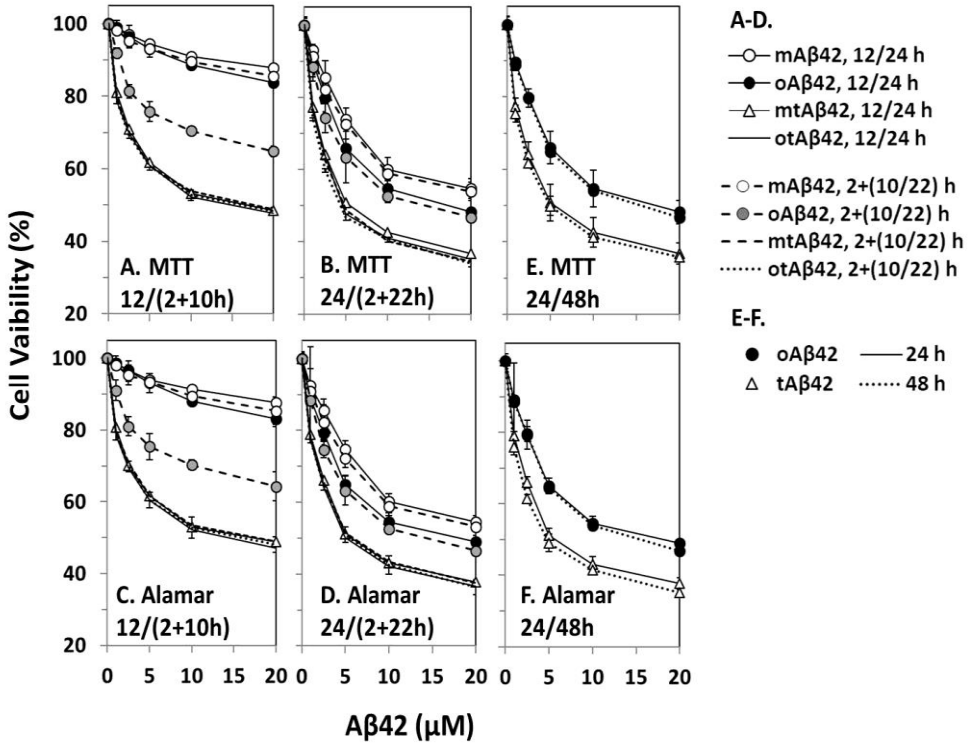

Fig 20: Cell Cytotoxicity of mAβ42, oAβ42, mtAβ42 and otAβ42 by MTT reduction Assay and Alamar Blue Assay. (A-D) SH-SY5Y cells (1.5×10^{4}) were treated at different concentrations for the indicated time with monomeric and oligomeric Aβ42, and tAβ42. **(A-F)** After treatment, cell viabilities were assessed with MTT reduction assay and Alamar Blue assay. Effect of monomeric and oligomeric form of Aβ42 and tAβ42 when cells are treated at different concentrations **(A-B)** for 12h or (2+10h) and **(C-D)** for 24h or (2+22h). **(E-F)** Effect of longer period of treatment for 48h at different concentrations on cytotoxicity induced by oligomeric form of Aβ42 and monomeric form of tAβ42. Control is cells incubated without peptides. The results are expressed as mean±standard deviation of 3 independent experiments. Here, mAβ42, oAβ42, mtAβ42 and otAβ42 represent monomeric Aβ42, oligomeric Aβ42, monomeric tAβ42 and oligomeric tAβ42.

III-2-5: DEVDase activity induced by Aβ42 and tAβ42

After that, we examined the DEVDase activity with monomeric and oligomeric form of Aβ42 and tAβ42 at 20μM, 5μM, 10μM for 24h, 2+22h, 36h and 2+34h treatment. Then, the activities of Caspases-3 of cell extracts were measured with respective synthetic substrates (10μMDEVE-AMC for caspase-3).

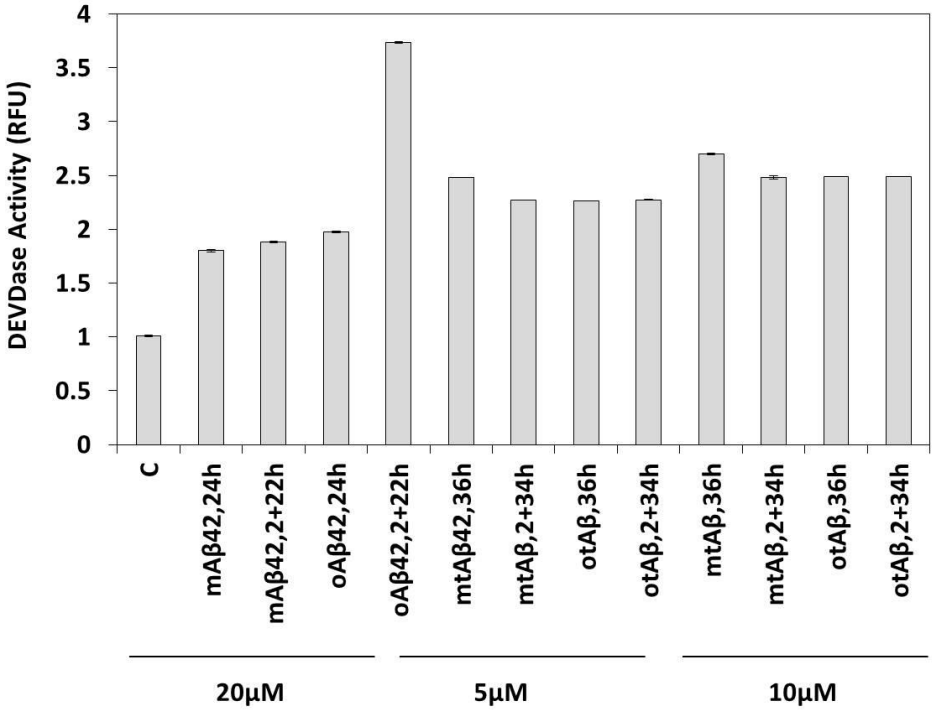

Fig 21: DEVDase Activity of mAβ42, oAβ42, mtAβ42 and otAβ42 in different concentration (5μM, 10μM, 20μM) in SH-SY5Y Cell. Cells (1.5×10^{4}) were treated with monomeric and oligomeric form of Aβ42 and tAβ42 at the indicated concentrations and time. Then, the activities of

Caspases-3 of cell extracts were measured with respective synthetic substrates (10μM DEVD-AMC for caspase-3).

III-2-6: Inhibition of Aβ42 cytotoxicity by AF, MBP and DnaK

AF, MBP and Dnak showed protective effect against Aβ42 mediated cytotoxicity. To examine whether the AF, MBP and Dnak accumulated oligomeric Aβ are toxic or non-toxic, cell survival have been measured in human neuroblastoma SH-SY5Y cells by MTT assay. Aβ (20M) alone caused -40% cell deaths after 24h of treatment (Fig 20A). Surprisingly, in case of dose dependent manner 90% of cell survival was found in presence of AF. Moreover, significant cell death inhibition was observed in dose dependent manner and more than 30% cell survival enhanced at higher molar ratio of MBP (Fig 22B). The results were also consistent with cell viability measured in presence of pre-incubated with Aβ species by Dnak (Fig 20C).

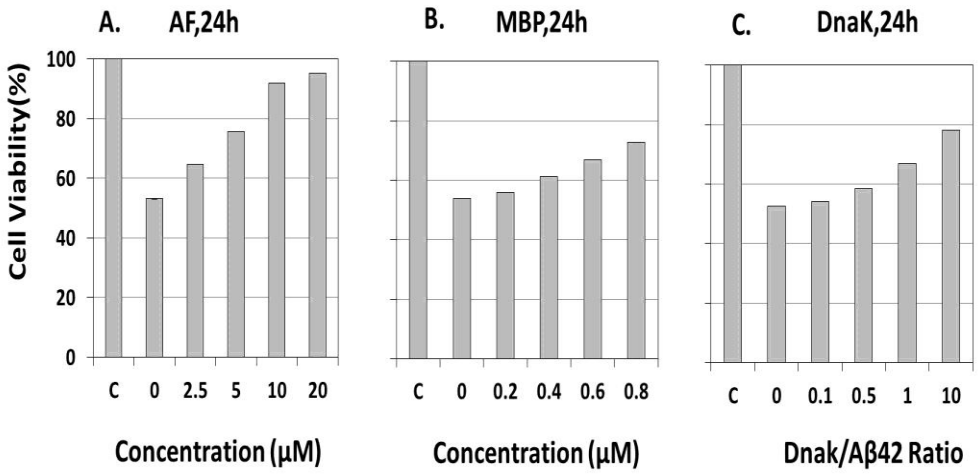

Fig 22: Cell Cytotoxicity of Aβ42 (20μM) with AF, MBP and DnaK by MTT reduction Assay in SH-SY5Y cell. (A-C) SH-SY5Y cells (1.5×10^{4}) were treated at different concentrations of AF, MBP and DnaK with Aβ42. After treatment, cell viabilities were assessed with MTT reduction assay. Control is cells incubated without peptides. The results are expressed as mean±standard deviation of 3 independent experiments.

III-2-7: Ratio of cells with intracellular Aβ (%) with or without inhibitor and cell death (%) in SH-SY5Y cell

We also observed the ratio of cells with intracellular Aβ (%) and cell death (%) from the cell death assay and confocal microscopy. In every cases cell death was not depended on entry of peptide into the cell except tAβ42. The entry of peptide into the cell was very less in case of mAβ42 than oAβ42 and tAβ42 (Fig 23). The highest entry of cell was observed in case of tAβ42 at 12 h cell treatment. However, when cells were treated with oAβ42 and Tat-Aβ42 along with Aβ inhibitor like AF and MBP, the entry of peptide was less than cell death (Fig 23).

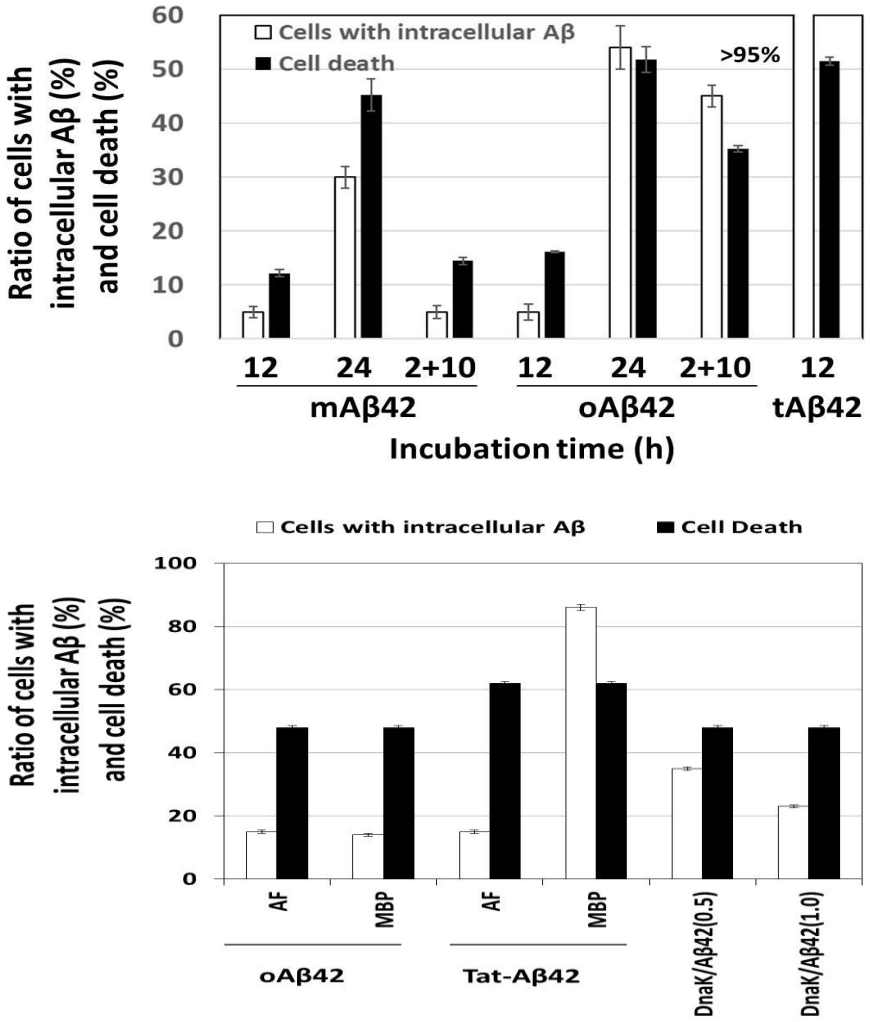

Fig 23: Ratio of cells with intracellular Aβ (%) with or without inhibitor and cell death (%) in SH-SY5Y cell. Summary of the comparison of the number of cells with intracellular Aβ42, tAβ42, AF, MBP and DnaK and cell death is presented. The number of cells with intracellular Aβ was calculated using the results shown in Fig 18, Fig 19 and the data for cell death were obtained from Fig 20 and Fig 22. Data are presented as the mean ± standard deviation of values from three independent experiments.

III-2-8: Comparison of internalization process of extracellular Aβ42 and intracellular Aβ42

From the current research, we can compare the internalization process of extracellular and intracellular Aβ42 in cells, which is shown in Fig 24.

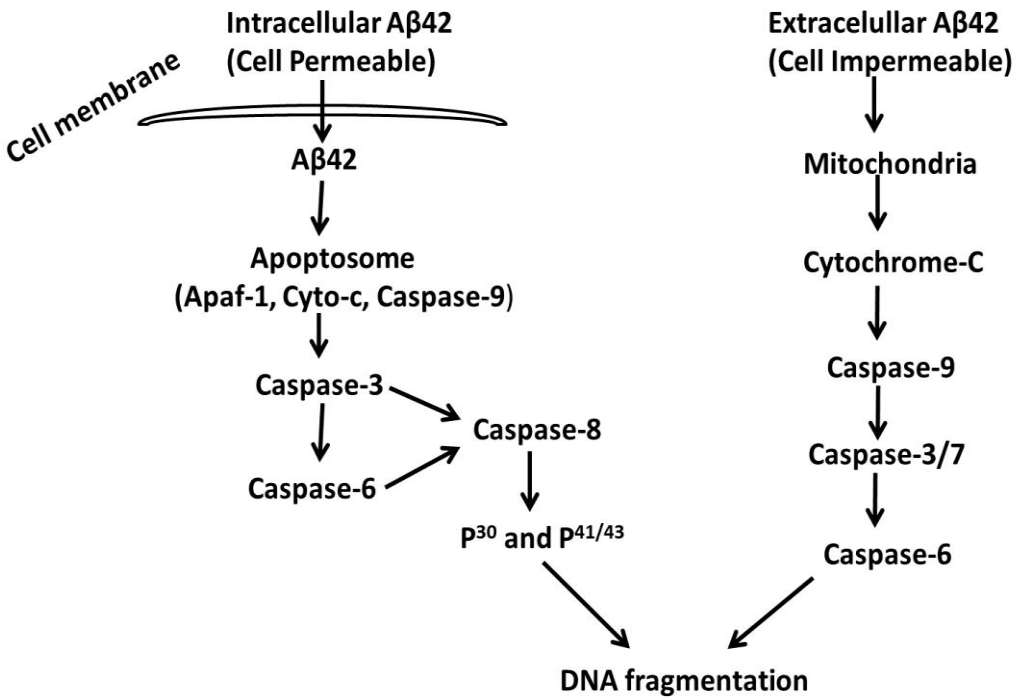

Fig 24: Schematic figures for the internalization of extracellular Aβ42 and intracellular Aβ42

III-2-8: DISCUSSION

We previously explored that Aβ42 inhibited an apoptotic pathway by interacting with procaspase-9 [134]. However, accumulating evidence points to apoptosis as a mechanistic feature of Aβ42-induced cell death [135-138]. Although extensive studies over decades have made significant progress in understanding the apoptotic pathway related to Aβ42, cytotoxic properties of Aβ42 and its mechanistic features have been difficult to assess due to variability in culture systems and methods associated with Aβ42 preparation and treatment [30]. In this study, we searched for a condition that activated caspases and explored the role of apoptosis pathways in that condition. As a result, the experimental condition involving double or more treatments of the peptide to cells may be close to physiological conditions. Hopefully, a complete understanding of the nature of the multiple treatments and related cell death pathways will shed new light on Aβ42-related pathology and AD control.

IV. FUTURE RESEARCH

Mixtures of Wild type Aβ42 and Flag-Aβ42 reduces cell death

To explore the mechanism Aβ42 mediated cell death, we used a combination treatment of Aβ42 and Flag-Aβ42 to treat HeLa cell to see whether there is any synergistic effect or not in terms of cytotoxicity. When concentration of fAβ42 was increased in the combination with the wild type Aβ42, cytotoxicity was little bit reduced (Fig 25). About 37% cell deaths were observed when the cells were treated with mAβ42 (10μM). But when mfAβ42 (10μM, 20μM) were mixed with mAβ42 (10μM) for cellular treatment, 33% and 28% cell death were found (Fig 25) respectively, which is less than the cytotoxicity caused by mAβ42 alone. There might be several regions behind this cell death scenario. The one reason may be fAβ42 interact with mAβ42 and inhibit its entrance into the cell. Thus it inhibits the cytotoxicity as the importance of entering into the cell. The clear mechanism behind this has to be explored that might help to design the treatment plan for fAβ42 associated Alzheimer's disease.

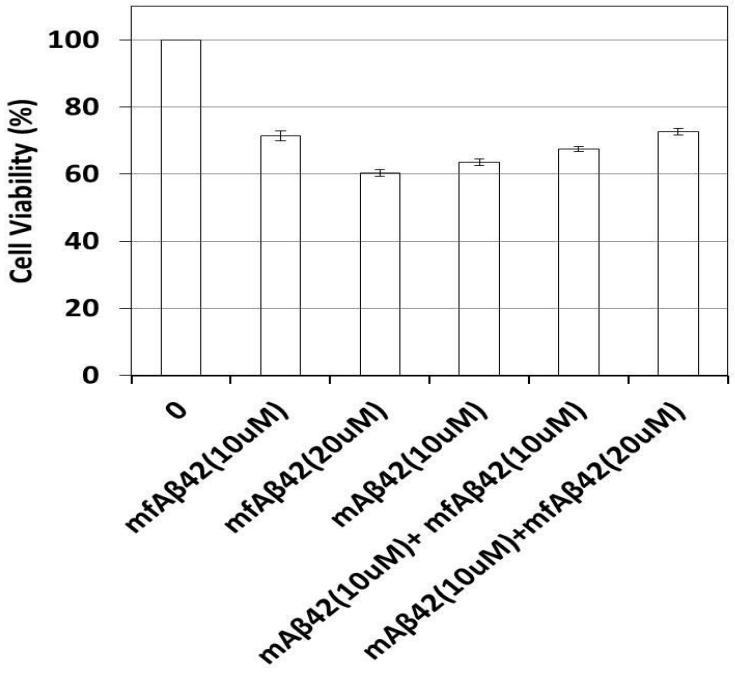

Fig 25: Cytotoxicity of Aβ42 and Flag-Aβ42 combination treatment. Cytotoxicity was measured by MTT assay. The results are expressed as mean ± standard deviation of 3 independent experiments.

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ABREVIATIONS USED

The abbreviations used are:

Aβ42;Amyloid β (1-42)
APP; Amyloid precursor protein
AD; Alzheimer's disease
mAβ42; Monomeric Amyloid beta(1-42)
oAβ42; Oligomeric Amyloid beta (1-42)
Tat-Aβ42; TAT-Amyloid beta (1-42)
AD; Alzheimer's disease
fAβ42; Flag-Amyloid βeta (1-42)
MTT; 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide
PBS; phosphate-buffered saline
SDS-PAGE; Sodium dodecylsulfate-polyacrylamide gel electrophoresis
STS; Staurosporine
SEC; Size exclusion column chromatography
DMEM; Dulbecco's modified Eagles medium
DTT; Dithiothreitol
FBS; Fetal bovine serum
CD; Circular dichroism
DISC; death-inducing signaling complex
ThT; Thioflavin T
RFU; Relative Fluorescence Unit
PMSF; Phenylmetanesulphonylchloride
Ac-DEVD-AMC; N-acetyl Asp-Glu-Val-Asp-amino methyl coumarin
Ac-LEHD-AMC; N-aetylLeu-Glu-His-Asp-amino methyl coumarin
PVDF; Poly vinylidenedifluoride

LA/LC; Lamin A/C

LB; Lamin B

AF; Amentoflavone

MBP; Maltose Binding Protein

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