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Ph.D. Dissertation

Involvement of calcium released from endoplasmic reticulum and TLCK and TPCK sensitive-serine proteases in amyloid beta-induced lamin fragmentation

Graduate School of Chosun University

Department of Biomedical Sciences

Md. Selim Hossain

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Amyloid-β 에 의해 유도되는 라민 단백질의 절단화와 관련된 소포체 유래 칼슘과 TLCK/TPCK 에 의해 저해되는 단백질 분해효소에 대한 연구

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Advisor: Prof. Il Seon Park

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CONTENTS

LIST OF FIGURES

한글 요약

Amyloid-β 에 의해 유도되는 라민 단백질의 절단화와 관련된 소포체 유래 칼슘과 TLCK/TPCK에 의해 저해되는 단질 분해효소에 대한 연구

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β-아밀로이드(Aβ)는 알츠하이머병 (AD)에서 확인된 아밀로이드 플라크의 주요 성분이다. 이 홀마크 펩타이드는 비아포토시스 및 카스파아제 의존성 아포토시스 세포사를 유도한다. AD 병리를 이해하기 위해서는 Aβ 유도 세포사 경로에 대한 더 많은 연구가 필요하다. 본 연구에서는 Aβ 유도 카스파아제 비아포토시스 경로에 초점을 맞췄다.

Aβ는 36~43개의 아미노산을 함유하고 있다. Aβ42 올리고머는 Aβ펩타이드의 독성 형태이며 세포사망 시 라민 절단를 일으키는 것으로 입증되었다. Aβ42는 카스파아제 비의존성으로 라민 A(LA)와 라민 B(LB)을 절단한다. 이 메커니즘의 핵심 주제 중 하나는 이러한 핵 라민을 절단하는 단백질 분해 효소를 확인하는 것이다. 이 단백질 분해효소의 성질을 알아보기 위해 여러 단백질 분해효소 억제제를 선별하여 TLCK, TPCK 및 Z-FF-

FMK가 Aβ42 유도 라민 절단을 차단할 수 있음을 발견했다. 또한 TLCK, TPCK 및 Z-FF-FMK는 Aβ42에 의해 유도되는 라민의 형태학적 변화를 억제하였다. 한편 TLCK와 Z-FF-FMK가 Aβ42 유도 세포사멸을 막았지만 TPCK 자체가 독성이었기 때문에 TPCK는 세포사멸을 감소시키지 못했다. Z-FF-FMK가 카테프신 L 억제제인 반면 TLCK 및 TPCK 단백질분해효소 억제제는 비특이적이었기 때문에 라민 절단에 대한 특정 단백질 효소는 카테프신 L이었다. 또한 TLCK 또는 TPCK의 존재 하에서 카테프신 L 활성을 측정하여 시스테인 단백질 분해효소에 대한 세린 단백질 분해효소의 영향을 조사하였고 TLCK 또는 TPCK의 존재 하에서 카테프신 L 활성의 감소를 관찰하였다. 결과적으로 카테프신 L 이전에 세린 단백질 분해 효소가 참여할 수 있다고 가설을 세웠다.

다음으로 카테프신 L이 어떻게 활성화되는지 궁금했고 문헌을 검색하여 카테프신 L이 칼슘(Ca^{2+}) 의존성 단백질 분해효소라는 것을 알게 되었다. 그리고 라민절단을 유도하는 Aβ42에 대한 Ca^{2+}의 역할을 조사하였다. 처음에 우리는 Aβ42가 세포사멸을 매개하는 세포내 Ca^{2+}를 유의하게 증가시켰다는 것을 발견했다. 이후 Ca^{2+} chelator (BAPTA-AM)를 사용하여 Ca^{2+} 증가와 라민절단간의 상관관계를 설정했다. 또한 Aβ42 처리 세포에서 BAPTA-AM에 의한 핵 형태 변화도 관찰됐다. 이 발견은 세포 내 Ca^{2+} 상승이 Aβ42 유도 라민 절단을 중재하는데 중요하다는 것을 나타낸다. 그

리고 Aβ42 처리된 세포에서 Ca^{2+} 과부하를 초래하는 Ca^{2+}의 출처를 확인했다. Aβ42에 의해 유도되는 소포체 (ER) Ca^{2+} 방출을 Cyclopiazonic Acid (CPA)를 사용하여 검사한 결과, CPA에 의한 ER Ca^{2+} 증가를 차단하는 것이 세포사뿐만 아니라 라민절단을 억제하는 것으로 나타났다. 이 데이터는 ER Ca^{2+}가 분비되어 Aβ42 처리 세포에서 세포질 Ca^{2+} 증가를 일으킨다는 것을 보여준다. 한편, 카스파아제 매개 라민절단과 세포 사멸 감소에 BAPTA-AM의 영향을 관찰했다. 결과는 BAPTA-AM의 존재 하에서 카테프신 L 활성이 유의미하게 감소했음을 보여주었다. 따라서 카테프신 L의 활성은 칼슘 의존성을 나타내는 세포질 Ca^{2+} 농도에 의해 조절되는 것으로 결론내렸다. 분리된 핵에 $CaCl_2$를 처리하면 Aβ42 처리시 보이는 라민절단 산물이 생성되었다. 분리된 핵에서 다양한 단백질 분해효소 억제제가 존재하는 상태에서 Ca^{2+} 유도 라민 분열을 관찰한 결과 카테프신 L 억제제(Z-FF-FMK)만이 이 라민절단 과정을 억제할 수 있음을 최종 확인했다. 따라서, Aβ42 처리 세포에서 라민절단에 Ca^{2+} 의존 카테프신 L이 관여하는 것이 확실해 보인다. TLCK, TPCK, BAPTA-AM 및 Z-FF-FMK의 추가적인 특징은 Aβ42 유도 팬 카스파아제 활성화 방지였다. 이 부분은 추후 자세히 조사할 필요가 있다.

Aβ42 유도 라민절단 메커니즘을 조사하기 위해 준비한 세포 투과성 Tat-tagged Aβ42(tAβ42)는 Aβ42 특이적 라민절단을 유도하였으며 위의 메커니즘과 유사하였고 이는 세포투과성과 특정 라민절단의 밀접한 연관성

을 암시한다. 또한 Aβ 독성 억제제로 커큐민시 커큐민이 세포 내 Ca^{2+} 방출을 억제함으로써 Aβ42 특이적 라민 분해를 막았다는 것을 암시했다.

따라서, 우리의 결과는 라민 단백질 절단을 위한 이 경로가 Aβ42에 특이적이며 알츠하이머병의 Aβ42 유도 세포사 메커니즘을 명확히 하는데 도움을 줄 수 있다는 것을 보여준다.

ABSTRACT

Involvement of calcium released from endoplasmic reticulum and TLCK and TPCK sensitive-serine proteases in amyloid beta-induced lamin fragmentation

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β-Amyloid(Aβ) is the main constituent of amyloid plaque identified in Alzheimer's disease. This hallmark peptide induces both non-apoptotic and caspase-dependent apoptosis cell death. To understand the AD pathology, more investigations on Aβ induced cell death pathway are needed. In this study, we focused on Aβ induced caspase-independent non-apoptotic pathway.

Aβ contains 36-43 amino acids. Aβ42 oligomer is the toxic form of Aβ peptide and is proved to cause lamin fragmentation during cell death. Aβ42 produces caspase-independent lamin A(LA) and lamin B(LB) cleavage. The mechanism of this lamin cleavage by Aβ42 remains inconclusive. One of the key events of this mechanism is to know the protease(s) responsible for this nuclear lamin damage. To find out the nature of this protease, we screened different protease inhibitors and found that TLCK, TPCK, and Z-FF-FMK were able to block Aβ42 induced lamin cleavage. In addition, TLCK, TPCK, and Z-FF-FMK shielded morphological changes of lamin induced by Aβ42.

Moreover, our cell viability data showed that TLCK & Z-FF-FMK prevented Aβ42 induced cell death but TPCK failed to reduce cell death because TPCK itself was toxic. From our findings, the specific protease for lamin cleavage was cathepsin L since the Z-FF-FMK is a cathepsin L inhibitor whereas TLCK & TPCK protease inhibitors were non-specific. We also examined the influence of serine proteases on cysteine protease by measuring cathepsin L activity in the presence of TLCK or TPCK and observed the cathepsin L activity reduction in the presence of TLCK or TPCK. We hypothesized that this could be due to the activation of serine protease before cathepsin L.

Next, we were curious about how cathepsin L is activated and by searching literature we came to know that lysosomal cathepsin release is calcium (Ca^{2+}) dependent. Then, we investigated the role of Ca^{2+} for Aβ42 inducing lamin cleavage. Initially, we found that the Aβ42 significantly increased the intracellular Ca^{2+} which mediates cell death. Later, we established a correlation between Ca^{2+} increase and lamin fragmentation using a Ca^{2+} chelator (BAPTA-AM). We also observed the prevention of nuclear morphology changes by BAPTA-AM in Aβ42 treated cells. This finding indicates that intracellular Ca^{2+} rise is important to mediate Aβ42 induced lamin damage. Then, we checked the source of Ca^{2+} in Aβ42 treated cells liable for cytosolic Ca^{2+} overload. Endoplasmic Reticulum (ER) Ca^{2+} release induced by Aβ42 was examined by using Cyclopiazonic Acid (CPA) and found that blocking ER Ca^{2+} mobilization by CPA inhibited lamin fragmentation as well as cell death. Our data demonstrate that ER Ca^{2+} causes cytosolic Ca^{2+} increase in Aβ42 treated cells. Similarly, we observed the effect of BAPTA-AM in other cell death-inducing agents where caspase-mediated lamin fragmentation occurred and there was no impact of BAPTA-AM in the reduction of lamin

fragmentation or cell death. However, the measurement of cathepsin L activity in Aβ42 treated cells was performed in the presence or absence of a Ca^{2+} chelator. Results revealed that cathepsin L activity was reduced significantly in the presence of BAPTA-AM. So, the cathepsin L activity is regulated by cytosolic Ca^{2+} concentration indicating its calcium-dependency. Incubation of isolated nuclei in the presence of $CaCl_2$ generated laminar fragments similar to those of Aβ42 treated lamin products. Furthermore, the observation of Ca^{2+}-induced lamin cleavage in the presence of various protease inhibitors in isolated nuclei ultimately confirmed that only cathepsin L inhibitor(Z-FF-FMK) was able to inhibit this lamin fragmentation process. Therefore, the involvement of Ca^{2+} dependent cathepsin L release to cleave lamin in Aβ42 treated cells has been justified. An additional feature of TLCK, TPCK, BAPTA-AM, and Z-FF-FMK was the prevention of Aβ42 induced activation of pan caspases. This part needs to be investigated in detail.

To support Aβ42 induced lamin fragmentation mechanism, cell-permeable Tat-tagged Aβ42(tAβ42) was prepared for the study. This highly cell-permeable and soluble tAβ42 induced Aβ42 specific lamin cleavage and the mechanism was similar. Furthermore, we used curcumin as an Aβ toxicity inhibitor. Our results implied that curcumin prevented Aβ42 specific lamin fragmentation by inhibiting intracellular Ca^{2+} release.

So, altogether, our results indicate that this pathway for lamin protein cleavage is specific for Aβ42 and could help to clarify the Aβ42 induced cell death mechanism in Alzheimer's disease.

I. INTRODUCTION

I-1. Alzheimer's Disease Overview

Alzheimer's disease (AD) is the most usual form of dementia and life-threatening neurodegenerative disorder in human that results in dysfunction of memory, thinking ability, regular activities, and learning capability. According to recent data, around 5.3 million people in United State (U.S) and 26.6 million all over the world are suffering from AD and these numbers are expected to increase four-fold by 2050 [1–3]. AD was first diagnosed in 1906 by German Psychiatrist Alois Alzheimer in the brain of a woman namely Auguste Deter who died of unusual mental problems [4]. After doing the autopsy, brain tissues were analyzed and many unusual clumps and tangled bundle fibers were identified. Abnormal clumps were named amyloid or senile plaque whereas bundle fibers were said neurofibrillary tangles. Since then, those two brain lesions have become the main causative factor of AD [5–7]. In the 1980s, the composition of plaques and fiber tangles were identified and found that amyloid plaques are consist of ~ 4 Kda amyloid beta-peptide(Aβ) while neurofibrillary tangles are made of the hyper-phosphorylated form of microtube, associated Tau protein [8–15]. Tau was formerly discovered as a protein binding to microtubules and helping in their formation and stabilization [16]. However, onto phosphorylation, it loses its ability to bind to microtubules and the unbound tau protein clumps in together as neurofibrillary tangles [17]. Both Aβ and tau proteins are presented in a highly ordered and aggregated form known as 'amyloid fibrils' and tangles respectively. Many researchers proved that amyloid plaques and neurofibrillary tangles occur independently and there is no relation to each other. Besides, in the absence of Aβ deposition, the presence of neurofibrillary tangles was found in much less common

neurodegenerative diseases [18,19]. So, neuro tangles have been suggested as the secondary cause of neuronal injury in AD [20,21]. Therefore, Aβ is considered as main marker in the development of AD.

I-2. Aβ hypothesis in Alzheimer disease

Aβ is a 4.5 kDa small peptide present in both cerebrospinal fluid and blood at the very low physiological concentration (nanomolar or less) [22]. Aβ peptides having 36-43 amino acid length are generated by sequential cleavage of a transmembrane protein, amyloid precursor protein (APP) by -α, -β & -γ secretases. Though the main physiological function of this precursor protein remains unknown, the protein is found in many tissues including in the synapses of neurons. APP undergoes post-translational processing involving its cleavage by various proteases and secretases [23–25]. APP has two amyloidogenic pathways (one is benign and another one is malignant). In the non-amyloidogenic pathway (about 90% of APP is processed in this way), APP is sequentially cleaved by α-secretase and γ-secretase producing in two relatively benign membrane-associated C-terminal fragments, p3 and AICD (Amyloid Precursor Protein Intracellular Domain). In the amyloidogenic pathway, APP is cleaved by β secretase and generate soluble APP fragments(β-APP) and membrane- bound C-terminal fragment(C99). α-secretase cannot cleave C99 rather γ-secretase acts on it and produce toxic Aβ and AICD. The Aβ-peptides are then accumulated extracellularly [26,27]. Variability in the cleavage site of γ-secretase generates several types of Aβ peptide. The variability in the membrane-bound C-terminal fragment(C99) cleavage site occurs due to the fact that the γ-secretase enzyme has multiple cleavage sites within the transmembrane domain of APP [28,29]. Among different types of detected Aβ peptides, Aβ40 and Aβ42 are most commonly found in the brain

of Alzheimer's patients. It is worth mentioning that Aβ42 is more hydrophobic as well as toxic than Aβ40. Thus, it is easily oligomerized and aggregated. The aggregated peptides are deposited outside of the cells and form plaque known as an amyloid plaque or senile plaques. On the other hand, less dense aggregates are diffused in the wall of small blood vessels of the brain known as amyloid angiopathy [30–32].

I-3. Different forms of Aβ and cytotoxicity

Though it has been proved that Aβ is neurotoxic and is involved with AD but detailed mechanism still remains obscure. In the main amyloid hypothesis, amyloid plaques or fibrils were considered the causative factor of AD. Recent studies are not supporting the previous hypothesis. For instance, APP was overexpressed in transgenic animals where cognitive deficits and neuronal abnormalities were observed before detecting Aβ plaques [33–37]. Therefore, it is important to study various Aβ species-induced cell death in order to find out a clear pathway of AD. Based on length, molecular weight, and microscopic dimension, Aβ species are classified as monomer, very short oligomers (dimers and hexamer form of Aβ), small oligomers (molecular weight ranges from 17 to 42 kDa) and protofibrils (a prior form of mature amyloid fibrils) [38,39]. The relationship between Aβ plaque thickness and clinical severity of dementia has been found tiny [40,41]. Surprisingly, in many severe AD patients, few Aβ plaques were detected while a notable amount of Aβ plaques was found in the brain tissues without AD. Some researchers have agreed with the original Aβ hypothesis proving the deposition of fibril in particular parts of the brain at a particular time (long before autopsy) could be critical [42]. Another hypothesis differed from the original amyloid concept in AD. They explained that small oligomeric species are formed before maturing

amyloid plaque which they considered the real toxic elements in AD whereas Aβ plaque could scavenge Aβ toxic species and might play the role of protective agent [43]. Monomeric Aβ especially Aβ40 and Aβ42 are produced in various cell types of the body and there is no report on monomeric Aβ42 cytotoxicity at physiological applicable concentrations. Many studies reported that insoluble Aβ aggregation, Aβ plaques and vascular Aβ possess less toxicity in vitro [44,45] Besides, studies also demonstrated that Aβ plaques could remove Aβ toxic species in vivo. One study in 1995 suggested that soluble Aβ not amyloid plaques could trigger neurotoxicity in AD. Another hypothesis found that the most toxic form of Aβ is soluble oligomer and induced acute synaptoxicity as well as neurodegeneration [46–49].

I-4. Aβ induced apoptosis and cell death

Programmed cell death (PCD) also known as apoptosis is very essential for many biological functions such as cell renewal, immune regulation, neuronal development, removing unwanted cells and individual morphogenesis. Deregulation of Program cell death results in neuronal development disorders of the nervous system and subsequently lead to neurodegenerative diseases. In AD, gradual neuronal loss and cognitive disorder happen [50]. Caspases (cysteine aspartic specific proteases) are well-known apoptotic proteases responsible for cell death [51]. Evidence demonstrated that different types of caspases have been detected in the brain of AD patients. Increased activity of initiator caspases such as caspase-2, -8, -9 and effector caspases e.g. caspase -3, -6, -7 was found in the case of AD as compared to control which suggest that there are some connections of apoptosis in AD [52]. Aβ interacts with different extra and intracellular proteins and results in the induction of the cell death process through the activation of caspases. This clearly indicates the

involvement of apoptosis in Aβ induced cell death process [53,54]. In the case of apoptosis, a series of biochemical events occur and those events are either based on mitochondria or death receptors. In the death receptors interaction mechanism, extracellular ligands such as TNF-α, TRAIL, CD95 bind with death receptors and form death-inducing signaling complex (DISC) which in turn activates initiator caspase-8 [55]. On the other hand, apoptosome (a multiprotein complex) is formed in mitochondria-mediated apoptosis whereas apoptosome is composed of cytochrome c release from mitochondria, caspase-9, Apaf-1, dATP and caspase-9. DISC and apoptosome activate initiator caspases procaspase-8 & -9 respectively which subsequently activate executioner caspase -3, -6 and -7 to produce downstream effect during apoptotic process [56]. Several studies exhibited the involvement of apoptosis in Aβ induced cell death process both in vitro and in vivo models. Several hypothesizes have been proposed to describe Aβ induced apoptosis mechanism, for instance-one hypothesis suggested that the release of mitochondrial apoptosis-inducing factor (APF) and cytochrome c in Aβ treated neurons which consequently initiated caspase activations to cause DNA fragmentation and chromatin condensation [57]. While another study showed the direct effect of Aβ as an inducer of apoptosis by demonstrating caspase-3 activation and processing in Aβ treated rat cortical neurons. Furthermore, a lot of investigators reported that blocking caspase inhibited Aβ mediated apoptosis but cell death remained the same by the peptide [58]. This means that Aβ follows different pathways to kill the cells. When one pathway is stopped, it finds another pathway to kill the cells. Therefore, the actual mechanism of Aβ induced apoptosis and its relation to cell death remain inconclusive. This needs to be studied more intensively to find out a clear pathway of cell death by Aβ in AD.

I-5. Aβ42 induced lamin fragmentation

Neuronal lamin disruption is considered a recent concept in AD pathology. The nuclear lamina is a thick fibril type of protein layer of nuclear envelope and chromatin. Lamin proteins undergo several changes during pathological conditions. Two types of lamin proteins (lamin A/C and lamin B1/B2) are located in the nuclear envelope. Lamin A/C proteins are encoded by LMNA gene and lamin B1/B2 proteins are encoded by the LMNB1 and LMNB2 respectively. Any defect of these proteins leads to laminopathy [59,60]. Lamin proteins are important for brain development and neuronal survival under various oxidative stress [61,62]. Some researchers observed the nuclear lamin invagination in tauopathy (neurofibrillary tangles formation in AD) [63]. So, it is inevitable to say that there is some connection between neuronal cell death and lamin damage [64,65]. Lamin fragmentation was also observed in Aβ42-treated cells and it is independent of caspase activation. The focus needs to be given on the agent responsible for lamin cleavage in Aβ42 treated cells. Literature showed that caspase-3 & -6 cleave lamin proteins. Besides, caspases, other proteases are also involved in lamin protein fragmentation such as nuclear scaffold proteases, granzyme A and B [66–69]. However, in the case of Aβ treated cells, lamin fragmentation is not mediated by caspase-6 because ~46 kDa N-terminal LA and ~21 kDa C terminal LB fragments were detected [69]. Thus, it can be said that another enzyme is involved for lamin fragmentation rather than caspase-6 in Aβ treated cells. This could be nuclear scaffold protease which has been searched in our current study.

I-6. Lysosomal enzymes in AD

The lysosome is an essential digestive organelle that involved in the pathogenesis of Alzheimer like a neurodegenerative disease. Cathepsin is considered the main class of lysosomal proteases. The endosomal-lysosomal system of neurons suggests that the cathepsin proteases are capable of initiating and executing cell death programs during pathological conditions as well as in aging [70]. In addition to the caspases, lysosomal proteases such as cathepsin B, D and L have been revealed to act as mediators of apoptosis in different cell systems [71–74]. Several investigations revealed that cathepsins including cathepsin B, L and D were involved in caspase- independent cell death. A group of scientists in 1990 reported a proximate relationship between lysosomal membrane disruption and AD [75]. It has also been denoted that increased lysosomal proteins especially cathepsin L family protein is observed in AD through upregulation of lysosomal system [75]. Another study showed the significant role of cathepsins in oxidative stress as well as age-related neurodegeneration [76,77]. Besides, cathepsin D plays a significant role in the processing of amyloid precursor proteins [78]. One report demonstrated that treatment of neuronal cells with cytotoxic Aβ42 generated rapid free radicals within lysosomes and disruption of lysosomal protein gradients lead to cell death [79]. Based on previous studies, it can be said that lysosomal enzymes have an important role in AD. In our current study, we checked the effect of lysosomal proteases on Aβ42 induced lamin fragmentation.

I-7. Aβ induces calcium dyshomeostasis in AD

Calcium ion is a significant messenger that controls a variety of signaling pathways involved in cell survival, proliferation, differentiation, transcription and apoptosis. In mammalian cells, calcium dynamics and intracellular calcium levels studying are very important to have a better understanding of a variety of illness conditions. A large number of evidences found in literature suggest that oligomer Aβ mediates neurotoxicity in AD and disruption of calcium homeostasis may have contributed to this pathological state. The AD calcium hypothesis explains that Aβ peptides affect calcium homeostasis conditions and lead to synaptic loss, neuronal connection disruption and cognitive function deficit [80–82]. One evidence reported that increasing of calcium level may be an early pathological event in AD. Moreover, changes in long-term potentiation were also observed in Aβ induced cells through activation of calcium -dependent molecular events. Besides, a report on transgenic mice also proved that oligomer Aβ increased calcium in the intact brain. The study also proposed that imbalance in calcium homeostasis occurs through NMDA receptors. The previous study demonstrated that oligomer Aβ either generated within the synapse or entering the outside affects synaptic function through calcium dyshomeostasis [83]. Although the mechanism of intracellular calcium rise by Aβ peptide is a complex and debated issue. It has been proved that Aβ plaque and oligomer Aβ causes dysregulation of calcium in the AD mouse model [84]. It is worth mentioning that different researchers proposed individual hypotheses regarding Aβ induced calcium dysregulation, for example- one hypothesis brought forward is that Aβ peptides elevate calcium inside of the cell by activating different calcium channels in the plasma membrane. Another mechanism showed that Aβ may cause synaptic terminal

dysfunction by unsettling calcium homeostasis through lipid membrane peroxidation and eventually neuronal degeneration as well as the cognitive deficit. There is also a report about calcium release from the ER in Aβ treated cells [85–87]. Based on the hypothesis of researchers, oligomer Aβ overload cytosolic calcium through several mechanisms such as: (i) decreased phosphatidylinositol-4,5-bisphosphate availability (ii) enhanced expression of ryanodine receptor (RyR) that intensify the inositol 1,4,5-triphosphate (IP_3) mediated intracellular calcium release (iii) increased IP3 receptor and RyR receptor channel sensitivity [88–91]. Evidence demonstrated that dysregulation of ER calcium(ca^{2+}) is an early event in human AD fibroblasts and consequently contributed to disease progression [92,93]. Different enzymes like kinases and phosphatases are essential for maintaining the homeostasis of neurons and those are regulated by Ca^{2+}. Aβ mediated calcium dysregulation occurs in neurons and ultimately leads to neuronal toxicity [94–97]. Report on AD model reveals that any reduction of ER stress removes memory impairment because it is believed that calcium is released from ER and modulate calcium homeostasis [98]. In addition, mitochondria can catch a significant amount of calcium when a large amount of calcium is released from ER [99]. The enormous inflow of calcium into mitochondria can cause the membrane potential to collapse [100]. As a result, apoptotic factor such as cytochrome c is released into the cytosol and binds with apoptosis-inducing factor (AIF) to activate caspase-9 [101]. This process activates caspase-3, which cleaves numerous cellular substrates, resulting in cell death [102]. Therefore, based on the previous study it can be said that the early release of calcium from ER causes apoptosis.

I-8. Prevention of Aβ42 induced cell death by chemical compounds

Different chemicals were identified as inhibitors of Aβ42 induced cell death and those inhibitors either inhibited the Aβ oligomer or fibril formation pathway [103]. Several studies looked into how thioflavin T and the sulfonate dye Congo red and it's derivative attach to Aβ in the tissue section and stain it [104,105]. Flavonoids which are commonly found in plants have been demonstrated to lessen the incidence of age-related dementia [106]. Research on Ginkgo biloba extracts and the effects of HE208 and EGb761 compounds revealed the need for flavonoid molecules in neuronal cells for anti-amyloidogenic and antiapoptotic action [107]. Generally, flavonoids are classified into two types, and those are-monoflavonoids and bioflavonoids. When compared to monoflavonoids like apigenin, bioflavones like taiwaniaflavone (TF), amentoflavone, and sumaflavone are said to suppress Aβ cytotoxicity more effectively [108]. Curcumin is a polyphenolic compound found in abundance in the *Curcuma longa* plant and displays a neuroprotective effect [109,110]. Previous studies show that curcumin interacts with Aβ species and inhibits aggregation and fibril production in in-vitro, as well as having the potential to disaggregate mature fibrils, making it the most important chemical to be considered as a treatment agent for AD pathogenesis [111].

I-9. Outline of the Dissertation

This thesis focused on the protease(s) responsible for lamin cleavage induced by Aβ42 and investigated the molecular pathway of this lamin fragmentation. Initially, the thesis screened different proteases inhibitors to find out the enzyme that breaks lamin protein induced by Aβ42. The later portion identified the role of calcium to cause lamin cleavage in Aβ42 treated cells. The rationale of this dissertation is summarized below:

1. Protease inhibitor screening method was used to identify the inhibitor that can prevent Aβ42 induced lamin fragmentation as well as cell death. Our results reveal that TLCK, TPCK and cathepsin L inhibitor(Z-FF-FMK) can prevent production of lamin fragment in Aβ treated cells. So, lysosomal cathepsin L is involved in Aβ42 induced lamin damage.

2. Aβ42 induced lamin fragmentation and cathepsin L activity were observed in AD mice.

3. Regulation of cathepsin L and caspases activity by TLCK and TPCK was identified in Aβ42 treated cells.

4. Effect of cathepsin L on the activation of the apoptotic cascade in Aβ42 treated cells has been demonstrated in this thesis.

3. To understand the release of cathepsin L from the lysosome, a comprehensive study has been carried out. Cathepsins are calcium-sensitive proteases and from our current investigation, it was found that activation of cathepsin L is dependent on calcium. Intracellular calcium release was determined in Aβ42 treated cells and its role on lamin fragmentation was observed. In addition, inhibition of cytosolic calcium in Aβ42 induced cells

showed a protective effect against lamin damage and cell death.

5. Aβ induced ER calcium release has been analyzed in this study and its influence on lamin cleavage has been correlated.

6. Cell permeable TAT tagged Aβ42(tAβ42) exhibited a similar pattern of lamin cleavage like of Aβ42 and molecular events of this lamin damage were also the same for both the peptides.

7. curcumin as Aβ inhibitor was examined whether it inhibits Aβ42 induced lamin cleavage. Curcumin prevented Aβ42 specific lamin cleavage effectively.

II-MATERIALS AND MEHTODS

II-1. Materials

Urea, Ethylene Diamine Tetra Acetic Acid (EDTA) and Isopropyl β-d-1-thiogalactopyranoside (IPTG) were obtained from Thermo Fisher Scientific (USA). Tris-Cl and Dithiothreitol (DTT) were from Gene Ray Biotech Co; Ltd (Shanghai, China). Methanol and ethanol were from OCI company ltd (Seoul, Korea). Fetal Bovine serum (FBS) was purchased from ATLAS Biologicals Inc. (Fort Collins, USA). Dulbecco's modified Eagles medium with high glucose (DMEM/HG) and Penicillin-Streptomycin solution (P/S) were procured from Welgene (Daegu, Korea). Caspase-9, Caspase-8, Caspase-6 and Caspase-3 substrates : N-acetyl Leu-Glu-His-Asp-amino methyl coumarin (Ac-LEHD-AMC), Ac-IETD-AMC, Ac-VEID-AMC and Ac-DEVD-AMC, respectively, were bought from A.G. Scientific Inc. (San Diego, USA). Z-VAD-FMK from Alexis (Lausen, Switzerland). Phosphate buffer saline (PBS), cathepsin L inhibitor(Z-FF-FMK) and cathepsin L substrate(Z-F-R-AMC) were purchased from EMD Millipore Corp. (USA). Dimethyl Formamide (DMF), Bisacrylamide and ammonium persulfate (APS) were from VWR Life Sciences (USA). Sodium Dodecyl Sulfate (SDS), acrylamide, Triton X-100 and potassium chloride (KCl) were from USB(USA). Gylcine was from Junsei chemical (Japan) and glycerol from Fujifilm Wako pure chemical corp. (Japan). N^{α}-p-tosyl-L-lysine chloromethyl ketone (TLCK), N^{α}- p-tosyl-L-phenylalanine chloromethyl ketone (TPCK) were from Calbiochem-Novabiochem Corp. (USA). Fluo-8-AM was bought from Abcam (UK). Lamin-A/C, lamin B and β-actin primary antibodies and HRP conjugated secondary anti-mouse were acquired from Santa Cruz Biotechnology

(California, USA). For confocal microscopy, Alexa-Flour-488-FITC conjugated goat anti-mouse IgG and Alexa-Fluor-546- Tetramethylrhodamine (TRITC) conjugated goat anti-rabbit IgG were procured from Invitrogen (USA). All other chemicals like BAPTA-AM, Staurosporine (STS), ALLN, MG-132, leupeptin etc. were bought from Sigma (St. Louis, USA) Aldrich, unless otherwise mentioned.

II-2. Fusion proteins expression process

We used previously constructed pET28b-GroES-ubiquitin-Aβ42 and pET28-GroES-ubiquitin-TAT-Aβ42 vectors for the expression of fusion protein as described before [112]. In short, for the expression of protein in transformed E. coli BL21(DE3) pLysS cells, 400 µM IPTG was added in large culture whenever the optical density (OD_{600}) reached at around 0.6 and it was cultured at 30^0C for 4 h. Then cells were harvested by centrifugation at 2000 X g for 10 min. After collecting the cells, cells were lysed in a lysis buffer (20 mM Tris-Cl, pH 8.0, 150 mM NaCl, 0.1 mM PMSF, 0.1 mM EDTA and 1 mM DTT) and centrifuged at 11000 X g for 30 minutes. Fusion protein was obtained as inclusion body. Solubilization buffer (50 mM Tris-Cl, 150 mM NaCl, 6 M urea and 1 mM DTT) was used to make soluble of inclusion body and centrifuged at 36000 X g to remove undissolved proteins. Supernatant was preserved for further purification process.

II-3. Aβ42 and tAβ42 purification

The protein sample (saved supernatant) was diluted with buffer A (50 mM Tris-Cl, pH 8.0, 150 mM NaCl and 1 mM DTT) to maintain the final concentration of urea 3M in order to prevent precipitation of fusion protein immediately before digestion. Protein concentration was maintained up to 4-5

mg/ml during digestion. For digestion, final concentration of DTT was 2 mM and USP2cc enzyme was added at 1:100 ratio to incubate at 37^0C for 3 h. After the digestion, the protein sample was sonicated for 10 min on water bath followed by centrifugation at 36000 X g for 30 minutes. After that, solubilization buffer was added to make final urea concentration 4 M. Then, methanol at 1:1 ratio was added to the digested protein sample and kept at room temperature for 15 minutes. Supernatant was collected by centrifuging at 2000 X g for 10 minutes. Pure Aβ42 and tAβ42 were found in the soluble fractions. Supernatant was dried using rotary evaporator. The dried samples were washed with 100 % ethanol to remove unnecessary salt and pellet was collected by centrifuging at 2000 X g for 10 minutes. The pellet having pure peptide was solvated in 100% 1,1,1,3,3,3-hexafluoro-2-propanol (HFIP) having concentration of 0.5 mM in order to get monomer Aβ42. After HFIP evaporation, peptide was preserved at -20^0C for long term use. Preparation of Usp2cc enzyme was based on our previous study [113].

II-4. Wild Aβ42 and tAβ42 preparation

The Aβ42 peptide was solvated in 0.1% NH_4OH having concentration of 2 mg/ml followed by 10 min bath sonication. After sonication, it was centrifuged at 16000 X g for 10 min. To prepare, tAβ42, the peptide was dissolved in 0.1 % HCl and sonicated followed by 10 min centrifugation at 16000 X g. Aβ42 oligomers were prepared as described before [114] with slight modification. Shortly, Aβ42 peptide was diluted in cell culture media (DMEM) at a concentration of 100 μM, vortexed for 30 seconds and incubated overnight at 4ºC. During experiments, the peptide solution was diluted with cell culture media to the desired concentrations.

II-5. Cell culture

Hela cells (Human epithelial) obtained from Korean Cell line Bank were cultured in DMEM (Dulbecco's modified Eagle's medium having high glucose) medium supplemented with 10 % (v/v) Fetal Bovine Serum and 1 % penicillin-streptomycin antibiotics under 5 % CO_2 at 37^0C.The cell density for the experiments was determined using Hemocytometer.

II-6. Analysis of AD Mice brain tissues

We obtained brain tissues from the human Swedish mutant amyloid precursor protein transgenic mice [115] and analyzed LA & LB by western blotting. We also measured cathepsin L activity in brain cell lysates.

II-7. Cell viability study

Cell viability was evaluated by 3-(4,5-dimethylthiazol-2-yl)-2,5-di-phenyltetrazolium bromide (MTT) method described formerly [116]. Succinctly, Cells(15000/well) were grown in 96 well plates (Nunc, Denmark) for 24 h and subsequently, 12 h serum deprivation period was followed. After treatment, 20 µl MTT solution (5 mg/ml) was added to each group of the plate and 2 h incubation was maintained. After that,100 µl solubilization solution [20% SDS in 50% (v/v) DMF (pH 4.7)] was included and incubated for another 12-16 h to get absorbance at 570 nm with a plate reader Spectra Max (Molecular Devices, CA, USA). In case of alamar Blue test, similar cell density and treatment procedures were followed. After adding alamar Blue (10 µl) directly to plate, cells were incubated for 4-16 h and optical density was documented at 570 nm with the same plate reader [117].

II-8. Determination of caspase activity

Caspase activity was determined according to the protocol of the previous article as stated [118]. Cells(20000/well) were seeded in 96 well plates for 24 h culturing and 12 h serum starvation was followed. After treatment, cells were washed thrice with ice-cold phosphate buffer saline (PBS). Consequently, 40 µl caspase lysis buffer (20 mM HEPES-KOH, pH 7.0, 1mM EGTA, 20 mM NaCl,1mM EDTA, 0.25 % Triton X-100, 1mM dithiothreitol (DTT), 1mM phenylmethylsulphonyl fluoride (PMSF), 10 µg/ml leupeptin, 5 µg/ml pepstatin A, 2 µg/ml aprotinin, 25 µg/ml ALLN) was added to individual well and incubated for 30 min on ice. After that, caspase assay buffer (CAB) (final concentration- 20mM HEPES-KOH, with pH 7.0, 20 mM NaCl, 1.5 mM $MgCl_2$, 1mM EGTA, 1mM EDTA and 10 mM DTT) as well as caspase substrates were added to monitor 7-Amino-4-methyl Coumarin (AMC) release for maximum 2 h at an interval 2 min. The excitation and emission wavelength for the experiments were 355 and 460 nm respectively and fluorescence release was observed using a microplate spectrofluorometer (Molecular Devices, CA, USA). The results are expressed as the slope vs time.

II-9. Measurement of cathepsin L activity

Cathepsin L activity was determined by method [119] with minor modification. Closely, cells (2 $X10^4$) were taken in 96 well plates and grown for 24 h. After 12 h serum deprivation, cells were subjected to treatment for the indicated time. At the end of treatment, cells were washed three times with PBS and 40 µl lysis buffer (100 mM potassium phosphate, PH 6.0, 100 mM NaCl, 0.25 % Triton X-100) was added in each well. Following 30 min incubation on ice, 2x cathepsin L buffer [40 mM Sodium acetate (NaOAc), 8 mM EDTA, 16

mM Dithiothreitol, 0.8 mM urea] & 10 μM cathepsin L substrate(Z-F-R-AMC) were then added to the mixture. Subsequently, the release of AMC from the substrate was monitored at 30ºC every 2 min for maximum 2 h with a microplate spectrofluorometer (Molecular devices, CA, USA, excitation wavelength 355 nm, emission wavelength 460 nm).

II-10. Screening of protease inhibitor

MTT test was applied to identify the protease inhibitors that can inhibit the cell demise induced by Aβ42. Cells were taken at a density of 1.5 X 10^4 cells/well in 96 well plate and cultured for 24 h followed by serum deprivation for next 12 h. Cells were then exposed to Aβ42 in the presence of different protease inhibitors as indicated in figure (2) and MTT reagent was added to determine viable cells.

II-11. Determination of intracellular free calcium

Intracellular calcium was determined following a method [120] with slight modification. Briefly cells (1 X10^5) were taken in 6 well plates and grown for 24 h in calcium-free DMEM medium. After keeping the cells 12 h in serum-free medium, cells were treated with Aβ42 at the indicated concentration for the mentioned period. Performing three-time washes with PBS, cells were incubated with Fluo-8-AM (10 μM) in calcium-free DMEM medium for 1 h at 37^0C, 5% CO_2. After two-time washes with PBS, a fluorescence image was acquired with a Carl Zeiss 510 confocal laser scanning microscope (Jena, Germany). Images were processed using company-provided LSM 510 software. To get reproducible data, the experiment was repeated three times.

II-12. Western Blotting Assay

After treatments, cells were washed more than two times with ice-cold PBS and harvested by scraping. The cell pellet was suspended properly in a lysis buffer (50 mM Tris-HCl, P^H 8.0, 1 % NP-40, 150 mM NaCl, 5 mM EDTA, 5 mM EGTA, 0.5 % Sodium Deoxycholate, 0.1 % Sodium Dodecyl Sulphate) with protease inhibitors and kept on ice for 30 min. The supernatant was collected via centrifugation at 18000 X g for 15 min at 4^0C. Bradford assay was used to measure protein concentration and equal amounts of protein were resolved in 12-15 % SDS-PAGE [69]. After separation, the resolved proteins were transferred to a PVDF membrane. The PVDF membrane was blocked using skim milk (5%) in TBS-T buffer (Tris buffer saline with 0.2% Tween 20) and immunoprobed with primary antibodies and HRP conjugated secondary antibodies. Primary and secondary antibodies were incubated overnight at 4^0C and 2 h at room temperature (R.T) respectively. Membranes were washed for 1 h with TBS-T after treating with antibodies. Target proteins were visualized with a Western blotting detection kit (WESTAR ŋC ULTRA 2.0) from Cyanagen (Bologna, Italy).

II-13. Immunocytochemistry (ICC)

Cells were seeded on glass coverslips in 12 well plate at 60-70% confluency and grown for 24 h. Additionally, cells were kept for 12 h in serum-free media and after that followed Aβ42 treatment in the absence or presence of inhibitors at the indicated concentration for mentioned time. After finishing the treatment period, cells were washed with PBS and fixed in 98 % cold methanol. For permeabilization and blocking, 0.3% Triton X-100 and 0.1% BSA were used respectively. Overnight blocking was maintained at 4^0C. The

next day, cells in the coverslips were incubated with suitable primary antibodies overnight at 4^0C After overnight primary antibody incubation, coverslips were washed with PBS and incubated with Alexa-Fluor-488-FITC conjugated goat anti-mouse IgG and Alexa-Fluor-546- Tetramethylrhodamine (TRITC) conjugated goat anti-rabbit IgG antibodies (dilution ratio: 1:200) for 2 h at room temperature wrapped in Aluminum foil followed by 1 h washing with PBS. Hoechst was used to stain nuclei. Coverslips were mounted on glass slides to capture images with Carl Zeiss LSM 510 microscope. Image brightness and contrast were also adjusted by LSM 510 company's software.

II-14. Isolation of nuclei and cell-free experiments

The nuclei were isolated from freshly harvested Hela cells as described before [121]. After collecting fresh Hela cells, it was washed 2 times with PBS and resuspended in TM buffer (10 mM Tris-HCl, PH 7.4, 0.5 mM PMSF, 2mM $MgCl_2$ and 0.5% Triton X-100). The cell suspension was incubated for 5 min each at room temperature and on ice. The cells were sheared via vortexing (three times) for 40 sec and centrifuged at 13000 X g for 5 min to get nuclei as a pellet. The pellet was resuspended in TM buffer without Triton X-100 to remove traces of Triton X-100. The nuclear pellet was used immediately for further experiments. For the cell-free experiments, isolated nuclei were incubated at 30^0C for one hour in a reaction buffer solution having 20 mM HEPES-KOH (pH 7.5), 0.1 mM EDTA, and 1 mM DTT with the designated chemicals or inhibitors. The reaction was stopped by including 2X SDS-PAGE gel loading buffer to the reaction sample. Nuclear proteins were separated on 12% SDS-PAGE and immunoblotted for the target proteins.

III-RESULTS AND DISCUSSION

III-1. TLCK &TPCK sensitive serine proteases modulate cathepsin L mediated Aβ42 induced lamin fragmentation

III-1-1. Aβ42 induced LA and LB cleavage, cell death and caspase activation under various treatment conditions

The current study was started with human neuroblastoma SH-SY5Y cells but due to excessive cell death and fluctuations of our experimental molecular events like lamin cleavage, caspase activation etc., we did not consider it for the present experiments (data not shown). So, we used epithelial Hela cells for the subsequent experiments because it removed the problems associated with SHSY5Y cells.

For our experiments, we searched a treatment condition which would produce lamin fragments as well as activate caspases robustly. Aβ42 used in this study was oligomeric Aβ42 because from the previous published paper we came to know that oligomeric Aβ42 was effective for lamin proteins reduction and fragmentation [69,121]. Hela cells were treated with Aβ42 at the indicated concentrations for 12 and 24 h respectively and no reduction or cleavage of lamin proteins was observed (Fig-1A-B). Subsequently, we examined the cell death and caspase activity in 12 h sample and found less cell death and no caspase activation (Fig. 1E &I). Similarly, cell death and caspase activity were investigated in Aβ42 treated sample incubated for 24 h. Although cell death increased in 24 h incubation time but showed negligible caspase activation (Fig. 1F & J). We next used, double treatment following our previous publications [1,2] to get lamin cleavage and caspase activation. Although prominent reduction of LA & LB was observed in Aβ42 treated cells for 2+10 h but

caspase activation remained absent (Fig.1C & 1K). Our cell viability data by MTT method showed that cell death was increased in the 2+10 h incubated sample compared to 24 h sample (Fig. 1G). This result revealed that the lamin reduction in Aβ treated cells for 2+10 h was caspase independent. Next, we chose double treatment with longer incubation period (2 +22 h) to observe both lamin cleavage and robust caspase activation. Our finding showed that 46 and 21 kDa products from LA and LB respectively were produced in Aβ42 treated samples (Fig. 1D). Moreover, we observed more than 50 % cell death and strong caspase activation in Aβ42 treated samples at the indicated concentrations incubated for 2 + 22 h (Fig. 1H &L). So, the double treatment is important for Aβ42 induced lamin fragmentation, robust caspase activation and cytotoxicity. In this study, Aβ42 induced lamin fragmentation was independent of caspase activation because obtained lamin fragments size were not caspase-6 mediated. 28 and 46 kDa fragments from LA & LB respectively are produced in caspase-6 mediated lamin damage [66]. Therefore, searching for the protease (s) responsible for lamin cleavage in Aβ42 treated cells would be our current study target.

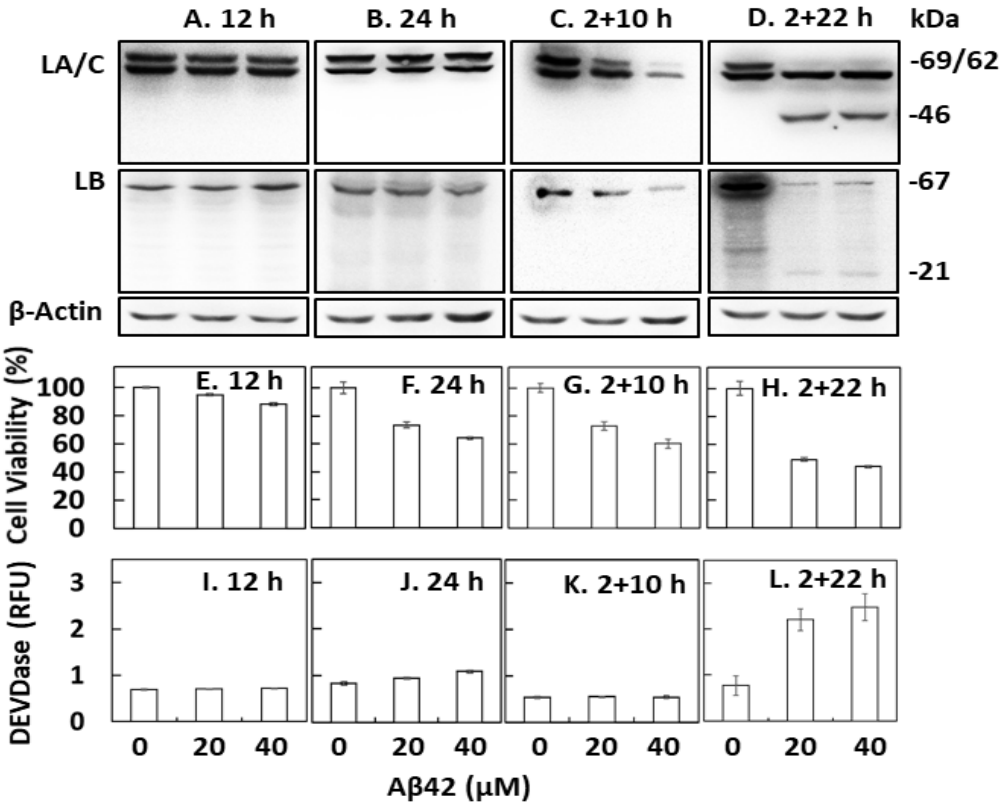

Figure 1. Amyloid beta induced cell death, nuclear lamin fragmentation & caspase activation. (A-B) Hela cells were treated with oligomeric amyloid beta(Aβ42) at the mentioned concentrations for the indicated time period. (C-D) The cells were first treated with the stated concentrations of Aβ42 for 2 hours, rinsed with the culture media to remove the added peptide, and then incubated with the indicated concentrations of Aβ42 for another 10 h (2+10 h sample) or 22 h (2+22 h sample). Cleavages of lamin-A & lamin B were detected using western blotting. β-actin was used as loading control. Fragments 46 for lamin A(LA) and 21 Kda for lamin B(LB) were observed. (E-H) Hela cells (15 $X10^3$) were approached with Aβ42 at the mention concentrations and for the referred time point to get cell death percentage by MTT method. (I-L)

Hela cells (2 X10^4) were exposed to Aβ42 at different concentrations for the indicated times and effector caspase-3 activity was measured using 10 μM DEVD-AMC substrate. RFU indicates relative fluorescence Unit.

III-1-2. Identification of TLCK, TPCK and Z-FF-FMK as inhibitor of Aβ42 induced lamin cleavage through screening protease inhibitors

Nuclear fragmentation occurs when caspase cascade is activated and results in programmed cell death [122]. In Aβ42 treated cells, lamin fragmentation is specific and is independent of caspase activation. Previous study proposed that nuclear scaffold (NS) protease could be liable for Aβ42 induced lamin cleavage [69]. In our investigation, we used AAPF-CMK (NS protease inhibitor) as positive control as it inhibited Aβ42 induced lamin damage (Fig. 2A) shown in previous study [69]. So, we designed following experiment to recognize the NSP protease involved in lamin protein fragmentation. In this study, to identify the protease (s) responsible for Aβ42 induced lamin damage, initially different protease inhibitors were screened for the inhibition of cell death (Data not shown). Proteases inhibitors such as ALLN (calpain inhibitor), leupeptin (serine protease inhibitor), MG-132 (proteasome inhibitor), pepstatin A (cathepsin D inhibitor), aprotinin (serine protease inhibitor), Z-VAD-FMK (pan caspase inhibitor), Z-FA-FMK (cathepsin B inhibitor), AEBSF (serine protease inhibitor), E-64 (cysteine protease inhibitor), 3,4-dichloroisocoumarin (DCI)(serine protease inhibitor), PMSF (serine protease inhibitor), UCF-101 (mitochondrial serine protease inhibitor) and benzamidine (serine protease inhibitor) were ineffective in inhibiting Aβ42 induced lamin fragmentation (Fig. 2A-C), excluding the involvement of those proteases in the process. We also checked the effects of TLCK (serine protease inhibitor), TPCK (serine protease inhibitor) and Z-FF-

FMK (cathepsin L inhibitor) on Aβ42 induced lamin damage. Our western blot results showed that TLCK, TPCK and Z-FF-FMK at the 50 μM concentration completely suppressed Aβ42 induced LA and LB cleavage suggesting the participation of those corresponding proteases in the lamin fragmentation process (Fig. 2D-E). The concentration below 50 μM of those protease inhibitors was not effective to inhibit lamin cleavage (Fig. 2D-E) and higher concentration of the inhibitors did not increase significant cell viability (data not shown) in Aβ42 treated cells.

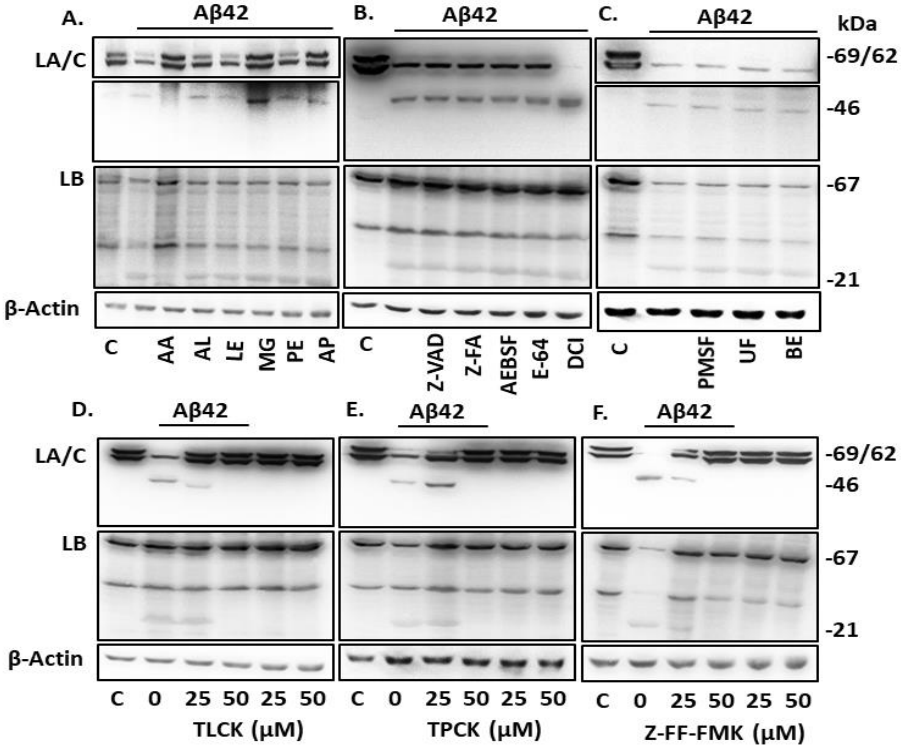

Figure 2. Protease inhibitors screening & prevention of Aβ42 induced lamin cleavage by TLCK, TPCK & Z-FF-FMK. (A-C) Hela cells (4 X10^5) were treated with Aβ42 for 2+22 h at 20 μM concentration. Inhibitors were added 1h before Aβ42 treatment. Effect of 40 μM suc-AAPF-CMK (AA), 20 μM ALLN (AL), 20 μM Leupeptin (LE), 5 μM MG-132 (MG), 20 μM Pepstatin (PE), 20 μM Aprotinin (AP), 20 μM Z-VAD-FMK(ZVAD), 50 μM Z-FA-FMK(Z-FA), 50 μM AEBSF, 10 μM E-64, 50 μM 3,4-dichloroisocoumarin (DCI), 100 μM phenylmethyl sulfonyl fluoride (PMSF), 50 μM Ucf-101(UF) and 100 μM Benzamidine(BE) on Aβ42 treated cells was examined by analyzing LA and LB cleavage using western blot. C means control cells without treatment. (D-F) Inhibition of lamin cleavage by Nα-p-

tosyl-L-lysine chloromethyl ketone (TLCK), Nα- p-tosyl-L-phenylalanine chloromethyl ketone (TPCK) and cathepsin L inhibitor(Z-FF-FMK) was observed by treating the cells with TLCK, TPCK and Z-FF-FMK at the indicated concentrations for 1 h at first, then further adding 20 μM Aβ42 for 2+22 h. Lamin bands and cleaved fragments were examined by western blot. Marks in the right side of the figures indicate the relative molecular weights (in kDa). β-actin was used as western control. Results are representative of three individual experiments.

III-1-3. TLCK, TPCK and Z-FF-FMK inhibit Aβ42 induced nuclear deformation

In this experiment, at first, we observed nuclear structural changes to examine the effect of Aβ42 on lamin. Nuclear abnormalities are most likely caused by changes in LA and LB protein level. As a result, we used Hoechst staining and immunofluorescence microscope for LA/C and LB to examine the nuclear integrity of the 20 μM Aβ42 induced sample. Our results showed the significant frequency of lamin deformation in Aβ42 treated cells. LA/C and LB antibodies were used to identify changes in the lamin epitope as observed in nuclear deformation. Deformation of the nuclear envelope in Aβ42 treated cells was previously documented [69]. So, our finding justifies the previous report. Next, we examined nuclear changes of Aβ42 treated cells in the presence of TLCK, TPCK and Z-FF-FMK. The nuclear deformation in the level of LA and LB were dramatically inhibited in samples treated with Aβ42 and TLCK/TPCK/Z-FF-FMK as shown in representative images (Fig. 3). Taken together, our finding indicated that TLCK, TPCK and Z-FF-FMK were able to prevent Aβ42 induced lamin deformation.

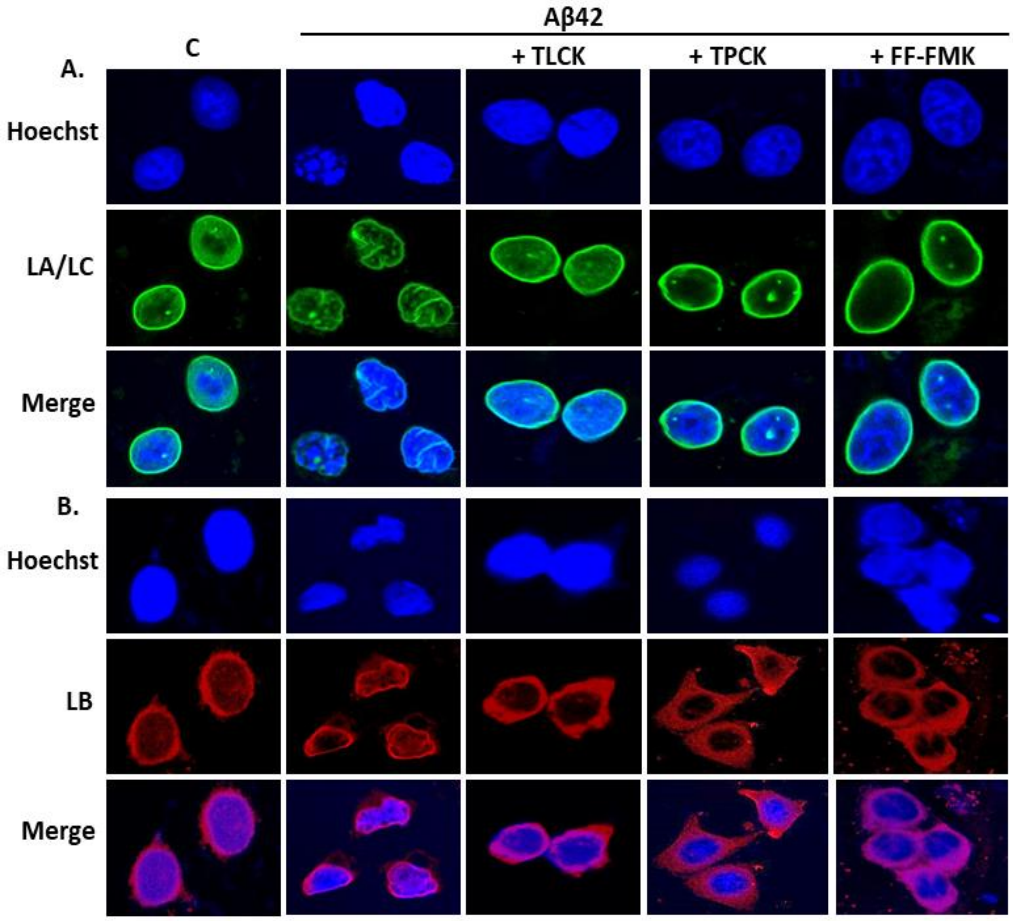

Figure 3. Suppression of Aβ42 induced nuclear deformation by TLCK, TPCK & Z-FF-FMK respectively. (A & B) Hela cells were treated with 20 μM Aβ42 for 2+22 h in the presence or absence of 50 μM TLCK, TPCK and Z-FF-FMK to capture the confocal images.1 h pre-treatment was followed in case of inhibitor and Aβ42 group. LA/C (green) was detected using primary mouse LA/C antibody and secondary anti-mouse IgG-FITC antibody. (B) For the detection of LB (red), primary rabbit LB antibody and secondary anti-rabbit IgG-TRITC antibody was applied. Hoechst was used to stain nuclei. Images shown here are only representative images.

III-1-4. TLCK & TPCK sensitive proteases regulate C-L activity in Aβ-42 treated cells

From figure 2(F), it was known that cathepsin L (C-L) caused LA and LB cleavage in Aβ-42 treated cells but question came how TLCK & TPCK inhibited lamin fragmentation. To solve this issue, in this study, we speculated a hypothesis and proved the connection of serine proteases and lamin fragmentation. At first, we measured the C-L activity in Aβ42 treated cells and our results demonstrated that Aβ42 increased C-L activity in dose dependent manner (Fig. 4A). Z-FF-FMK inhibited Aβ42 induced increase in C-L activity (Fig. 4B) indicating role of C-L in Aβ42 treated sample. However, in the presence of TLCK and TPCK, C-L activity was abrogated in Aβ42 treated cells (Fig. 4C) suggesting the influence of those two-serine protease inhibitors on C-L. So, this finding proved that C-L activity was controlled by TLCK and TPCK sensitive serine proteases. So, we inferred that C-L is responsible Aβ42 induced lamin cleavage and involvement of serine proteases could be upstream cellular event of this pathway.

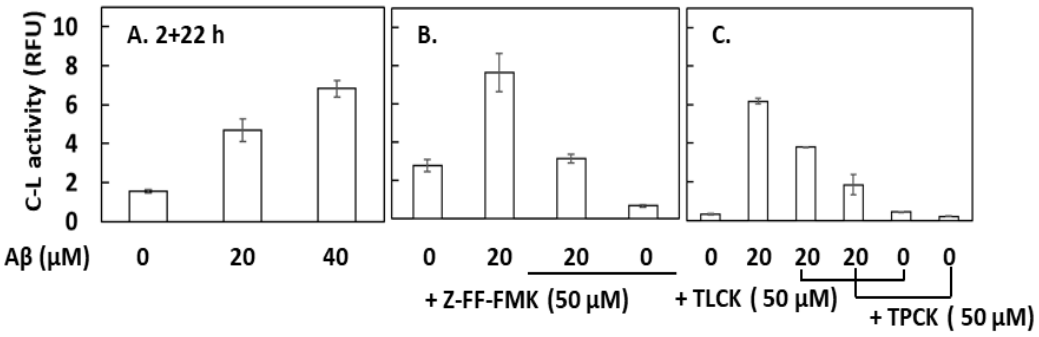

Figure 4. Effect of Z-FF-FMK, TLCK and TPCK on cathepsin L(C-L) activity in Aβ42 treated cells. (A) Hela cells (2 X10^4) were treated using Aβ42 at the mentioned concentrations and time. After treatment, cells were lysed and 10 μM cathepsin L substrate(Z-F-R-AMC) was added to measure C-L activity. Aβ42 significantly increased the C-L activity. (B & C) C-L activity was measured after treating Hela cells using Aβ42 in the absence or presence of Z-FF-FMK, TLCK or TPCK at the indicated concentration for 2+22 h. Inhibitors were added 1 h before the Aβ42 treatment. Z-FF-FMK, TLCK and TPCK markedly reduced C-L activity. Results are average of triplicate and expressed as mean ± standard deviation (SD).

III-1-5. Impact of TLCK, TPCK and Z-FF-FMK on Aβ-42 induced cell death

The effect of TLCK, TPCK and Z-FF-FMK identified by protease inhibitor screening method as inhibitors of lamin cleavage was re-examined on the Aβ42 induced cell death. We used MTT and alamar Blue assay to check the protective effect of TLCK, TPCK and Z-FF-FMK in Aβ42 treated cells. Viable cells were measured after double treatment of Aβ42 followed by 1 h pretreatment of inhibitors. It is worth mentioning that AAPF-CMK was used as positive control since previous published article reported the shielding effect of this serine protease inhibitor in Aβ42 treated cells (Fig. 5A) [69]. In our study, Aβ42 treated cells showed less than 50% cell viability which was increased to more than 80% upon adding TLCK and Z-FF-FMK (Fig. 5A & C). In contrast, TPCK attenuated cell viability in Aβ42 induced cell death although TPCK prevented Aβ42 mediated lamin fragmentation. Unexpectedly, TPCK itself induced cell toxicity (Fig. 5B). So, our results depicted that TLCK and Z-FF-FMK had protective effect against Aβ42 induced cell death

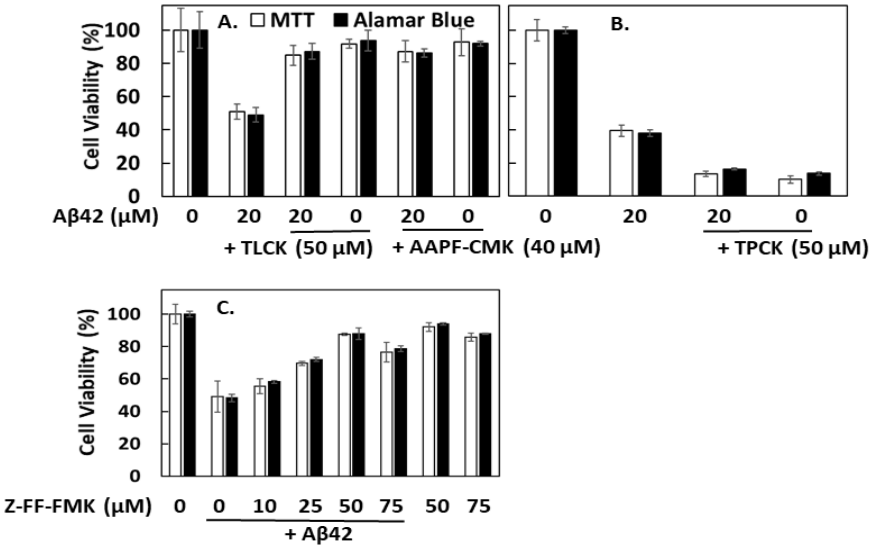

Figure 5. Influence of TLCK, TPCK & Z-FF-FMK in Aβ42 induced cell death. (A-B) Hela cells (1.5 X 10^4) were medicated with Aβ42 in the absence or presence of TLCK, AAPF-CMK & TPCK inhibitors at the concentrations for 2+22 h. AAPF-CMK was used as positive control as it has protective effect against Aβ42 mediated cell death which has been proved from previous publication of our lab. 1hr pretreatment of the cells with inhibitors was maintained in all Aβ42 and inhibitors treated groups. (C) Hela cells (1.5 X 10^4) were approached with Aβ42 in the absence or presence of C-L inhibitor(Z-FF-FMK) for 2+22 h. MTT reduction and alamar Blue assay were applied to get the cell viability data. Three individual experiments were carried out to get the mean value.

III-1-6. Lamin fragmentation and a rise in C-L are observed in the 3xTg (mutant of APP, presenilin1 and tau gene) model of AD

In in vitro Aβ42 toxicity model, we observed C-L mediated LA and LB cleavage. In this study, we used 8-month-old triple transgenic (3xTg) mice having increased Aβ42 deposition to check the status of lamin in the brain tissues of those mice. After preparing cell lysate of brain tissues, we determined the nuclear lamin integrity by using western blotting and confirmed 46 and 21 kDa fragments for LA and LB respectively in 3xTg mice (Fig. 6A). Unexpectedly, LC band was missing both in control and 3xTg mice and the reason behind it was unknown. This could be the future research interest to provide more clues in AD pathology. Previous study showed the involvement of LB in AD pathology suggesting that neurodegenerative laminopathy was acquired in AD [64,123]. In addition, researcher discovered tau induced laminopathy in Drosophila model [64]. so, our results are justified. Lysosomal dysfunction and lysosomal enzymes are known to have a role in AD related neuronal cell death [124–126].Following increased C-L activity in our Aβ42 toxicity study (Fig. 4), we measured C-L activity in brain tissues of 3xTg mice. Our results revealed that C-L activity rose significantly in transgenic mice samples compared to control (Fig. 6B).

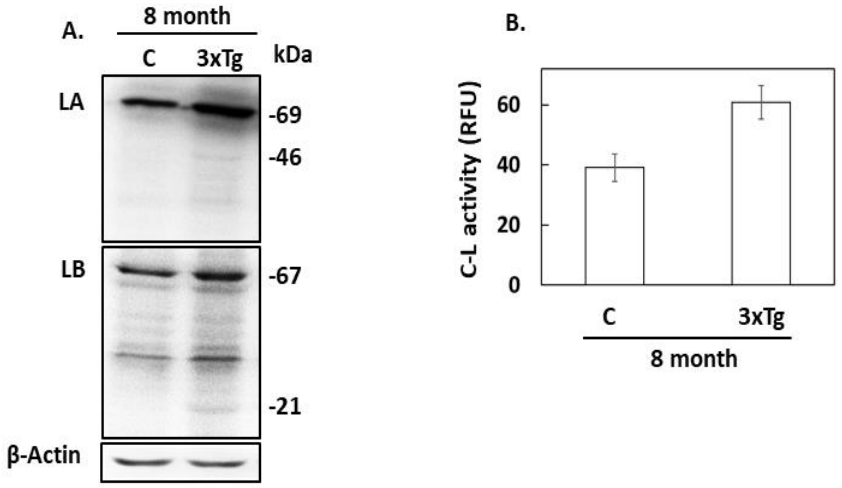

Figure 6. Lamin cleavage and cathepsin L activity in 3xTg mouse brain tissue. For this experiment, 8-month 3xTg mice were used. (A) lysates of brain tissue were prepared and lamin fragments (46 kDa for lamin A and 21 kDa for lamin B) were observed by western blotting. Both in control and 3xTg mice, lamin C were not found and the reason for this is not understood. (B) Cathepsin L activity of control and 3xTg mice brain were measured by Z-F-R-AMC substrate. Results are expressed as mean ± SD. SD and RFU indicate Standard Deviation and Relative Fluorescence Unit respectively.

III-1-7. TLCK and TPCK are potent inhibitors of Aβ42 induced caspase activation

It was observed previously that double treatment of the Aβ42 could lead to robust caspase activation [116]. Other researchers reported that twice treatment was important for Aβ42 mediated cell death [127,128]. Our current study also supports the robust caspase activity of Aβ42 treated cells. Initially, we checked the role of TLCK and TPCK in suppressing lamin cleavage but in this experiment, we examined their preventive effect on Aβ42 induced caspase activity. Aβ42 induced sample incubated for 2+22 h showed strong caspase-3 like DEVDase activity which was reduced in the presence of TLCK and TPCK (Fig. 7A). TLCK attenuated caspase-3 activity significantly whereas TPCK completely removed the Aβ42 induced DEVDase activity. This experiment showed that TLCK and TPCK itself could not activate the caspases in healthy Hela cells. In case of caspase-6 like VEIDase activity, TLCK reduced more caspase-6 activity compared to TPCK in Aβ42 treated cells (Fig. 7B). Moreover, both TLCK and TPCK decreased similar pattern of caspase-8(IETDase) and caspase-9 (LEHDase) activity in Aβ42 treated cells (Fig. 7C & D). These results solidly suggested that both TLCK and TPCK had inhibitory effects on Aβ42 induced caspase activity and caspase activation is later stage event in Aβ42 treated sample.

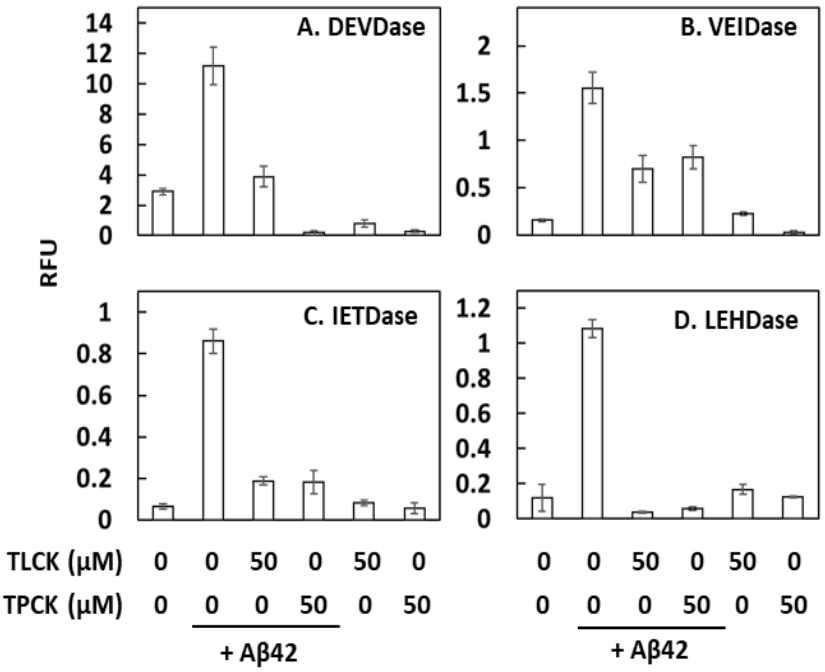

Figure 7. Effect of TLCK & TPCK on caspase processing in Aβ42 treated Hela cells. (A-D) Cells (2X10^4) were treated with 20 μM Aβ42 for 2+22 h. Inhibitors (TLCK & TPCK) were added before 1 h of Aβ42 treatment at indicated concentrations. After treatment, processing of different caspases (-3, -6, -8, -9) was evaluated using their respective substrates (10 μM DEVD-AMC, 50 μM VEID-AMC, 50 μM IETD-AMC & 50 μM LEHD-AMC). Results are mean of three individual experiments and expressed as mean ± SD.

III-1-8. Discussion

LA/C and LB are the major structural component of nuclear scaffold (NS) and NS supports the nuclear envelope [129]. Nuclear lamin fragmentation and deformation are common phenomena in dying cells [130,131]. Previous study showed that LA and LB reduction as well as cleavage in oligomeric Aβ42 treated cells were independent of caspase activation but protease (s) involved in the process have not characterized yet [69]. In the present study, we have recognized that C-L mediates lamin cleavage in Aβ42 induced cytotoxicity (Fig. 2F). We also observed that C-L is also associated with significant nuclear structural changes in Aβ42 treated cells (Fig. 3). Therefore, C-L inhibitor prevented Aβ42 induced lamin fragmentation and deformation suggesting promising role of C-L in lamin damage (Fig. 2F & 3). Surprisingly, Aβ42 induced LA and LB cleavage and increased C-L activity were also observed in 3xTg mice (Fig. 6). Therefore, the 3xTg mouse model of AD provided more evidence for C-L involvement in nuclear alterations. C-L's relevance has been demonstrated in a range of biological processes, including cell division and cell death [132]. In our observation, Z-FF-FMK also significantly reduced cell death providing evidence the role of C-L in Aβ42 induced cell death (Fig. 5C). Previous study reported that C-L regulates cell proliferation in NIH3T3 cells via processing the CDP/Cux transcription factor and is targeted to nuclear compartment [132]. Moreover, inhibiting C-L has been proven to stop cancer cells from proliferating [133]. Another example of C-L nuclear activity has been demonstrated in the differentiation of mouse embryonic stem cells, which is regulated by histone H3 cleavage [134]. C-L activation has also been associated to DNA repair machinery degradation, including 53BP1, pRb, and p107 [135,136].One study showed that effect of Aβ on C-L is upstream event

in the neurodegenerative process [137].C-L's ability to degrade cathepsin D, which is an activator of caspase-3 mediated apoptosis, has previously been shown to have an anti-apoptotic role [138]. Therefore, the role of C-L as a mediator of Aβ42 induced lamin damage and cytotoxicity found in our study is supported by available literature. Eventfully, TLCK and TPCK also completely inhibited Aβ42 induced lamin fragmentation and both of them are serine proteases inhibitors (Fig. 2D & E). From literature review we found that TLCK and TPCK prevented chromatin degradation [139]. Previous study demonstrated that TLCK & TPCK blocked apoptosis through inhibiting caspases and cytochrome c release [140,141]. TLCK and TPCK have hydrophobic region and they interact with other proteins so easily [142,143]. Previous published article also showed that the serine protease inhibitor, TPCK and NS protease specific inhibitor, AAPF-CMK blocked the lamin fragmentation in isolated nuclei. The size of lamin fragments was similar to the lamin cleavage produced by Aβ42 treated cells. They also showed that both compounds (TPCK & AAPF-CMK) have phenylalanine residues which indicate identical inhibitor selectivity [144]. Our finding proved that TLCK and TPCK reduced C-L activity significantly in Aβ42 treated cells demonstrating regulatory effect of those compounds on C-L (Fig. 4C). It is worthy to mention that C-L activity was increased in Aβ42 treated cells which was decreased in the presence of Z-FF-FMK (Fig. 4). So, based on our results we propose that TLCK and TPCK sensitive proteases work as upstream event of C-L activation and thus inhibit Aβ42 induced lamin fragmentation. Previous study also showed the protective effect of TLCK against colchicine induced neuronal cell death [145]. In this investigation, we examined the effect of TLCK and TPCK on Aβ42 induced cell death. Although TLCK prevented the Aβ42 induced cell

death but TPCK failed to do it because the concentration of TPCK used to block Aβ42 induced lamin fragmentation was toxic itself whereas TLCK did not show remarkable toxicity (Fig. 5A & B). So, TPCK has ability to prevent lamin cleavage but it itself induces cell death in different pathway. This needs to be studied in detail. Our results also revealed that both TLCK and TPCK suppressed the caspase-3,6,8,9 activities in Aβ42 treated cells very effectively (Fig. 7), diminished caspase activity could be due to impairing caspase processing or inhibition of mature caspases. Previous report showed that TLCK and TPCK were potent inhibitors of caspases [146]. So, our results are consistent with the previous study. Therefore, it can be concluded that TLCK and TPCK modulate C-L mediated Aβ42 induced lamin fragmentation and activation of caspases is later stage event in this regard. This investigation was performed in cell lines and reproducibility of this finding needs to be justified in animal model as well.

III-2. Involvement of calcium (Ca^{2+}) in Aβ42 induced lamin fragmentation

One of the aims for AD treatment is the suppression of Aβ42 induced cell damage. The nature of the toxic form of Aβ42 involved in AD pathology is a controversial issue. Aβ42 have three forms and those are monomer, oligomer and fibrillar. Oligomer Aβ42 rather than monomer and fibrillar are thought to mediate neurotoxicity in AD [147,148]. Oligomer Aβ42 triggers molecular events to cause neuronal cell damage and one of them is disruption of calcium homeostasis which may have contributed to the pathological effect [83]. Moreover, oligomer Aβ42 produces Aβ42 specific lamin fragmentation and in our study, we proved that cathepsin L is responsible for this lamin cleavage (Fig. 2F). From the literature review, we came to know that the release of cathepsin from lysosome is calcium-dependent [149]. In the current study, we have correlated the two molecular events (one is lamin fragmentation and another is calcium dysregulation) induced by oligomer Aβ42.

III-2-1. Aβ42 induces intracellular Ca^{2+} increase to mediate cell death

We started this study with neuroblastoma (SHSY5Y) cells but due to unstable apoptotic events like lamin fragmentation, caspase activity (data not shown), we designed our experiments with Hela cells. Calcium (Ca^{2+}) imaging experiment was performed to measure the intracellular Ca^{2+} induced by Aβ42. We used oligomer Aβ42 for this investigation because other forms of Aβ42 did not induce lamin cleavage, proved in the previous study [121] and oligomer Aβ42 interferes with calcium homeostasis [83]. Therefore, a correlation between calcium dysregulation and lamin fragmentation has been observed in this study. Here, a calcium-sensitive dye (Fluo-8-AM) was applied to measure

the changes in intracellular calcium induced by Aβ42 and the image was viewed with a confocal microscope system. Hela cells treated with Aβ42 for 24 h did not show any change in intracellular calcium level as compared to control. Next, we followed a double treatment procedure and cells treated for 2+22 h elevated cytosolic Ca^{2+} significantly compared to control and 24 h treatment period (Fig.8A). So, our images showed that double treatment was important for the significant uptake of Ca^{2+} by Fluo-8-AM and increased intracellular Ca^{2+}.

In our subsequent experiment, we looked for a relation between Aβ42 induced Ca^{2+} mobilization and cell death. An MTT assay for cell viability showed that Aβ42 at a concentration of 20 μM caused ~ 50% cell death for 2+22 h in Hela cells. To evaluate the contribution of increased intracellular Ca^{2+} in Aβ42 induced cell death, we pretreated Hela cells with cell-permeable Ca^{2+} chelator 1,2-Bis (2-amino phenoxy) ethane-N, N, N′, N'- tetraacetic acid tetrakis (acetoxymethyl ester) (BAPTA-AM) followed by Aβ42 treatment. The pretreatment of Hela cells with BAPTA-AM prevented Aβ42 induced cell death in a dose-dependent manner. BAPTA-AM at a concentration of 25 μM increased the viability of cells remarkably in the Aβ42 treated cells. More than 80% of viable cells were observed at 25 μM BAPTA-AM pre-treated cells followed by Aβ42 for 2+22 h, indicating the role of Ca^{2+} in Aβ42 induced cell death process. Besides, BAPTA-AM alone at a concentration of 50μM produced negligible cell death, providing its safe index (Fig. 8B). We obtained similar type of cell viability data using alamar Blue assay. Therefore, our results confirmed that Aβ42 induced Ca^{2+} rise was one of the molecular events in mediating cell death.

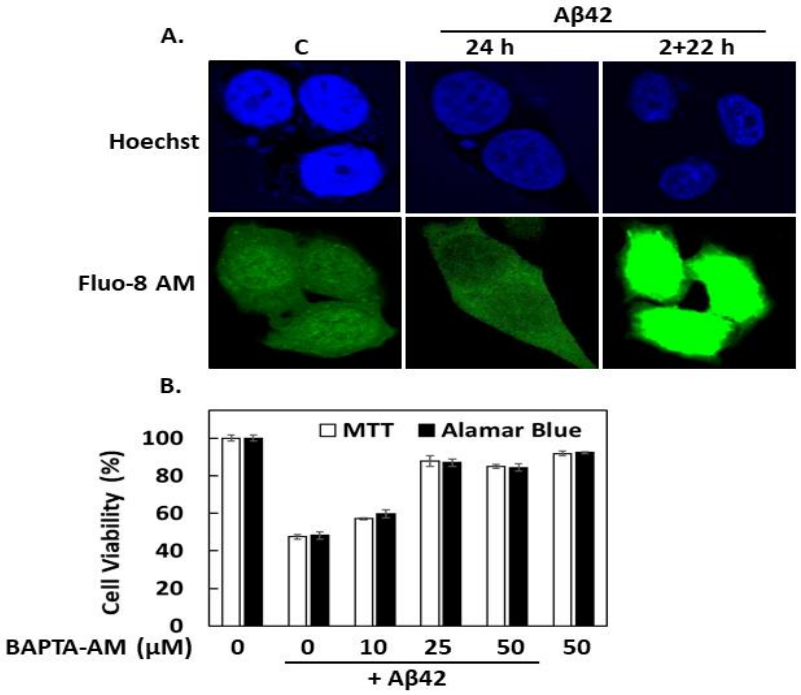

Figure 8. Relationship between Aβ42 induced cell death and calcium increase. (A) Hela cells (1 X 10^5) were treated with Aβ42 for 24 and 2+22 h respectively. After treatment, fluorescent images of Fluo-8/AM loaded cells (control & treated) were taken. (B) Cell viabilities were evaluated by MTT method and alamar Blue assay in the cells treated with Aβ42 in the absence or presence of indicated concentrations of BAPTA-AM for 2+22 h. BAPTA-AM was added 1h before treating the Hela cells with Aβ42. Three individual experiments were carried out to get mean ± SD.

III-2-2. Ca^{2+} chelator inhibits Aβ42 induced lamin fragmentation

In this experiment, administration of Aβ42 resulted in the appearance of 46 and 21 kDa LA & LB fragments respectively. we examined the effect of BAPTA-AM on Aβ42 induced lamin cleavage and found that pretreatment of Hela cells followed by Aβ42 prevented lamin fragments events effectively (Fig.9A). We used 25 μM BAPTA-AM for this and other experiments because this concentration was most effective to prevent Aβ42 induced cell death. BAPTA-AM concentration above 25 μM did not increase cell viability significantly (Fig. 8B). 25 μM BAPTA-AM alone neither reduced nor generated fragments of lamin proving no impact of it on lamin protein. It should be mentioned here that lamin C was also reduced in Aβ42 treated cells and this type of reduction was removed by BAPTA-AM. However, the effect of Aβ42 on lamin C reduction is uncertain, so it is not discussed further and the 46 kDa fragment in Aβ42 treated cells is a cleave product of lamin A. These data suggested that intracellular calcium is playing a role to cause lamin cleavage induced by Aβ42. Nuclear abnormalities may be linked to changes in LA and LB levels. We used Hoechst staining, LA and LB antibodies to examine the nuclear integrity of the cells by confocal microscope in the Aβ42 treated cells as well as in other experimental groups. Our representative images showed clear nuclear deformation in Aβ42 treated cells. We then examined whether pretreatment of Hela cells with BAPTA-AM prevented lamin invagination or not in Aβ42 treated cells. Our results confirmed that BAPTA-AM effectively prevented Aβ42 induced lamin invagination or deformation (Fig. 9B). we applied 25 μM BAPTA-AM to protect lamin from Aβ42 induced damage. Here, it can be said firmly that cytosolic calcium is an important molecular event in Aβ42 induced lamin cleavage and nuclear deformation.

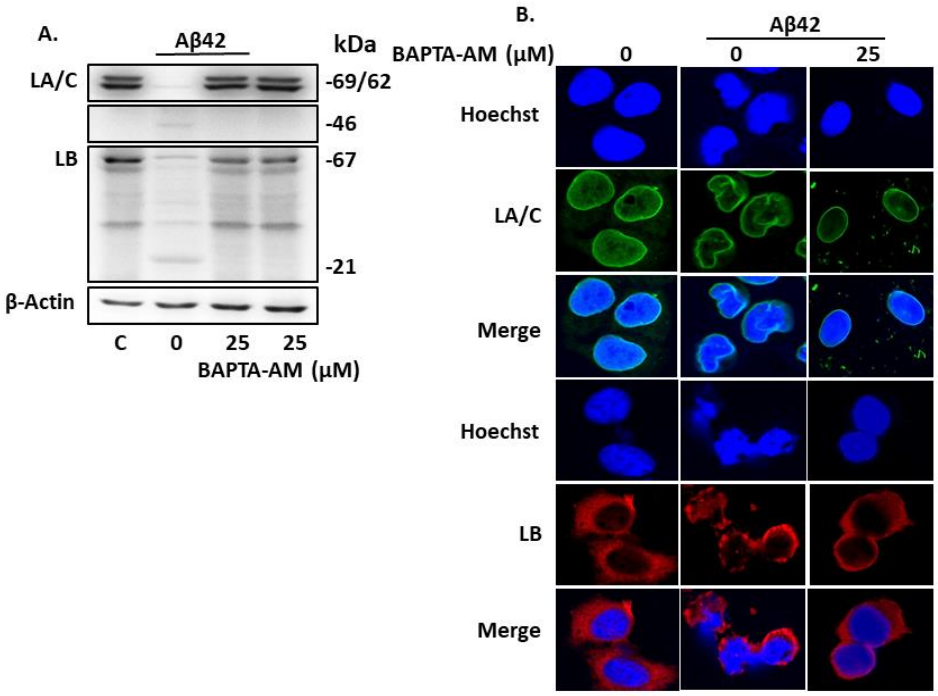

Figure 9. Effect of calcium on Aβ42 induced lamin cleavage. (A) Hela cells (4 X 10^5) were subjected to 20 μM Aβ42 treatment with or without BAPTA-AM at the indicated dose for 2+22 h. For inhibitor & Aβ42 group,1 h pretreatment was maintained. Western immunoblot analysis was used to monitor cleaved fragments. All data are representative of three single experiments. (B) Suppression of lamin deformation was observed by confocal images after treating Hela cells two times with 20 μM Aβ42 in the presence or absence of BAPTA-AM at the indicated concentrations for 2+22 h. Mouse anti-LA/C and rabbit anti-LB antibodies were used to detect LA/C(green) and LB (red) which were projected using secondary anti mouse Ig-G-FITC and anti-rabbit Ig-G-TRITC antibodies respectively. To stain nuclei, Hoechst (blue) was employed.

III-2-3. Ca^{2+} are released from Endoplasmic Reticulum (ER) in Aβ42 treated cells and contribute to mediating lamin cleavage

Prevention of Aβ42 induced lamin cleavage by BAPTA-AM proved the role of Ca^{2+} in mediating lamin cleavage. Therefore, we focused on the source of intracellular Ca^{2+} release. ER is the main component of intracellular calcium storage. A study showed that calcium was released from ER in Aβ42 treated cells [150]. Therefore, we tested the participation of ER to upload cytosolic calcium in Aβ42 treated samples. For our experiment, we used cyclopiazonic acid (CPA), a sarcoplasmic Ca^{2+}-ATPase inhibitor that prevented Ca^{2+} mobilization from ER. In the beginning, we evaluated whether blocking of ER-based Ca^{2+} mobilization by CPA affected Aβ42 induced cell death. Cell viability was assessed by the MTT reduction and alamar Blue method. Hela cells were treated with different concentrations of CPA in the presence or absence of 20 μM Aβ42. Treatment with 20 μM CPA significantly decreased Aβ42 induced cell death whereas other doses used here were not that effective. It was noted that CPA itself at the concentration of 10-40 μM was not toxic and had negligible contribution in cell death (Fig.10A). So, in our subsequent experiment, we used 20 μM CPA concentration. We here concluded that ER Ca^{2+} was involved in the cell death process in Aβ42 treated samples.

We then investigated the effect of CPA on Aβ42 induced lamin protein damage. Our results showed that 20 μM CPA completely blocked Aβ42 induced LA and LB cleavage compared to control. CPA alone did not produce any lamin fragmentation (Fig. 10B). So, we presumed that Ca^{2+} was released from ER Ca^{2+} store to mediate lamin cleavage in Aβ42 treated cells.

Finally, we checked the effect of CPA on Aβ42 induced apoptosis. In Hela cells, application of Aβ42 caused an increase of more than 2-fold caspase-3 activity compared to control. However, pre-treatment of cells with CPA reduced caspase-3 activity indicating the significance of ER Ca^{2+} release in Aβ42 induced apoptosis. It was mentioned that CPA itself did not affect apoptosis (Fig. 10C).

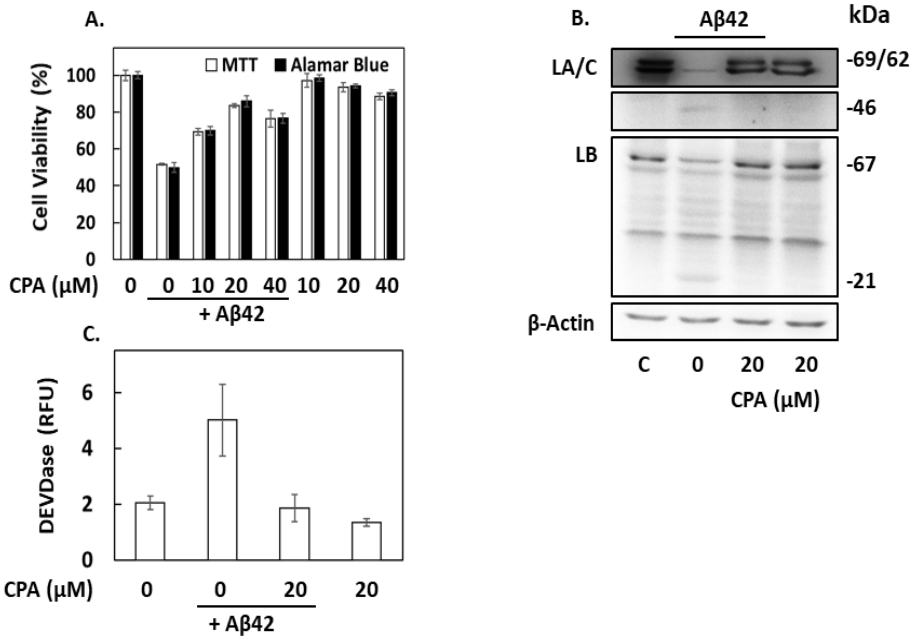

Figure 10. Aβ42 induced lamin fragmentation is mediated by mobilization of Endoplasmic reticulum (ER) calcium. (A) Hela cells were treated with 20 μM Aβ42 in the absence or presence of cyclopiazonic acid (CPA) at the indicated concentrations for 2+22 h. 1hr pretreatment was followed by Aβ and CPA group. After treatment, cell viability was measured by MTT reduction method and Alamar Blue assay. (B) Lamin fragmentations of LA/C and LB were checked after treating Hela cells with Aβ42 with or without CPA. Western blot was used to examine lamin bands. (C) (2 X 10^4) Hela cells were taken to measure caspase-3 activity of untreated, Aβ42 treated and Aβ42 + CPA treated groups. DEVD-AMC was used as substrate to determine the activity. There independent experiments were performed to get average. Results are expressed as mean ± SD. Here, CPA prevents mobilization of calcium from ER.

III-2-4. The implication of Ca^{2+} in inducing lamin fragmentation is specific to Aβ42 treated cells

To examine whether Ca^{2+} plays role in mediating lamin cleavage in different cell death-inducing agents, we observed lamin cleavage in the presence of BAPTA-AM. Staurosporine (protein kinase inhibitors), etoposide (a topoisomerase II inhibitor), TNF-α (a ligand of TNFR), brefeldin A and thapsigargin (endoplasmic reticulum-associated damage) were used as cell-damaging agents. We detected 28 and 46 kDa fragments from LA and LB respectively in our investigated cell death agents indicating fragmentation of lamin was caspase-6 mediated. Cells were also treated with 25 μM BAPTA-AM in the presence of different cell death-inducing agents and observed caspase-6 mediated lamin cleavage. 46 kDa LB fragment by etoposide was increased in the presence of BAPTA-AM whereas no remarkable change of fragments by other cell death-inducing agents was noticed (Fig.11A & B). So, the effect of BAPTA-AM in blocking lamin cleavage is Aβ42 specific.

We then focused on the cell death induced by those damaging agents in the presence or absence of BAPTA-AM, and cell viability was checked by MTT assay. STS and TNF-α showed in average 62 % and 86 % cell death respectively. This cell death percentage was almost similar in the presence of BAPTA-AM meaning no effect of BAPTA-AM on STS and TNF-α induced cell death. However, BAPTA-AM increased cell death in the case of etoposide, brefeldin, and thapsigargin treated cells (Fig. 11C). Therefore, our cell viability results proved that BAPTA-AM did not inhibit cell death induced by damaging agents.

Figure-11(A & B) showed that our examined damaging agents induced lamin cleavage was caspase-mediated. We explored the effect of BAPTA-AM on caspase-3 activity induced by cell death-inducing agents, our results revealed that caspase-3 activity was increased in the case of STS, etoposide and thapsigargin treated groups in the presence of BAPTA-AM indicating no effect of Ca^{2+} chelator in reducing caspase-3 activity (Fig. 11D). Although we noticed a slight reduction of caspase activity by BAPTA-AM in respect of TNF-α and brefeldin A it had no impact on removing lamin fragments. So, we were not further interested to find out the reason for negligible caspase activity reduction.

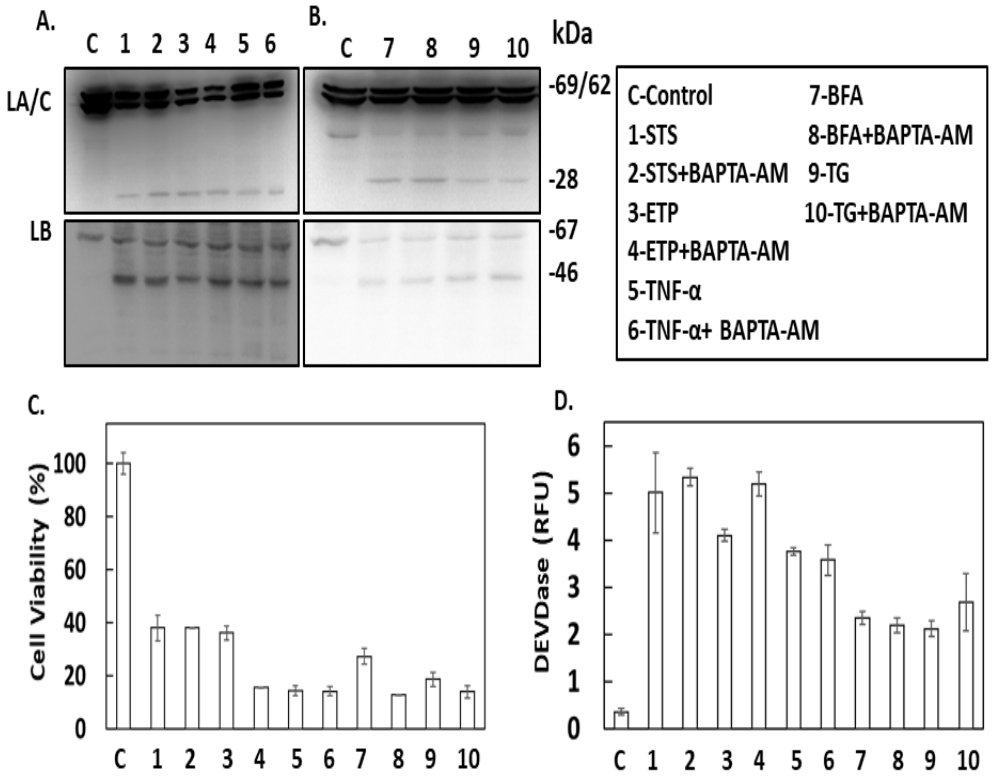

Figure 11. Lamin fragmentation with calcium is specific to Aβ42 treated cells. (A & B) Hela cells were treated with different cell death inducing agents such as Staurosporine (STS), Etoposide (ETP), Tumor Necrosis Factor-α (TNF-α), Brefeldin A (BFA), Thapsigargin (TG) in the presence or absence of 25 μM BAPTA-AM. In case of STS, 6h treatment period was followed, in other cell damaging agents, it was 24 h. After preparing cell lysates, generation of lamin fragments were visualized by performing western blotting. β-actin was not used as loading control because lamin fragments were observed in each cell death inducing group as well as inhibitor and cell damaging group (C) The rates

of cell death were examined by an MTT reduction method in cells treated with different cell death inducing agents as indicated. (D) Caspase-3 activity was assessed with 10 μM DEVD-AMC in Hela cells treated with cell death inducing agents as mentioned in figure. Data represents mean ± SD from 3 individual experiments.

III-2-5. BAPTA-AM regulates Aβ42 induced cathepsin L activity as well as the expression

Our protease inhibitor screening experiments showed that Z-FF-FMK inhibited Aβ42 induced lamin fragmentation (Fig. 2F). This finding indicated that cathepsin L was the main player in cleaving LA & LB in Aβ42 induced lamin damage. We measured cathepsin L activity in Aβ42 treated cells and found in average 3 times more increase of fluorescence value than control. We also applied BAPTA-AM in Aβ42 treated cells and quantified cathepsin L activity in the same way. Our investigation showed a significant reduction of cathepsin L activity in the BAPTA-AM and Aβ42 treated group. BAPTA-AM itself was used as a control and produced no remarkable cathepsin L activity (Fig. 12A). In parallel with the cathepsin L activity as observed in 20 μM Aβ42 treated cells, we also found an increase in cathepsin L expression by western blotting. Following Aβ42 exposure of Hela cells, cathepsin L precursor (40 kDa) and processed cathepsin L (27 kDa) increased significantly. In contrast, cathepsin L activity was decreased in the presence of BAPTA-AM in Aβ42 treated cells compared to only Aβ42 treated sample (Fig. 12B). Taken together, these results suggested that cytosolic Ca^{2+} modulated cathepsin L activity induced by Aβ42.

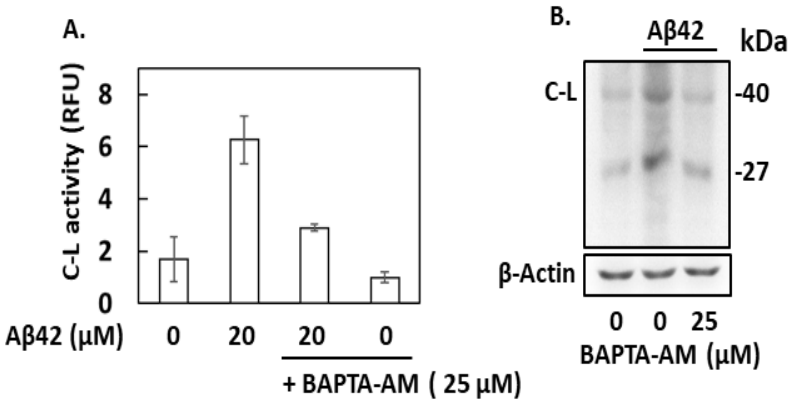

Figure 12. Increased intracellular calcium is upstream event in cathepsin L mediated Lamin cleavage induced by Aβ42. (A) Hela cells were pretreated with BAPTA-AM for 1 h and treated with Aβ42 for 2+22 h. After treatment, C-L activity was assessed by monitoring cleavage of fluorogenic substrate Z-F-R-AMC (10 μM). (B) Hela cells were treated with 20 μM Aβ42 in the presence or absence of BAPTA-AM at the indicated concentration for 2+22 h and the cell lysates were subjected to western blotting to evaluate the expression of C-L. 40 & 27 kDa upper and lower band indicate precursor and processor form of C-L respectively.

III-2-6. Z-FF-FMK suppresses Ca^{2+}induced lamin cleavage in isolated nuclei

Previous studies showed that Ca^{2+} promoted fragmentation of lamin in isolated nuclei and Ca^{2+} dependent proteases were involved [151]. The estimated size of the cleaved product induced by Ca^{2+} was equal to those observed in our previous study [69]. We used 2.5 mM calcium chloride ($CaCl_2$) for the current study following our previous report [69]. Incubation of isolated nuclei in the presence of $CaCl_2$ produced ~46- and ~21 kDa fragments from LA and LB respectively. Our inhibitor study proved that Z-FF-FMK blocked Aβ42 induced lamin cleavage and Ca^{2+} was an upstream event in mediating this fragmentation. To confirm the participation of Ca^{2+} in mediating lamin cleavage, we used different concentrations of Z-FF-FMK to observe whether cathepsin L inhibitor blocks lamin cleavage in isolated nuclei. Lamin fragmentation induced by the addition of Ca^{2+} was suppressed by Z-FF-FMK at 30 μM concentration (Fig.13A) confirming the participation of Ca^{2+}-dependent cathepsin L protease in the lamin fragmentation process. Other randomly selected protease inhibitors such as pepstatin (cathepsin D inhibitor), aprotinin (serine protease inhibitor), Z-FA (cathepsin B inhibitor), and MG-132 (a proteasome inhibitor) did not inhibit Ca^{2+} induced LA and LB cleavages in isolated nuclei (Fig. 13B) indicating corresponding proteases were not involved in lamin fragmentation. So, our nuclei results justified that Ca^{2+} plays role in mediating lamin cleavage through activation of Ca^{2+} dependent cathepsin L.

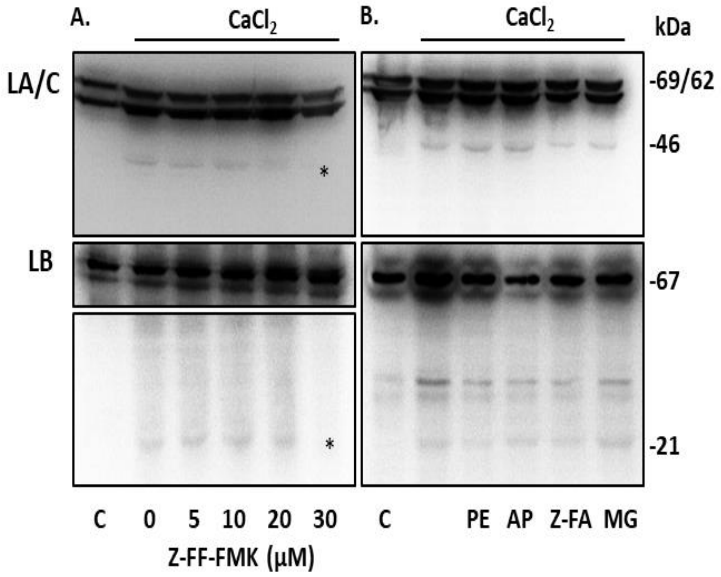

Figure 13. Effect of Z-FF-FMK as well as various protease inhibitors on calcium induced lamin cleavage in isolated Hela nuclei. (A) Nuclei were isolated from harvested Hela cells and effect of Z-FF-FMK at the indicated concentrations was observed on 2.5 mM calcium induced lamin fragmentation in isolated nuclei following experimental conditions. (B) Isolated nuclei from cells were treated for 1 h with 2.5 mM $CaCl_2$ in the presence or absence of indicated protease inhibitors. Inhibitor concentration as mentioned in figure-2 was also applied here. To check lamin fragments, western blotting was performed.

III-2-7. Aβ42 induced caspases activation involves cathepsin L

Double treatment of the Aβ42 could lead to a higher level of caspase activation [116]. Others have claimed that nucleation-dependent polymerization was required for Aβ42 mediated cell death in the doubly treated cells [127,128]. As a result, we predicted that the polymerization process in the twice-treated samples would offer a greater signal for caspase activation. Aβ42 double treatment-induced lamin fragmentation in our study and this cleavage was mediated by cathepsin L (Fig. 2F). Our inhibitor study also showed that all caspase inhibitors (Z-VAD) had no effect to prevent Aβ42 induced lamin cleavage (Fig. 2B). Therefore, lamin fragmentation in Aβ42 treated cells is caspase-independent. We used the same treatment condition to check caspase activity and found that 20 μM doubly treated Aβ42 produced robust caspase activation. We hypothesize that cathepsin L mediated lamin fragmentation is an early event in the apoptotic process and caspase is activated later. We also speculate that cathepsin L plays role in controlling caspase activity. 20 μM Aβ42 showed on average 2 times more caspase-3 activity than control. We measured the DEVDase activity of Aβ42 treated cells in the presence of Z-FF-FMK and observed a dramatic reduction of caspase-3 activity (close to the control value). It was noted that Z-FF-FMK itself did not produce any caspase-3 like activity (Fig.14A). Next, we were interested in caspase-6 activity since Aβ42 induced lamin cleavage was not mediated by caspase-6 in 2+22 h incubation time. IETDase activity was the highest in the 2+22 h sample and this activity was abolished in the presence of Z-FF-FMK. We observed on average a 3 times reduction of caspase-6 activity than Aβ42 treated cells (Fig.14B). The Aβ42 induced caspase-8 like IETDase activated in the 2+22 h incubation sample. In control, caspase-8 activity was less than 1 RFU and this

was significantly increased to about 3 RFU by Aβ42. Cathepsin L inhibitor reduced Aβ42 induced caspase-8 activity remarkably (Fig.14C). Similarly, we measured caspase-9 like LEHDase activity in the 2+22 h incubated Aβ42 sample and found 2 times more activity than control. In the presence of Aβ42 and Z-FF-FMK, caspase-9 activity was reduced. In the presence of cathepsin L inhibitor alone, caspase-9 activity was like that of control (Fig.14D). So, our results demonstrated that the stimulatory effect of Aβ42 on caspases is blocked by the cathepsin L inhibitor (Z-FF-FMK) suggesting that activation of caspases involves upstream stimulation of cathepsin L.

III-2-8. Aβ42 induces caspases activation upon ER Ca^{2+} release

Our study proved that intracellular calcium release was an upstream event in cathepsin L mediated lamin fragmentation (Fig. 12A &B). In addition, activation of cathepsin L stimulated caspases (Fig. 14A-D). In this investigation, we examined whether BAPTA-AM could regulate caspases activities in Aβ42 treated cells. The activity of initiator caspases (-8, -9) and effector caspases (-3, -6) was measured in Hela cells treated with Aβ42. An increase in caspase-3, -6, -8, -9 activity was detected after Aβ42 exposure. BAPTA-AM alone did not affect caspase activity. Our results showed that BAPTA-AM almost completely inhibited Aβ42 induced caspase -3 and -6 activity. Besides, a significant reduction of caspase -8 and -9 activity was observed in BAPTA-AM followed by Aβ42 treated cells (Fig. 14E-H). So, based on our findings, it can be said that intracellular Ca^{2+} release modulates pan caspases activity in Aβ42 treated cells.

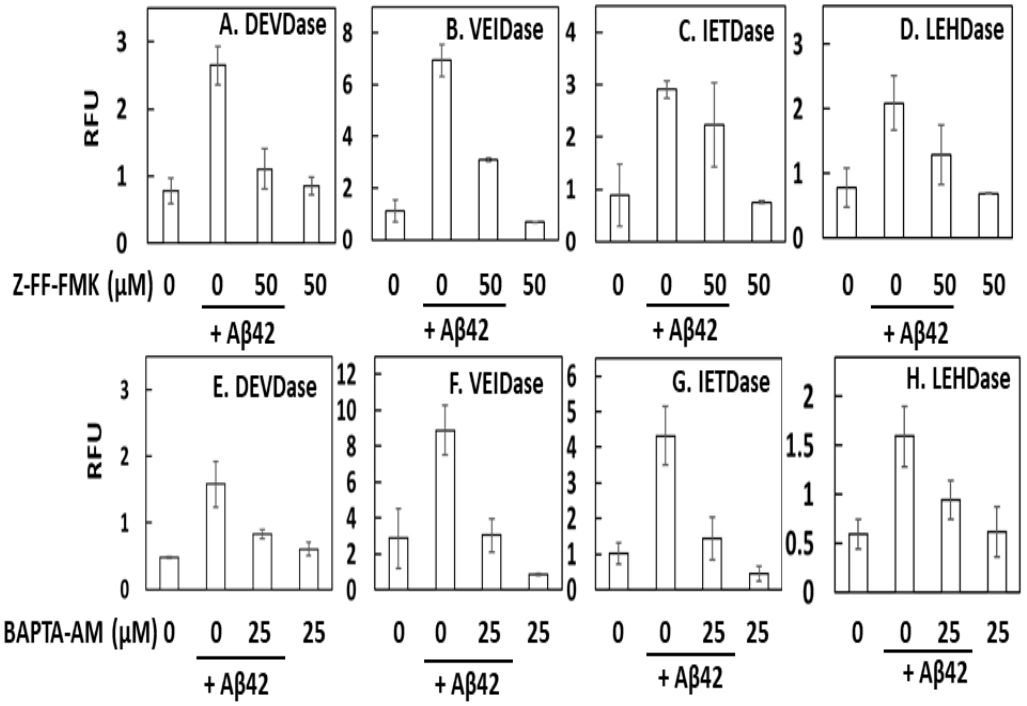

Figure 14. Intracellular calcium increases the caspase activity in Aβ42 treated cells. (A-H) Hela cells (2 X 10^4) were exposed to 20 μM Aβ42 with or without Z-FF-FMK and BAPTA-AM at the indicated concentrations and for 2+22 h. Aβ42 treatment was started 1 h later after adding inhibitors. After treatment, Caspase-3,6,8 & 9 enzymes activities were assessed with 10 μM DEVD-AMC, 50 μM VEID-AMC, 50 μM IETD-AMC and 50 μM LEHD-AMC synthetic substrates respectively. RFU denotes Relative Fluorescence Unit. The results are mean of three equivalent experiments and expressed as mean ± SD.

III-2-9. Discussion

In this present study, we have identified that cytosolic Ca^{2+} rise is the upstream event in Aβ42 induced lamin fragmentation. It was suggested that Ca^{2+} plays a central role in apoptosis and synaptic plasticity. Several studies showed that Ca^{2+} dysregulation had a role in the pathogenesis of Alzheimer's disease [152,153]. Previous study showed that single-treated oligomer Aβ42 induced Ca^{2+} dysregulation in rat cortical neurons [86]. In our investigation, double treatment of oligomer Aβ42 rose cytosolic Ca^{2+} in Hela cells instead of a single treatment (Fig. 8A). So, double treatment is important for Ca^{2+} overload in the cytosol. Moreover, double treatment of Aβ42 demonstrated more cell death and potent caspase activation compared to a single treatment. However, there is a discrepancy between our result and the previous study in terms of Ca^{2+} rise by Aβ42. One potential reason could have differed in experimental conditions. In our case, we used Hela cells while the aforementioned study used rat cortical neurons or brain cells [154]. In addition, Aβ42 concentration and treatment period were also different. In our previous study, we found that a single treatment of Aβ42 did not produce lamin fragmentation and cell death was less. Besides, the study also showed that a single treatment of oligomer Aβ42 was unable to generate caspase activation [69]. Therefore, we chose double treatment because doubly treated Aβ42 produced lamin fragmentation, highest cell death, and strong caspase activation. Our cell viability data exhibited that intracellular Ca^{2+} was a mediator for Aβ42 induced cell death. Ca^{2+} chelator BAPTA-AM at the 25 μM concentration reduced the Aβ42 induced cell death very effectively (Fig. 8B). In a previous study, the cortical neurons were pretreated with BAPTA-AM to minimize Aβ induced cell death suggesting that intracellular Ca^{2+} was one of the mediators

for Aβ induced cell death [154]. BAPTA-AM reduced Aβ induced cell death in this study [154]. Although the experiment used Aβ40 and Aβ25-35 [154], our results are consistent with their result because they evaluated cytosolic Ca^{2+} involvement in cell death. The previous reports showed that double treatment of Aβ42 induced the Aβ42 specific lamin fragmentation pathway [69]. Our finding provided evidence that cathepsin L was involved in lamin cleavage (Fig. 2F). Interestingly, both intracellular Ca^{2+} rise and lamin cleavage molecular events were observed in Aβ42 induced cells. Therefore, we postulated a relation between those two events. Evidence shows that lysosomal cathepsin release is dependent on Ca^{2+} and calpain are Ca^{2+} sensitive proteases [149,155]. It was noted that calpain inhibitor (ALLN) was not effective in hindering Aβ42 induced lamin damage (Fig. 2A). Moreover, cathepsin B inhibitor (Z-FA-FMK) and cathepsin D inhibitor (Pepstatin A) did not inhibit lamin breakdown (Fig. 2A & B). The reason why calpain and others cathepsin proteases are not causing lamin cleavage in Aβ42 treated although they are Ca^{2+} dependent warrant further investigation. So, the involvement of Ca^{2+} dependent cathepsin L release in lamin cleavage is specific for Aβ42 treated cells. Then, we carried out further experiments to prove the involvement of Ca^{2+} in lamin fragmentation. BAPTA-AM was used to block the effect of the Ca^{2+} and the lamin cleavage was observed by western blotting. Intriguingly, BAPTA-AM completely inhibited Aβ42 induced LA and LB fragmentation (Fig. 9A). Furthermore, BAPTA-AM prevented nuclear morphological changes of LA & LB induced by Aβ42 (Fig. 9B) indicating the role of Ca^{2+} in disrupting lamin integrity. This proved that intracellular Ca^{2+} rise sensitized lysosomal cathepsin L release to cause damage of lamin protein in Aβ42 treated cells. The report showed that oligomer Aβ42 induced ER Ca^{2+} release [150]. So, in our study,

we assumed that ER could be the storage of Ca^{2+} and Aβ42 induced this organelle to release Ca^{2+} into the cytosol. We blocked Ca^{2+} release from ER by CPA and CPA at the 20 μM concentration inhibited Aβ42 induced cell death at the highest percentage (Fig. 10A). This indicated that Ca^{2+} was released from ER mentioning the importance of this signaling event in cell death. For further confirmation, lamin cleavage was checked in the presence of CPA by western blotting and data showed that CPA completely abolished Aβ42 induced lamin cleavage suggesting that Aβ42 facilitates mobilization of Ca^{2+} from ER (Fig. 10B). Eventually, inhibiting Ca^{2+} release from ER by CPA affected apoptosis which was justified by observing reduced caspase-3 activity in Aβ42 and CPA-treated samples (Fig. 10C). So, our finding suggested that Ca^{2+} was released from ER in Aβ42 treated cells.

We examined different cell death-inducing agents to check whether BAPTA-AM could prevent lamin fragmentation induced by those damaging agents. Our western blot data revealed that BAPTA-AM failed to block lamin cleavage induced by damaging agents. The previous reports showed the role of Ca^{2+} in the induction of apoptosis by STS, etoposide, and TNF-α [156–158]. In our study, we did find any effect of blocking cytosolic Ca^{2+} on lamin cleavage, cell death and caspase-3 activity in those cell-damaging agents (Fig.11A). There could be couple of reasons for this discrepancy for example, different cell lines and treatment conditions can be the possible reasons. More importantly. The previous study did not check the impact of blocking Ca^{2+} overload on lamin fragmentation [156–158]. LA and LB cleavage were produced by caspase-6 in those cell deaths inducing agents and caspase-6 activity is not Ca^{2+} dependent [156–158]. However, we disagree with their results because if caspases are activated by those agents how Ca^{2+} chelator inhibits cell death and apoptosis.

Those issues were not addressed. Further investigation should be carried out in this respect. Brefeldin A and thapsigargin produced ER stress to generate caspase-mediated lamin fragments and adding BAPTA-AM did not inhibit lamin cleavage, cell death, and caspase-3 activity (Fig. 11B-D). Based on our results, it can be concluded that the effect of Ca^{2+} on lamin cleavage is only Aβ42 specific. Our results also demonstrated that BAPTA-AM reduced cathepsin L activity and expression induced by Aβ42 (Fig. 12A-B). This provides evidence that cytosolic Ca^{2+} rise is the upstream event in Aβ42 induced lamin cleavage and cathepsin L release from lysosome is dependent on intracellular Ca^{2+}.

Previous studies reported the presence of cathepsin L in the nucleus [132,134]. We, therefore, wondered whether activating this nuclear cathepsin L would result in a similar LA and LB cleavage pattern of Aβ42 treated cells in isolated nuclei. A previous study showed that nuclei isolated from healthy Hela cells were unable to produce any lamin fragment [69]. The addition of Ca^{2+} to the nuclei generated 46 and 21 kDa LA and LB fragments respectively which was similar to Aβ42 induced lamin fragments. We used cathepsin L inhibitor in Hela nuclei in the presence of Ca^{2+} and found complete inhibition of LA and LB cleavage by Z-FF-FMK (Fig. 13A). So as expected, we observed cathepsin L mediated lamin fragmentation induced by Ca^{2+} in isolated nuclei. To exclude the appraisement of other proteases in lamin cleavage, we checked LA and LB fragmentation in isolated nuclei in the presence of different protease inhibitors. Except for cathepsin L, none of the protease inhibitors were able to block Ca^{2+} induced lamin cleavage in nuclei (Fig. 13B). These nuclei study strongly supports that Ca^{2+} is involved in cathepsin L mediated lamin damage induced by Aβ42.

Additionally, we examined the effect of Z-FF-FMK and BAPTA-AM in Aβ42 induced apoptosis. Our results provided evidence that both Z-FF-FMK and BAPTA-AM reduced caspase-3, -6, -8, -9 activities (Fig. 14A-H). Thus, the intracellular Ca^{2+} rise is an upstream event in Aβ42 induced apoptosis. Since the caspase activities are controlled by cytosolic Ca^{2+}, therefore, we propose that classical apoptosis is a later stage event in Aβ42 induced cell death.

In summary, the involvement of intracellular Ca^{2+} rise is an upstream molecular event in Aβ42 induced lamin fragmentation and this pathway is specific for Aβ42 treated cells. Moreover, our experimental evidence shows that cathepsin L is released from lysosome by cytosolic Ca^{2+} overload and mediate lamin cleavage. Our results also recognize that cytosolic Ca^{2+} rise and cathepsin L activation delay the onset of caspase- mediated apoptosis. So, our observation clearly shows that BAPTA-AM inhibits Aβ42 induced lamin cleavage and cell death which suggests blocking intracellular Ca^{2+} rise could be an effective way for controlling Aβ42 induced AD pathology.

It should be noted that the present study used only Hela cells to observe the molecular events of Aβ42 toxicity and SHSY5Y cells to evaluate cytotoxicity (data not shown). So, it is remained unclear whether this Aβ42 induced cellular event is followed in other cell lines. Further investigation is required in this regard. Accordingly, this is an in vitro toxicity study, and this must be complemented in animal models and AD patients to understand the chronic nature of neurodegeneration.

III-3. tAβ42 induced lamin fragmentation mechanism

III-3-1. tAβ42 induces cytotoxicity, lamin protein fragmentation and caspase activation in Hela cells

To explore the real cytotoxicity and cell death mechanism of Aβ42, we constructed cell permeable Aβ42 by attaching Trans Activator of Transcription (TAT) to the N terminus of Aβ42. We investigated whether cell permeable TAT Aβ42(tAβ42) is able to induce the similar pattern lamin cleavage as the wild type Aβ42. Initially, cell viability was monitored using MTT method in order to determine the concentration needed for lamin fragmentation. Hela cells were treated with different concentrations of freshly prepared tAβ42 for 24 h. We used freshly prepared tAβ42 for all our tAβ42 related experiment. At 5 μM concentration of tAβ42, less than 50% cell viability was observed in Hela cells (Fig.15A). This cytotoxic result indicates that tAβ42 is more toxic than oligomer Aβ42. 50 % cell death was determined at 20 μM oligomeric Aβ42(oAβ42) which is ~ 4 times more concentration than tAβ42 and double treatment of oAβ42 peptide is needed to get this much cell death. This actually proves that doubly treated oligomer Aβ42 gains similar structure of tAβ42.

Aβ42 peptide generates specific lamin cleavage and this lamin fragmentation occurs in cells treated twice with oAβ42. It has been reported that this lamin fragmentation is independent of caspase activation [69]. tAβ42 also induced specific cleavage of LA and LB in Hela cells. tAβ42 at the concentration of 2.5~10 μM produced lamin fragments. ~46 Kda N-terminal fragment from LA and ~21 Kda C-terminal fragments from LB were found in the tAβ42 treated cells for 24 h incubation. At 2.5 μM concentration, only LA cleavage was observed which could be due to more cell viability at this concentration (Fig.

15B). The fragmentation pattern was identical to those of Aβ42. Only cells treated with oligomer Aβ42 showed similar patterns tAβ42 induced fragmentation of lamin proteins.

The tAβ42 preparations induced caspase-3 activity (DEVD-AMC) in a dose dependent manner with the highest activity observed at 20 μM concentration for 24 h incubation (Fig.15C). Here only single treatment is enough for the activation of caspase. This also indicates the level of toxicity of tAβ42.

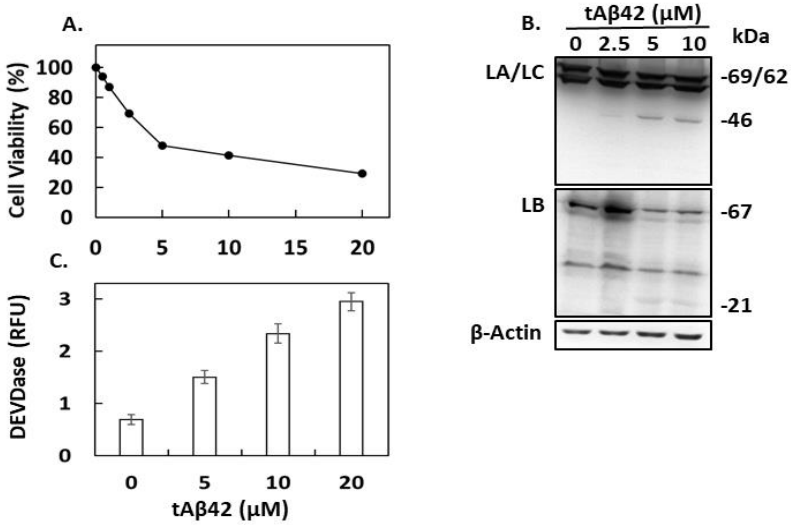

Figure 15. Tat Aβ42(tAβ42) induced cytotoxicity, lamin fragmentation and caspase activation. (A) Hela cells (15 X 10^3) were treated with tAβ42 at the indicated concentrations and time and the viability of the cells was determined by MTT reduction method. (B) Cells (4 X 10^5) were treated with tAβ42 at different concentrations for 24 h and lamin fragments were monitored by Western blotting. β-actin used here was loading control (C) Caspase-3 activity was measured using 10 μM DEVD-AMC synthetic substrate after treating Hela cells with tAβ42 at the indicated concentrations for 24 h.

III-3-2. Measurement of intracellular Ca^{2+}, participation of Ca^{2+} in cell death, calcium event in lamin fragmentation and caspase activation in tAβ42 treated cells

To understand the role of Ca^{2+} in Aβ42 induced cell death, we designed similar type of experiments like of Aβ42 in case of cell permeable tAβ42. We used Fluo-8-AM to measure the changes of intracellular Ca^{2+} induced by tAβ42. Fluorescence image depicts significant Ca^{2+} binding of Fluo-8-AM in tAβ42 treated cells compared to control (Fig. 16A). This reveals the tAβ42's role in the rise of intracellular Ca^{2+} levels. This experiment was done in absence of extracellular Ca^{2+} which also prove the release of Ca^{2+} from intracellular store in tAβ42 treated cells.

Next, we explored a relation between intracellular Ca^{2+} increase and cytotoxicity. Ca^{2+} chelator BAPTA-AM was used to inhibit the role of Ca^{2+} in cell death process. The protective effect of BAPTA-AM was quantified by MTT method. Application of tAβ42 caused more than 50 % cell death in Hela cells. Upon treating the cells with 25 μM BAPTA-AM in the presence of tAβ42 increased the cell viability to ~83 %. The pretreatment of BAPTA-AM up to 25 μM concentration prevented tAβ42 induced cell death in a dose-dependent manner. So, the cell permeable chelator BAPTA-AM prevented tAβ42 induced cell death which suggested that intracellular Ca^{2+} was one of the mediators in tAβ42 induced cell death.

We examined the effect of BAPTA-AM on lamin damage induced by tAβ42 because involvement of C-L was found on mediating lamin cleavage in our study and C-L activity is Ca^{2+} dependent. Lamin fragments were not detected in cells treated with tAβ42 in the presence of 25 μM BAPTA-AM (Fig. 16C).

We used 25 μM BAPTA-AM concentration because at this concentration maximum cell viability was observed in the presence of tAβ42 and the higher concentration of this chelator alone induced cell demise and the lower concentration was less efficient in preventing lamin cleavage (data not shown).

The effect of BAPTA-AM on caspase-3 activity was monitored in cells treated with tAβ42 for 24 h during which the activity was prominent. In the presence of 25 μM BAPTA-AM, caspase-3 like DEVDase activity was reduced in tAβ42 treated cells (Fig.16D). So, the influence of Ca^{2+} for inducing apoptotic caspase activation is obvious.

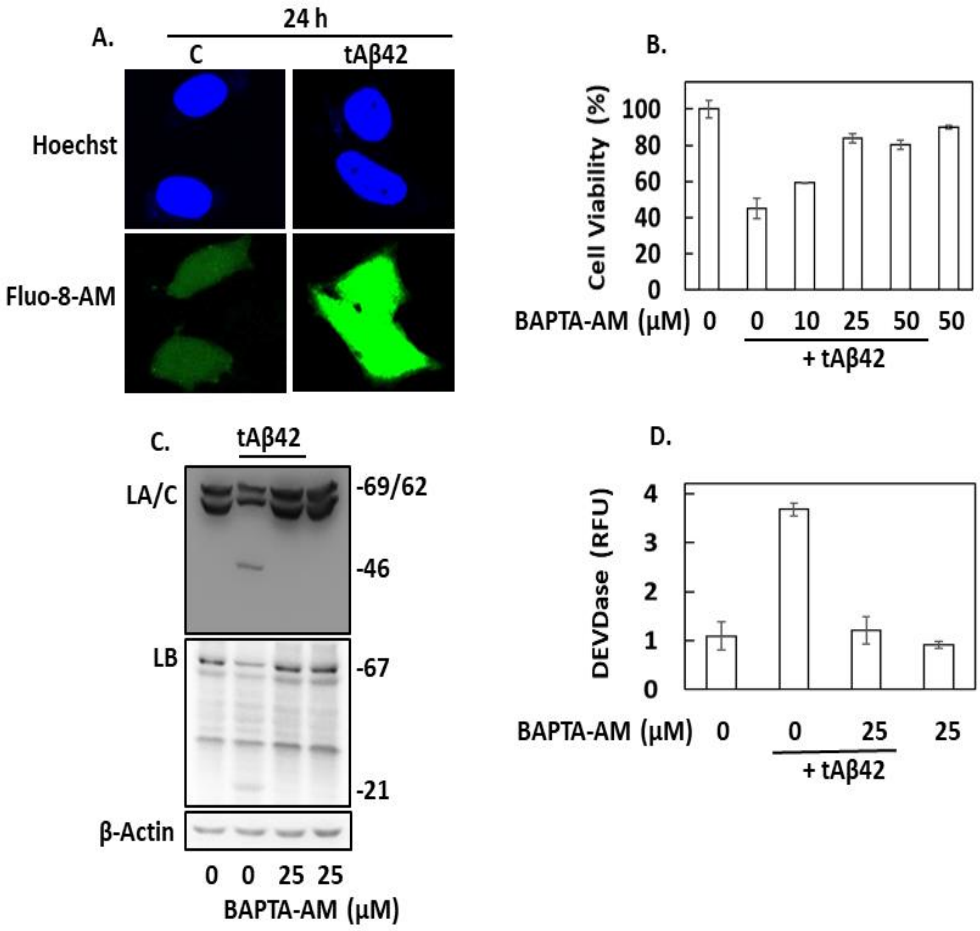

Figure 16. Role of calcium on tAβ42 induced lamin fragmentation. (A) Hela cells were treated with tAβ42 at the indicated concentration and time. After treatment, fluorescent images of Fluo-8/AM loaded cells (control & treated) were taken. (B & D) Hela cells were pretreated for 1 h with BAPTA-AM. Cells were then exposed to tAβ42 at different concentrations for 24 h and the percentages of viable cell were determined by MTT method. Similar treatment procedures were followed for measuring Caspase 3 activity. After treatment, DEVD-AMC substrate was added and the levels of DEVD

fluorescence were quantified. Results are expressed as Mean ± SD. (C) Hela cells (4 X 10^5) were treated with tAβ42 in the presence or absence of BAPTA-AM. After preparing cell lysates, western blotting was used to check LA/C and B fragments. All the experiments were carried out for three times. As loading control, β-actin was used.

III-3-3. C-L inhibitor suppresses tAβ42 induced lamin cleavage and caspase activation

Our finding proved that Aβ42 induced C-L mediated LA & LB cleavage and tAβ42 produced similar pattern of lamin fragmentation. For further confirmation of C-L involvement in tAβ42 induced lamin cleavage, we checked the effect of some selective protease inhibitors on tAβ42 induced lamin cleavage. MG-132(a proteasome inhibitor), ALLN (a calpain inhibitor), Z-FA-FMK (cathepsin B inhibitor), Leupeptin (a serine protease inhibitor) and Aprotinin (serine protease inhibitor) did not inhibit the tAβ42 induced lamin fragments production. Our results justified the participation of cathepsin L in tAβ42 induced lamin cleavage (Fig. 17A).

In this study, tAβ42 induced lamin fragments (LA & LB) were absent in the presence of 50 μM Z-FF-FMK which identified the involvement of C-L in lamin cleavage process. We did not focus on lamin C because effect of Aβ42 on lamin C is uncertain. Here it is worthy to say that doubly treated oAβ42 produced similar type of lamin fragments of tAβ42. It seems that doubly treated oligomer Aβ42 achieves the property of tAβ42 because both of the peptides followed same pattern lamin cleavage mechanism (Fig. 17B).

To rule out caspase involvement in tAβ42 induced LA and LB cleavage, we treated the cells with cell permeable pan caspase inhibitor Z-VAD-FMK. However, Z-VAD-FMK at 20 μM concentration did not inhibit lamin cleavages when exposed to tAβ42 (Fig. 17C). Study showed that Z-VAD-FMK up to 100 μM had negligible effect on Aβ42 induced lamin reduction [69]. Our data suggest that lamin are substrate of C-L in tAβ42 treated cells.

We checked the effect of Z-VAD-FMK on Staurosporine (STS) induced lamin cleavage. 20 μM Z-VAD-FMK inhibited STS induced caspase-6 mediated 28 kDa LA and 46 kDa LB production. This proves the involvement of caspase mediated lamin cleavage in STS treated cells (Fig. 17D).

Similarly, we also examined the effect of Z-FF-FMK on STS induced lamin cleavage. Z-FF-FMK at the 50 μM concentration did not inhibit the STS induced lamin fragments production. Our results implied that involvement of C-L to cleave lamin was specific to tAβ42 treated cells (Fig. 17E).

MTT assay was used to get the optimum concentration of C-L inhibitor(Z-FF-FMK) needed for the inhibition of tAβ42 induced lamin cleavage. In our investigation, we checked the cell viability of tAβ42 treated cells in the presence of different concentrations (10-75 μM) of Z-FF-FMK. 5 μM tAβ42 killed more than ~60 % cells. Z-FF-FMK up to 50 μM increased cell viability in the presence of tAβ42 in a concentration dependent manner. More than 80% cell viability was observed at the concentration of 50 μM Z-FF-FMK in the presence of 5 μM tAβ42. For our further experiments, we used 50 μM Z-FF-FMK concentration because above this concentration, cell viability is reduced and below concentration is ineffective for our target experiments. Our data also revealed that Z-FF-FMK at the concentration of 75 μM itself induced cell death.

Therefore, we limited our experiments to 50 μM Z-FF-FMK (Fig. 17F).

Study showed the role of C-L in Aβ42 mediated caspase activation [137]. Our investigation proved that 50 μM Z-FF-FMK inhibited tAβ42 induced DEVDase activity. In control cells, caspase-3 activity was in average ~1.15 RFU and this was significantly increased to ~ 4.0 RFU by 5 μM tAβ42. Z-FF-FMK at 50 μM concentration abrogated tAβ42 induced caspase-3 activation. In the presence of tAβ42 and Z-FF-FMK, caspase-3 activity was ~1.6 RFU whereas Z-FF-FMK alone produced ~1.41 RFU caspase-3 activity. So, our results confirmed the involvement of C-L in tAβ42 induced caspase activation (Fig. 17G).

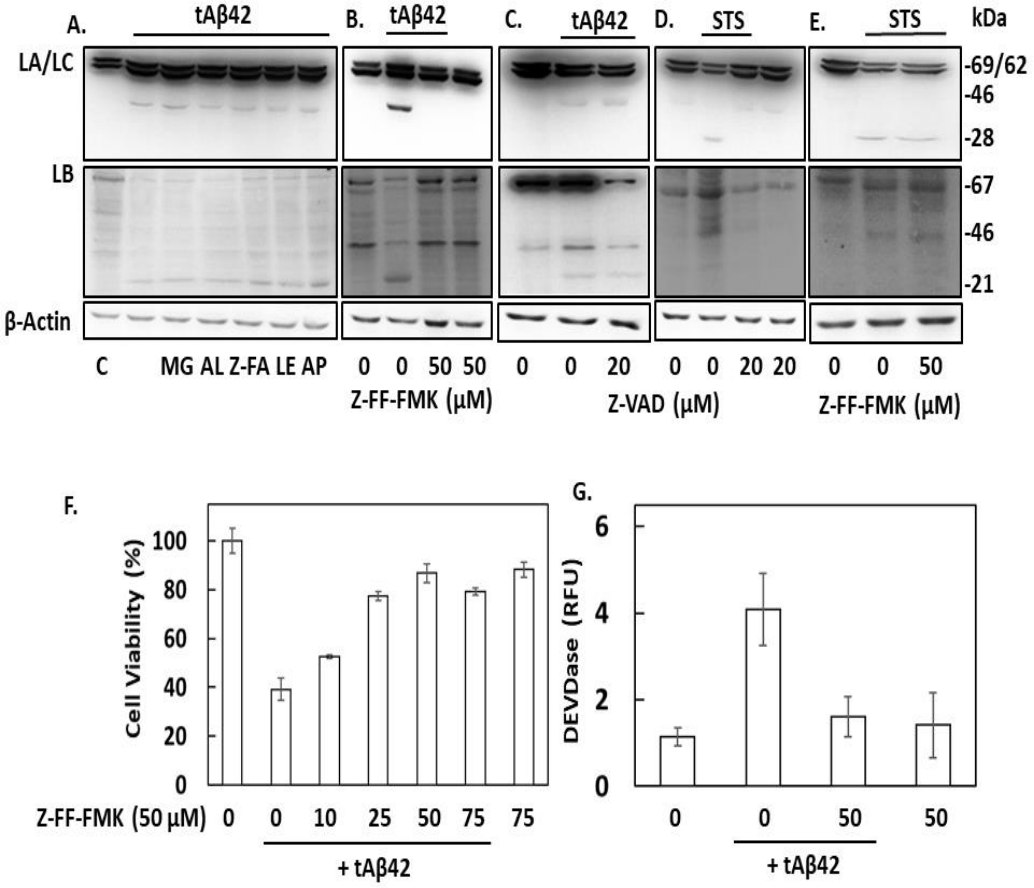

Figure 17. Involvement of C-L in tAβ42 induced lamin cleavage. (A) Hela cells were treated with indicated concentration of tAβ42 in the presence or absence of Z-FF-FMK and cytotoxicity was measured using MTT assay. (B) The efficacy of Z-FF-FMK in blocking lamin cleavages was showed by western blotting in tAβ42 treated cells. (C) Caspase-3 activity was measured using its synthetic substrate Ac-DEVD-AMC (10 μM) after treating the cells with indicated tAβ42 in the presence or absence of inhibitor. Inhibitor was added before 1 h of the treatment. Results are the average of three individual

experiments. (D) Effect of 5 μM MG-132(MG), 20 μM ALLN(AL), 25 μM Z-FA-FMK(Z-FA), 20 μM Leupeptin (LE), 20 μM Aprotinin (AP) was examined on lamin cleavage in tAβ42 treated Hela cells. C denotes to control. (E & F) Cells were pre-exposed with the indicated concentration of Z-VAD-FMK for 1 h and further treated with tAβ42 for 24 h or STS for 4 h at the indicated concentrations. Fragmentation of LA/C and LB was determined by western blotting. (G) Effect of Z-FF-FMK on STS induced lamin cleavage was checked by western blotting at the indicated concentration following 1 h pre-treatment of inhibitor.

III-3-4. tAβ42 augments C-L activity

Hela cells were treated with tAβ42 for 24 h and C-L activity was measured by fluorogenic substrate. tAβ42 at the concentration of 5 μM tAβ42 increased C-L activity around 2-fold compared to control. Results showed that tAβ42 elevated C-L activity in a dose-dependent manner (Fig. 18A). Results in figure18(B) demonstrated that presence of BAPTA-AM or Z-FF-FMK inhibited C-L activity in tAβ42 treated cells. This provides evidence that Z-FF-FMK is an inhibitor of cathepsin L and this inhibitor can prevent tAβ42 induced C-L activity. Moreover, data also proves that C-L activity is also dependent on intracellular Ca^{2+} in tAβ42 treated cells.

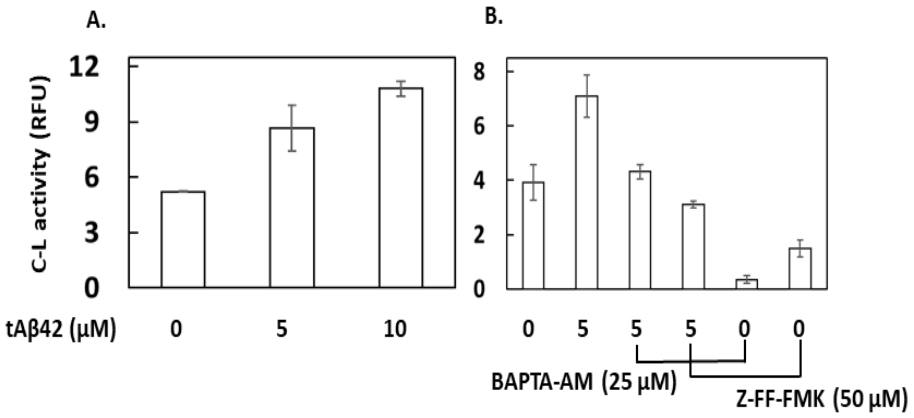

Figure 18. Intracellular calcium increase is upstream event in C-L mediated tAβ42 induced lamin fragmentation. (A) tAβ42 significantly increased C-L activity at the indicated concentrations as well as time and C-L activity was measured using 10 μM Z-F-R-AMC fluorogenic substrate. (B) Cathepsin L inhibitor(Z-FF-FMK) and calcium chelator (BAPTA-AM) at the mentioned concentrations reduced C-L activity significantly for 24 h.

III-3-5. Inhibition of tAβ42 induced nuclear lamin invagination by BAPTA -AM and Z-FF-FMK

From western blot data we came to know that lamin abnormalities occurred in tAβ42 treated cells. In this experiment, we examined the nuclear integrity using fluorescence microscope. LA and LB invaginations were observed in tAβ42 treated cells but those were suppressed effectively by BAPTA-AM and Z-FF-FMK respectively (Fig. 19). We recorded higher percentage of lamin damage in tAβ42 induced cells (data not shown). So, intracellular Ca^{2+} and C-L have role to cause lamin damage in tAβ42 treated cells.

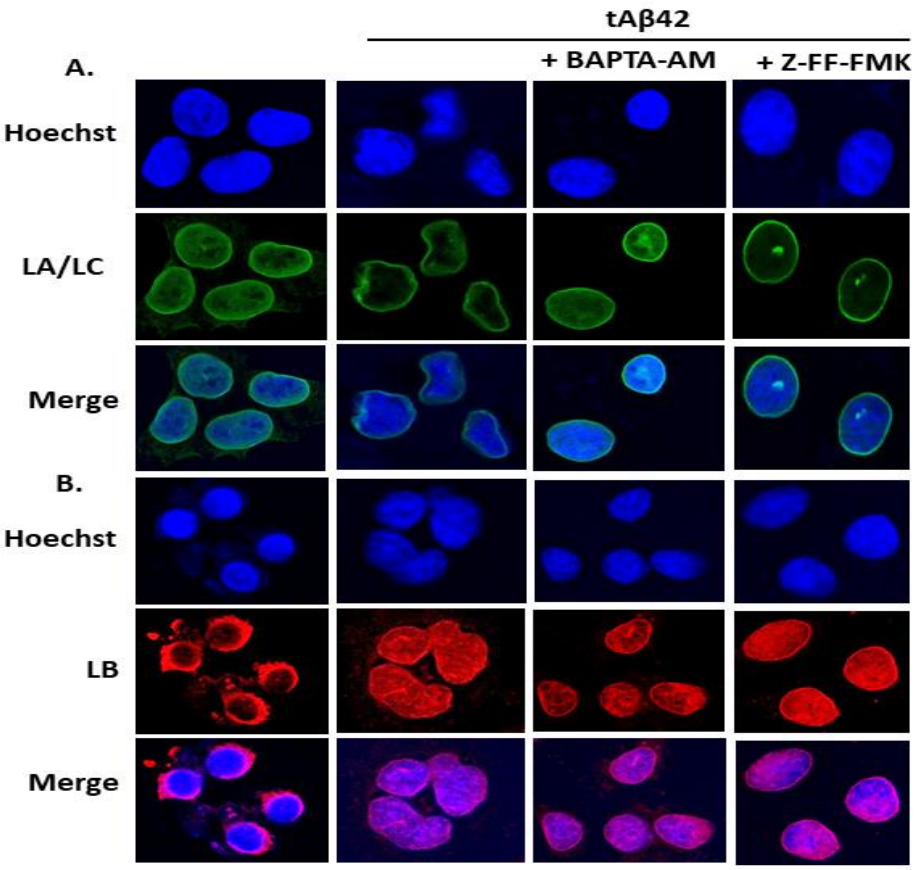

Figure 19. Suppression of tAβ42 induced nuclear deformation by BAPTA-AM and Z-FF-FMK respectively. (A & B) Hela cells were treated with the indicated concentration of tAβ42 for 24 h in the presence or absence of BAPTA-AM and Z-FF-FMK to take the confocal images. LA/C (green) was detected using primary mouse LA/C antibody and secondary anti-mouse IgG-FITC antibody. (B) For the detection of LB (red), primary rabbit L-B antibody and secondary anti-rabbit IgG-TRITC antibody was applied. Hoechst was used to stain nuclei. Representative images have been shown here.

III-3-6. Discussion

In the present study, we found that tAβ42 followed Aβ specific lamin fragmentation mechanism indicating that doubly treated oligomer Aβ42 gained similar kind of cytotoxic property of cell permeable tAβ42. Our data showed that nuclear integrity dependent on C-L activity in tAβ42 induced toxicity model (Fig. 19). Prevention of lamin damage by C-L inhibitor also justify the key role of C-L in tAβ42 induced lamin cleavage. Confocal images support the involvement of C-L in tAβ42 treated nuclear changes (Fig. 19). Besides, we also observed that C-L activity was controlled by the concentration of the intracellular Ca^{2+} (Fig18). Involvement of intracellular Ca^{2+} in losing nuclear lamin integrity is new concept. Our results revealed that intracellular Ca^{2+} was increased in tAβ42 treated cells. We noticed same observation in oligomer Aβ42 double treated cells. The main feature in tAβ42 induced lamin damage is that only single treatment is enough to get similar effect like of doubly treated Aβ42 and no need to prepare oligomers. So, tAβ42 supports the oligomer Aβ42 induced specific lamin cleavage pathway. Therefore, to understand the Aβ42 cytotoxicity mechanism in AD pathogenesis, tAβ42 concept could be useful.

III-4. Protection of curcumin against Aβ42 induced cell death through prevention of lamin fragmentation

Evidence shows that curcumin protects cells against Aβ induced cell death and damage by preventing intracellular Ca^{2+} rise [159]. Our study has demonstrated the involvement of intracellular Ca^{2+} in Aβ42 induced lamin fragmentation pathway (Fig. 9). In this study, we examined whether curcumin prevented Aβ42 induced lamin cleavage mechanism.

III-4-1. Curcumin protects Hela cells from Aβ42 induced cell death

The cytotoxicity of curcumin and Aβ42 alone towards Hela cells was primarily evaluated to determine the proper dosages for combination treatment. In this study, Aβ42 induced group decreased MTT reduction when compared to the control group (20 μM Aβ42 treated cells for 2+22 h showed ~50% cell death in comparison to control). To explore the protective mechanism of curcumin against Aβ42 induced cell death, 2 h pre-treatment of curcumin was employed. Curcumin at the given concentrations significantly inhibited Aβ42 induced reduction in cell viability in a dose-dependent manner. However, curcumin (10,20,40 μM) increased cell viability from 51% (Aβ42) to 67, 84, 82 % respectively. Curcumin alone at the 40 μM concentration showed no toxicity to Hela cells (Fig. 20A). Furthermore, Aβ42 treated cells became shrink, round, and disconnect from one cell to another. Curcumin improved morphological changes induced by Aβ42 indicating its protective effect (Fig. 20B). Taken together, our result indicated that curcumin reduced Aβ42 induced cytotoxicity and thereby increased cell viability.

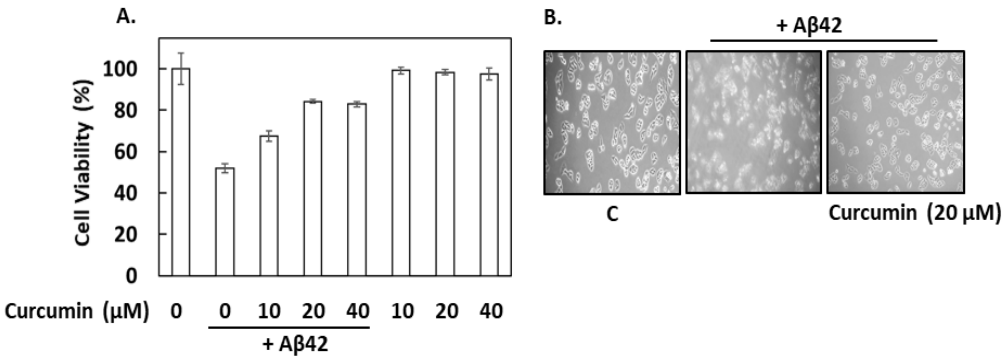

Figure 20. Protective effect of curcumin against Aβ42 induced cell death in Hela cells. (A) Hela cells ($1x10^4$) were treated with 20 μM oligomer Aβ42 in the presence or absence of indicated concentrations of curcumin for 2+22 h. Aβ42 was added after 2 h of curcumin treatment. Following treatment, MTT reagent was used to get the cell viability. Triplicate experiments were carried out to get the mean value. (B) Cell morphological observation. Cells were treated with 20 μM Aβ42 in the presence of indicated concentration of curcumin for 2+22 h and light microscope was used to observe the cells images. C represents control without treatment.

III-4-2. Effect of curcumin on Aβ42 induced lamin cleavage

Aβ42 induced lamin fragmentation pathway is very specific and inhibiting this mechanism could be an effective treatment plan in Aβ42 related AD. This hypothesis made us curious to check the Aβ42 toxicity inhibitors. Based on our literature screening, we explored that curcumin was very potent to prevent Aβ42 induced cell death [160]. In this study, we examined the effect of curcumin on lamin protein fragmentation induced by Aβ42. Aβ42 generates ~46 kDa N-terminal and ~21 kDa lamin fragments from LA and LB respectively. This specific protein fragmentation occurred in the cells treated twice with the Aβ42. Lamin cleavages were produced consistently in the group treated with 20 μM Aβ42 for 2+22 h. Those lamin fragments were not identified in the cells treated in the same way in the presence of curcumin (Fig. 21A). 20 μM curcumin diminished the lamin fragments indicating that it suppressed lamin fragmentation process. Although 10 μM of curcumin reduced Aβ42 induced lamin protein reduction, it was not enough to remove lamin damage completely.

III-4-3. Curcumin prevents Aβ42 induced nuclear deformation

Our western blot result showed that curcumin inhibited Aβ induced lamin fragmentation. So, we checked whether curcumin prevented nuclear morphological changes in Aβ treated cells. Nuclear lamin invaginations or abnormalities were observed in Aβ42 treated cells. Curcumin at the 20 μM concentration prevented Aβ42 induced LA & LB deformation indicating protecting effect of curcumin (Fig. 21B).

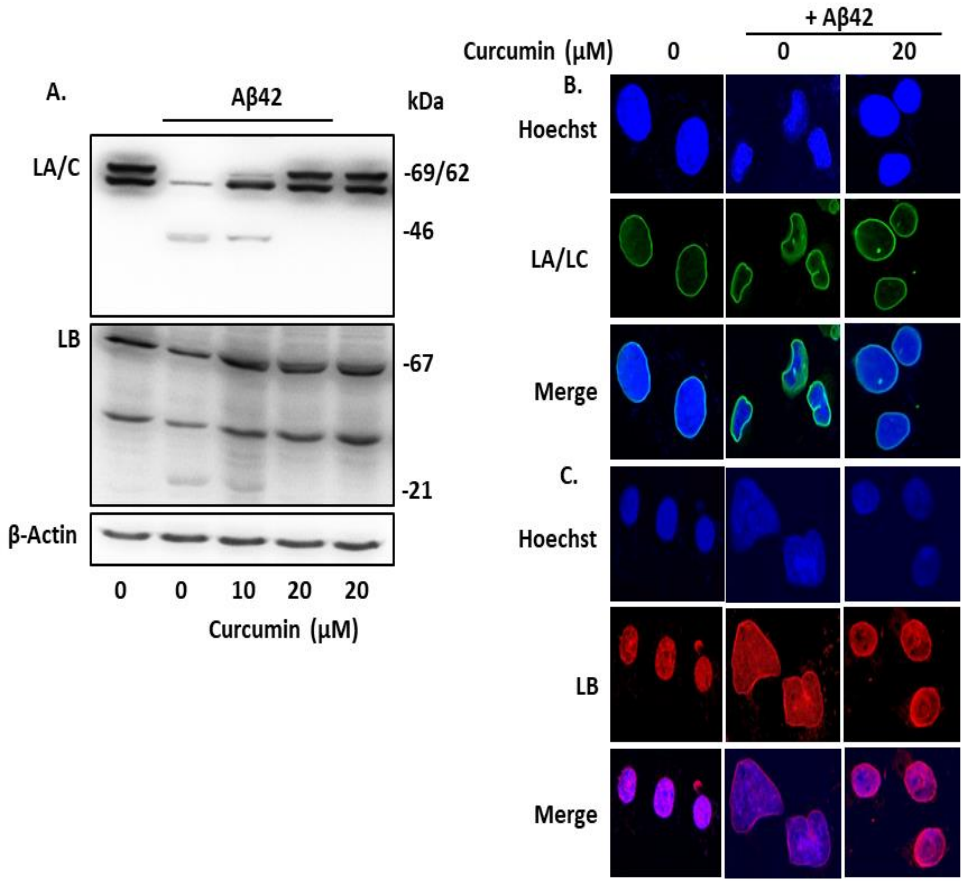

Figure 21. Effect of curcumin on Aβ42 specific lamin fragmentation or deformation. In this experiment, 20 μM oligomeric Aβ42 was used to treat the cells in the presence of indicated concentration of curcumin for 2+22 h (A) After treatment, cell lysates were prepared and LA or LB fragments were evaluated by western blotting. LA/C and LB antibodies were used to detect LA/C and LB proteins. β-actin was used as western control. (B) Nuclear deformation after treatment was observed by confocal microscope. Hoechst was applied as nuclei staining dye. Only representative images have been presented here.

III-4-4. Curcumin prevents intracellular Ca^{2+} increase caused by Aβ42

In our study, we proved the involvement of Ca^{2+} in Aβ42 specific lamin fragmentation (Fig.9). In this experiment, we examined the effect of curcumin on intracellular Ca^{2+} rise as observed in Aβ42 induced lamin cleavage pathway. The fluorescence intensity of the Ca^{2+} sensitive fluo-8-AM dye in the Aβ42 treated group was much higher when compared to the control. The fluorescence images are shown in figure 22 and those are only representative images. However, the curcumin protective group reduced fluorescence intensity significantly compared with the Aβ42 treated sample. Curcumin at the 20 μM concentration suppressed Aβ42 induced intracellular Ca^{2+} rise completely indicating the protective nature of curcumin. Therefore, fluorescence images showed that Aβ42 resulted in the rise of cytosolic Ca^{2+} immediately and curcumin inhibited those Ca^{2+} increases.

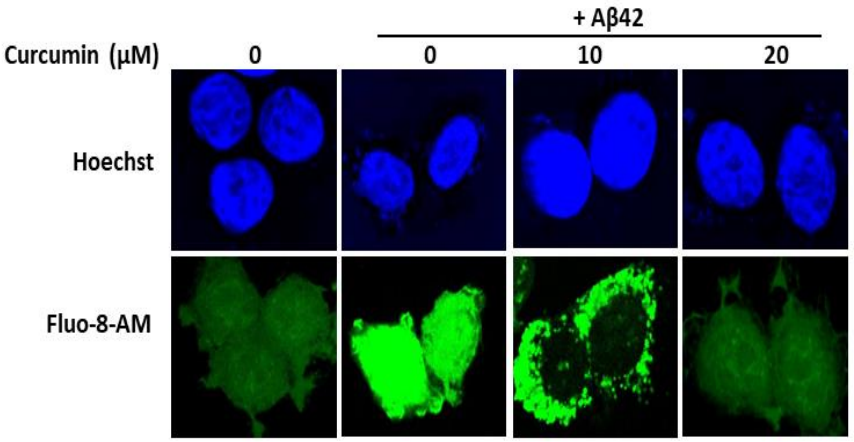

Figure 22. Curcumin prevents amyloid beta induced intracellular calcium release. Hela cells($1x10^5$) were treated with 20 μM Aβ42 in the presence of indicated concentration of curcumin for 2+22 h. For the curcumin and Aβ42 treated group, cells were treated with curcumin at first for 2 h and then followed Aβ42 treatment procedure to observe the intracellular calcium release. Fluo-8-AM, a calcium binding dye was used to observe the cytosolic calcium.

III-4-5. Aβ42 induced increased C-L activity are decreased by curcumin

In our study, we detected C-L as a mediator of Aβ42 induced lamin damage, and increased C-L activity was also observed in Aβ42 treated cells (Fig. 2F & 4A). In this observation, curcumin pre-treatment reduced Aβ42 induced C-L activity remarkably suggesting the C-L controlling effect of curcumin. It is noted that curcumin alone did not produce any effect on C-L (Fig. 23).

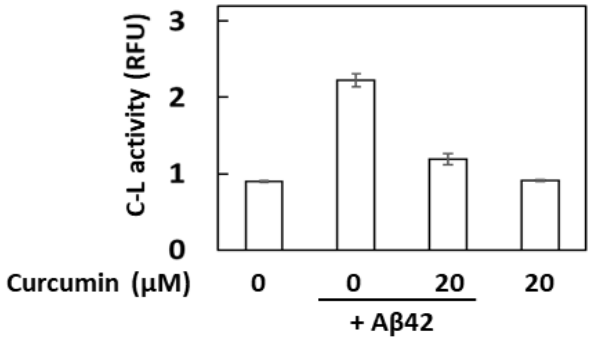

Figure 23. Curcumin inhibits Aβ42 induced cathepsin L activity. Hela cells (2 X 10^4) were treated with 20 μM Aβ42 in the presence or absence of indicated concentration of curcumin for 2+22 h. After treatment, cell lysates were prepared and 10 μM Z-F-R-AMC substrate was added to measure cathepsin L activity. Data are mean of triplicate value and expressed as mean ± SD. RFU indicated Relative Fluorescence Unit.

III-4-6. Curcumin destabilizes Aβ42 fibrillogenesis

Fibrils are the long insoluble structures that are formed by the polymerization of Aβ and bind easily with Thioflavin T (ThT) [161]. Curcumin is known to affect Aβ aggregation by slowing the polymerization of Aβ [166]. In our study, we showed that oligomer Aβ42 induced Aβ42 specific lamin fragmentation (Fig.1). In this study, we examined whether curcumin affects Aβ aggregation process in order to prevent lamin fragmentation. ThT fluorescence results of 20 μM Aβ42 exhibited a sigmoidal curve implying Aβ aggregation. The ThT fluorescence value indicates how many mature fibrils are formed through Aβ aggregation. However, the addition of curcumin in Aβ42 reduced the fluorescence of ThT effectively indicating the anti-aggregation property of curcumin (Fig. 24).

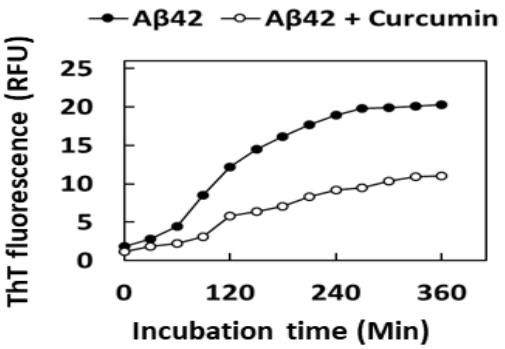

Figure 24. Effect of curcumin on Aβ42 polymerization. 20 μM Aβ42 was incubated in PBS at 37^0C in the presence or absence of curcumin. ThT fluorescence assay was carried out in a time dependent manner to get fluorescence. 20 μM curcumin was used to check anti-polymerization effect.

III-4-7. Curcumin protects against Aβ42 induced caspase activation

Caspases are a family of cysteine proteases that play an important role in cell death [162]. In this report, exposure of 20 μM Aβ42 in Hela cells for 2+22 h significantly activated caspase-3 indicating activation of the apoptotic pathway. Curcumin pretreatment prior to Aβ42 exposure inhibited caspase-3 like DEVDase activity suggesting the defensive effect of curcumin against apoptosis (Fig. 25) In this experiment, curcumin also did not induce apoptosis.

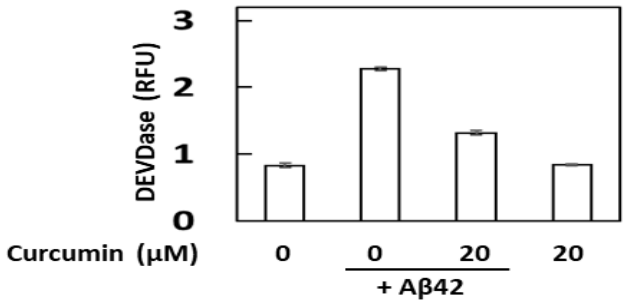

Figure 25. Attenuation of Aβ42 induced caspase-3 activity by curcumin. Hela cells were pre-treated with indicated concentration of curcumin for 2 h and then approached with 20 μM Aβ42 for 2+22 h. After treatment, cells lysates were prepared and 10 μM DEVD-AMC was added to measure caspase-3 activity. Readings were recorded as fluorescence unit.

III-4-8. Discussion

A unique observation in this study was the prevention of Aβ42 specific lamin fragmentation and nuclear deformation by curcumin (Fig. 21). The mechanism behind the inhibitory effect of curcumin against Aβ42 induced lamin cleavage was also revealed in this study. In the process of neuronal cell death, lamin dispersion and distortion are crucial [64,163]. Our results showed that curcumin prevented Aβ42 induced cytotoxicity (Fig. 20) and this could be due to the prevention of Aβ42 induced LA & LB cleavage. Previous observation reported that curcumin promoted cell viability in Aβ treated cells [160]. Therefore, our results are justified by the previous study. In Aβ42 induced lamin fragmentation mechanism, we discovered the involvement of intracellular Ca^{2+} in the lamin damage process (Fig. 9). Ca^{2+} dysregulation in neurons might have a significant role in AD pathogenesis [164]. Another study demonstrated that Aβ induced intracellular Ca^{2+} rises were inhibited by

curcumin [159]. Thus, we examined the effect of curcumin on cytosolic Ca^{2+} rise by Aβ42 and found that curcumin prevented Ca^{2+} increases (Fig.22). As expected, curcumin also reduced C-L activity (Fig.23). To reveal the protective mechanism of curcumin in this study, we speculated two possibilities- prevention of Aβ aggregation by curcumin or curcumin enters into cells and bind with Ca^{2+} induced by Aβ42 and therefore prevent lamin cleavage. A previous study reported that curcumin worked as chelators and therefore, it bound with Ca^{2+} [165]. In this study, only oligomer Aβ42 induced Aβ42 specific lamin fragmentation, and preventing oligomerization could be another way to prevent lamin damage. Our results proved that curcumin inhibited Aβ42 aggregation (Fig. 24). Many investigators demonstrated the anti-aggregation behavior of curcumin [111,166]. Moreover, curcumin also reduced caspase activity which is a later stage event in Aβ42 induced cell death pathway (Fig.25).

In conclusion, curcumin prevented Aβ42 specific lamin fragmentation by inhibiting Aβ aggregation or preventing intracellular Ca^{2+} elevation and this might provide new insights to control Aβ associated AD pathogenesis. However, this mechanism needs to be reproduced in the AD mice model and for that further investigation is needed.

IV. FUTURE RESEARCH

The mechanism behind the inhibitory effect of Taiwaniaflavone on Aβ42 induced lamin cleavage as well as cell death

We chose taiwaniaflavone as a protective agent because a previous report showed the protective nature of this flavonoid against Aβ [108]. Different concentrations of taiwaniaflavone was used to observe its cytoprotective effect in Aβ42 treated cells. Our MTT results showed that at the 10 μM concentration of taiwaniaflavone in Aβ42 induced cells, more than 80% cell viability were recorded. Taiwaniaflavone alone did not cause any significant cell death (Fig. 26A). Next, we observed the effect of taiwaniaflavone on Aβ42 induced lamin fragmentation process.

Studying lamin protein cleavage is a helpful tool for the discovery of Aβ42 specific cytotoxic mechanism because Aβ42 peptide produces unique lamin fragmentation. The fragmentations are only seen in cells that have been exposed to oligomer Aβ42 treated twice. Therefore, inhibiting this pathway might be useful for AD treatment. The presence of 10 μM taiwaniaflavone in the cells prevented Aβ42 induced lamin cleavage indicating the protective effect of taiwaniaflavone. So, taiwaniaflavone suppressed Aβ42 induced lamin fragmentation process (Fig. 26B). Our data imply that the preventive effect of taiwaniaflavone in terms of cell death and lamin cleavage is correlated to each other. Therefore, taiwaniaflavone inhibited Aβ42 induced lamin cleavage as well as cell death but how it was inhibiting the lamin fragmentation process which was not clear. This needs to be studied in detail in order to design AD treatment.

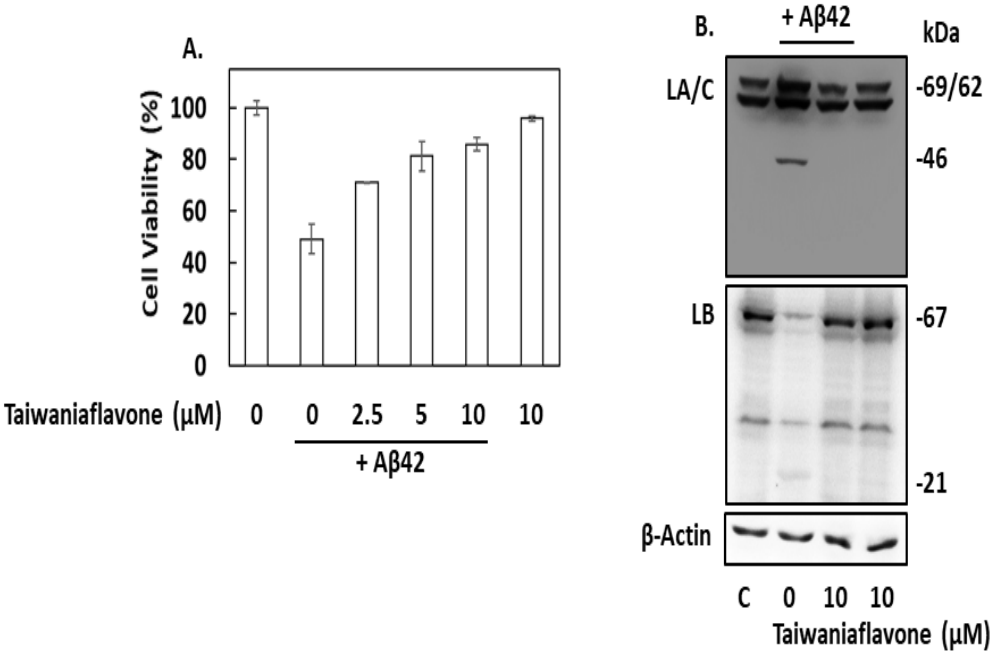

Figure 26. Effect of taiwaniaflavone on Aβ42 induced cell death and lamin fragmentation. Here 20 μM Aβ42 was used to treat the Hela cells for 2+22 h. (A) Hela cells (15 X 10^3) were treated with Aβ42 in the presence of indicated concentration of taiwaniaflavone. After treatment, survival rates were assessed by MTT reduction method. Results are average of three experiments and expressed as mean ± SD. (B) After treatment, cell lysates were prepared to check LA/C and LB by performing western blotting. For ensuring equal loading, β-actin was used. Molecular weights (in kDa) are donated at the ride side.

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