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NEUROPROTECTIVE ACTIVITY OF GUANOSINE IN AN *IN VITRO* MODEL OF ALZHEIMER'S DISEASE

PhD thesis introduced by
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INDEX

1. INTRODUCTION

The clinical picture of dementia as a deterioration in cognition in later years has been recognized for centuries but understood in its current formulation only for the past three decades. In the late 19th and early 20th centuries the shift in the conceptualization of mental illness away from moral causation towards disease etiologies extended to dementia, which was then conceived as an effect of aging and related to arteriosclerosis. Alzheimer's disease (AD) did not emerge as a distinct disease until the mid-20th century, which led to the recognition of multiple diseases of cognition with different etiologies. The dementias can be categorized according to clinical presentation, neuropathology and/or etiology. Parkinson's group (including Lewy Body disease, dementia of Parkinson's and Alzheimer's dementia with Parkinson's); the frontotemporal group (including Pick's disease and Semantic dementia); and the vascular group (including large and small vessel disease).

1.1 ALZHEIMER'S DISEASE

AD is the most common form of dementia, accounting for 50–60% of all cases. The prevalence of dementia is below 1% in individuals aged 60–64 years, but shows an almost exponential increase with age, so that in people aged 85 years or older the prevalence is between 24% and 33% in the Western world. Representative data from developing countries are sparse, but about 60% of patients with dementia are estimated to live in this part of the world. AD is very common and thus is a major publichealth problem. In 2001, more than 24 million people had dementia, a number that is expected to double every 20 years up to 81 million in 2040 because of

the anticipated increase in life expectancy. Besides ageing, which is the most obvious risk factor for the disease, epidemiological studies have suggested several tentative associations. Some can be linked to a decreased reserve capacity of the brain, including reduced brain size, low educational and occupational attainment, low mental ability in early life, and reduced mental and physical activity during late life. The brain reserve capacity is determined by the number of neurons and their synaptic and dendritic arborisation together with lifestyle-related cognitive strategies. A low reserve capacity has been linked with early presentation of some pathological changes of the disease. Moreover, several epidemiological studies have shown that head injury could be a risk factor (Blennow k, 2006).

1.1.1 CLINICAL FEATURES

Short-term memory loss and visual-spatial confusion are one of the earliest clinical manifestations in AD. As the disease progresses, symptoms include confusion, irritability and aggression, mood swings, language breakdown, long-term memory loss, and the general withdrawal of the sufferer as their senses decline and patients are unable to recognize familiar objects or persons. Gradually, bodily functions are lost, ultimately leading to death. Individual prognosis is difficult to assess, as the duration of the disease varies. AD develops for an indeterminate period of time before becoming fully apparent, and it can progress undiagnosed for years. The mean life expectancy following diagnosis is approximately seven years. Fewer than three percent of individuals live more than fourteen years after diagnosis (Oddo, 2008). Because AD cannot be cured and is degenerative, management of patients is essential. The role of the main caregiver is often taken by the spouse or a close relative. Alzheimer's disease is known for placing a great burden on caregivers; the pressures can be wide-ranging,

involving social, psychological, physical, and economic elements of the caregiver's life. In developed countries, AD is one of the most economically costly diseases to society.

1.1.2 ETIOLOGY AND PATHOGENESIS OF AD

AD is named after Dr. Alois Alzheimer, German doctor who in 1907 noticed changes in the brain tissue of a woman who had died of an unusual mental illness. There is a loss of nerve cells in areas of the brain that are vital to memory and other mental abilities. In AD, the progressive nature of neurodegeneration suggests an age-dependent process that ultimately leads to degeneration of synaptic afferent systems, dendritic and neuronal damage, and the formation of abnormal protein aggregates throughout the brain. The age-related susceptibility of the brain to neurodegenerative disease may be inherent in the susceptibility of individual neurons to various stressors. AD is accompanied by three main structural changes in the brain: diffuse loss of neurons, intracellular protein deposits termed neurofibrillary tangles (NFT) consisting of hyperphosphorylated tau protein and extracellular protein deposits termed amyloid (βA) or senile plaques, surrounded by dystrophic neurites. Genome scale analysis revealed marked differences in gene expression between AD and normal brain. Such analysis is a potentially powerful approach to listing genes related to the pathogenesis.

1.1.3 MOLECULAR MECHANISMS

Two major hypothesis have been postulated to explain the molecular mechanism of disease: the cholinergic hypothesis and the amyloid cascade hypothesis. Many studies support the cholinergic hypothesis, showing that a dysfunctional cholinergic system is sufficient to produce memory deficit in animal models that are analogous to Alzheimer’s dementia. Brains from

AD patients show degeneration of cholinergic neurons of the basal fore brain. A marked decline in cholinergic markers, choline acetyltransferase and acetyl cholinesterase has been reported in the cerebral cortex of AD brain. Although cholinergic deficits cannot fully account for the overall neuropathological features observed in AD, it represents a significant part of AD etiology and further research regarding the preferential vulnerability of this system in AD is warranted. The other hypothesis, the amyloid cascade hypothesis states that the neurodegenerative process observed in AD brains is a series of events triggered by the abnormal processing of the APP that causes production, aggregation, deposition and toxicity of its βA derivative. Currently the amyloid cascade hypothesis has received considerable support from molecular genetic studies. Several genes have been localized and/or identified in AD patients. It may be possible, for example, to correlate specific biochemical reactions with changes in these functions. At least four genes are implicated in the pathophysiology of AD such as mutations of the APP gene14 on chromosome 21, polymorphism of the apolipoprotein E (ApoE) on chromosome 19 and mutations in the presenilin 1 (PS1) and presenilin 2 (PS2) genes on chromosome 14 and 1, respectively. Mutation in either the APP gene on chromosome 21 or presenilin (PS) gene that increase the βA production have been described in a modest number of patients with familial early onset AD. This peptide is derived from the membrane bound APP which can be processed via two distinct processing pathways: the amyloidogenic pathway that liberates the βA peptide and the nonamyloidogenic pathway which precludes the formation of βA and instead generates a secreted form of APP, sAPPa. The proponents of the amyloid cascade hypothesis argue the increased production of the longer amyloid peptide βA_{1-42}, which forms the plaque core. This βA_{1-42} peptide goes on to induce all the subsequent pathology including tau aggregation, phosphorylation, neuronal attrition and chemical

dementia. However, all the molecular and cellular events occurring in different forms of AD cannot be explained by classical amyloid hypothesis. Mechanisms have emerged in which the fibrillar amyloid deposits does not act as the primary effector of this neurodegeneration. No correlation has been reported between senile plaques and the degree of dementia. Most of the experiments conducted on βA toxicity have been performed with a suspension of fibrils. Recent studies have detected novel and stable intraneuronal pools of insoluble βA deposits, which are generated in the endoplasmic reticulum/intermediate compartment (ER/IC), challenging the prevailing view that βA deposition is initiated in the extracellular space by the secretion of βA peptide. Accordingly, it has been observed that intracellular deposits of βA are mainly concentrated in detergent insoluble glycolipid enriched membrane domains. It is presently thought that the amyloidogenic process that converts soluble βA from its relatively inert form to its putative pathogenic state into amyloid fibrils, is a nucleation-dependent process that probably requires structural transitions of βA. An amyloid deposit might be seeded with βA derived locally from disintegrating neuronal membrane or from the insoluble pool rather than from diffuse βA deposits or from secreted βA This internalization could lead to a protection of the cells against βA peptide by its total clearance or to cell death through long term intracellular aggregation.

Amyloidogenesis:

Amyloid plaques in AD are not constituted by a single protein derived from APP. Many proteins, such as proteases, protease inhibitors, and microtubule-associated proteins (e.g., tau), are deposited in plaques. ApoE also associates with AD plaques. These additional plaque-associated proteins may contribute to the aggregation of the β-peptide into fibrils and the formation of insoluble βA arrays within the extracellular space.

Regardless of the underlying amyloid fibril protein/peptide or associated disease, isolated amyloid fibrils when examined by negative staining or high resolution electron microscopy are composed of multiple filaments or protofibrils twisted around each other forming nonbranching fibrils typically 7–10 nm in diameter with a crossed β-pleated sheet conformation. βA has the propensity of self-aggregation and facilitate nucleation-dependent polymerization of the shorter form. Studies have shown that aggregation might be modulated by some of the proteins present in senile plaques. Factors responsible for or inducing aggregation of peptide, as well as the cellular context in which it occurs, have yet to be established. All evidence in favor of fibrillation, over production of βA, decreased clearance of secreted βA and involvement of aggregating factors, might lead to neurodegeneration.

Role of APP and secretases:

Amyloid precursor protein is a 770 residue ubiquitous glycosylated transmembrane protein with a large hydrophilic aminoterminal extracellular domain, a single hydrophobic putative transmembrane domain and a small carboxy-terminal cytoplasmic domain. It is the source of βA peptide found in neuritic plaques of AD. A single transmembrane domain consisting of 23 residues. APP is coded by a single copy gene located on the mid portion of the long arm of human chromosome 21. The APP proteins mature in the endoplasmic reticulum and Golgi apparatus and exhibit post-translational modifications, including phosphorylation, glycosylation and sulfation. However, the abnormal processing of the APP triggers the aggregation, deposition and toxicity of its βA derivative. The production of βA from APP is dependent upon the activities of two enzymes β-secretase and γ-secretase. On its way to the cell surface, two different proteases, α- and β-secretase can cleave at different positions

within the APP molecule leading to the release of the large soluble N-terminal fragments, α-APPs and β-APPs, respectively (Fig. 1). Cleavage by α-secretase occurs within the region containing βA and thus precludes formation of βA. In contrast, β-secretase cleavage generates the free N-terminus of βA and is therefore considered the first critical step in amyloid formation. Two C-terminal fragments C83 and C99 are also produced by α-secretase and β-secretase, respectively. Both C83 and C99 can be further cleaved by γ-secretase, within the APP transmembrane domain which leads to the release and secretion of βA from C99 and of p3, a shortened, presumably nonpathogenic, βA from C83. Mutations in genes of APP and presenilins have been shown to modify the processing of APP, through modulation of secretase activities. This altered processing leads to an increase in βA. It might trigger aggregation and induce the neurodegeneration process.

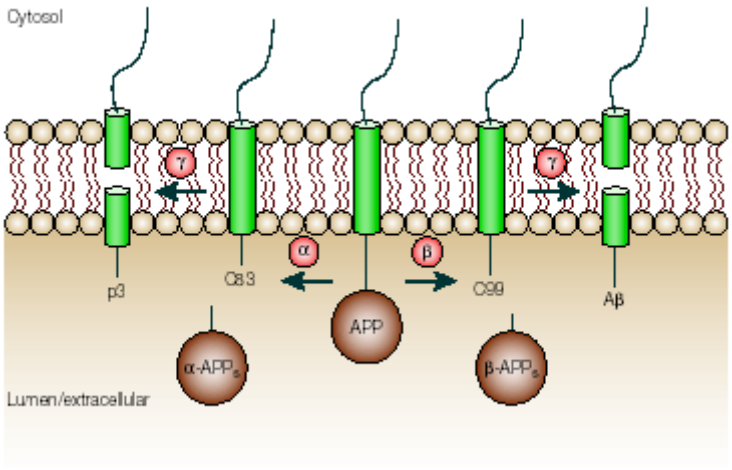


Figure 1. The amyloid-β precursor protein (APP) is processed by α-secretase (non-amyloidogenic pathway) or β-secretase (amyloidogenic pathway) with βA generation.

Role of tau proteins:

Almost in parallel with the identification of βA in plaques, tangles were shown to be composed of abnormally hyperphosphorylated tau protein. Tau is a normal axonal protein that binds to microtubules through its

microtubule-binding domains, thereby promoting microtubule assembly and stability. Tau phosphorylation is regulated by the balance between multiple kinases and phosphates. Tau hyperphosphorylation in Alzheimer's disease starts intracellularly and leads to sequestration of normal tau and other microtubule-associated proteins, which causes disassembly of microtubules and thus impaired axonal transport, compromising neuronal and synaptic function. Tau also becomes prone to aggregation into insoluble fibrils in tangles, further compromising neuronal function. Tau pathology starts early in the disease process in neurons in the transentorhinal region, spreads to the hippocampus and amygdala, and later to the neocortical association areas. Whether tau hyperphosphorylation and tangle formation are a cause or consequence of Alzheimer's disease is unknown (Blennow, 2006).

1.2 PATHOLOGICAL ROLE OF AMYLOID IN AD

Diverse lines of evidence suggest that βA plays a central role in the pathogenesis of neuronal dysfunction in AD, yet the βA hypothesis remains controversial, not least because the quantity and temporal progression of amyloid plaques do not show a simple relationship to the clinical progression of the disease.

1.2.1 THE EMERGING ROLE OF SOLUBLE βA

Recent studies suggest that the relatively weak correlation between plaque burden and severity of cognitive impairment may be explained by the activity of multiple different βA assembly forms and that early memory impairment may be mediated by soluble low-n oligomers. At ambient or body temperature and at concentrations 10–20 μM, both synthetic $\beta A_{1\text{-}40}$ and $\beta A_{1\text{-}42}$ self-associate to form low-*n* oligomers, PFs (protofibrils) and

fibrils (Fig. 2). An important caveat when considering the cellular effects of different βA assemblies is the highly dynamic nature of βA aggregation. Because intermediates can further associate into higher-ordered aggregates, it is difficult to unambiguously ascribe cytopathological activity to a discrete species. However, amyloid oligomers, which represent intermediates in the fibril formation process may be primarily responsible for amyloid pathogenesis, rather than the mature fibrils that accumulate as large aggregates (Irvine, 2008).

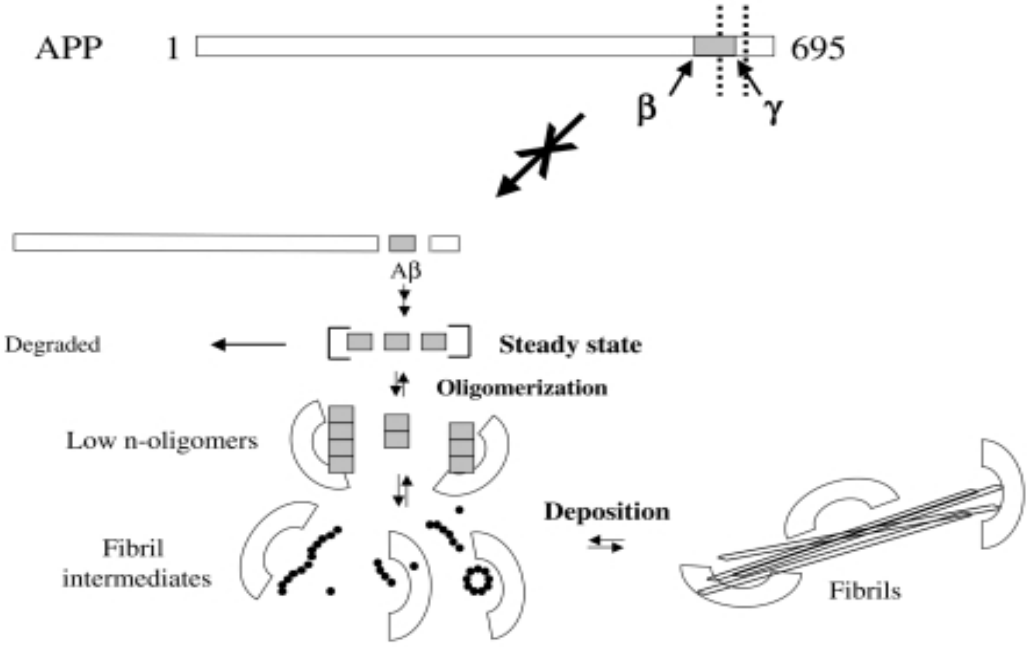


Figure 2: Production of βA by proteolytic cleavage from APP followed by association of βA to form oligomers and fibrils.

1.2.2 COMMON PATHWAY OF AMYLOID FORMATION

Amyloid fibrils accumulate in degenerative diseases as a consequence of the intermolecular hydrogen bonding of extended polypeptide strands that arise as a consequence of protein mis-folding. Amyloids from different diseases may share a common pathway for fibril formation. The initiating event is protein mis-folding or denaturation, which results in the acquisition

of the ability to aggregate in an infinitely propagating fashion. Quasi-stable intermediate aggregates ranging from dimers up to particles of a million Dalton or greater have been observed by a variety of methods. Soluble spherical aggregates of approximately 3-10 nm have been observed for many different types of amyloids by electron and atomic force microscopy and these spherical oligomers appear to represent intermediates in the pathway of fibril formation. These spherical particles have also been called micelles, prefibrillar aggregates, protofibrils and ADDLs. At longer aggregation times, curvilinear fibers form that have a beaded appearance form. These structures have also been called “protofibrils” because they appear to be formed by the coalescence of the spherical subunits. Finally, these structures either anneal or undergo a conformational change to form mature 6-10 nm cross-fibrils that have either a smooth or helical morphology (Glabe, 2006; Harper, 1997). Amyloids also have a number of structural features in common. Amyloid fibrils have a “cross-β” structure, which indicates that the backbone hydrogen bonding is parallel to the fibril axis. Amyloids also bind characteristic dyes, like Congo red and thioflavin dyes, which may be a reflection of their common cross-β structure (LeVine, 1993). Amyloid oligomers also display a common structural motif that is distinct from fibrils based on the observation that a conformation dependent antibody specifically recognizes a common epitope on amyloid oligomers, but not fibrils, monomers or natively folded proteins for many different types of proteins. This indicates that the antibody recognizes a generic polypeptide backbone epitope that is independent of the amino acid sequence, but yet is shared in common among all types of amyloid oligomers. The anti-oligomer antibody also generically inhibits the toxicity of soluble oligomers examined in vitro (Kayed, 2003).

Pathogenic pathways of amyloid oligomers

The permeabilization of membranes by amyloid oligomers that is a common component of amyloid toxicity may represent the primary common mechanism of amyloid pathogenesis. It may initiate a series of downstream pathological events that represents a common pathway of degeneration in amyloid-related diseases. These events that may lie immediately downstream from membrane permeabilization may constitute a core group of common pathological events that ultimately result in cell dysfunction and death. Membrane permeabilization by amyloid oligomers and the concomitant increase in intracellular calcium may be the proximate initiator of several pathogenic pathways, including reactive oxygen species (ROS) production, altered signaling pathways and mitochondrial dysfunction. Many signaling pathways are regulated directly or indirectly by intracellular Ca^{2+} levels or membrane depolarization, including pathways leading to up regulation of autophagy and cell death. In particular, accumulation of Ca^{2+} in the matrix of mitochondria leads to an increase in ROS production, cytochrome C release and apoptosis. Amyloid oligomers may also directly permeabilize the mitochondrial membrane (Hashimoto, 2003). Thus, the increase in energy demand necessary to maintain ion homeostasis and membrane polarization may also be a source of mitochondrial stress. Mitochondrial dysfunction may also feed back to upstream pathways that regulate the level of mis-folded proteins because many chaperones and the proteasome system utilize ATP. Chronic inflammation may also be a component of the core degenerative pathway as it is frequently observed in neurodegenerative diseases. The failure to efficiently clear amyloid aggregates may also contribute to pathogenesis by stimulating autophagic programmed cell death.

1.2.3 THE INVOLVEMENT OF THE UBIQUITIN-PROTEASOME SYSTEM IN AD

The accumulation of βA and tau makes AD a proteins-misfolding disease and suggests that alterations in protein quality control mechanisms may be directly or indirectly involved in the disease pathogenesis. Protein clearance by the UPS occurs in two sequential steps, a tagging reaction and a subsequent degradation of the tagged proteins by the proteasome system (Oddo, 2008). Ubiquitin is a small, highly conserved peptide present in all eukaryotic cells that is conjugated to the proteins that needs to be targeted to the proteasome. This process occurs in three steps. First an ubiquitin monomer is activated in an ATP-dependent reaction by the ubiquitin-activating enzyme (E1). Subsequently ubiquitin is transferred to an ubiquitin conjugating enzyme (E2). In the final step, ubiquitin is transferred to the target protein viaan ubiquitin ligase (E3). The E3 ligase binds both the target protein and the complex E2-ubiquitin and facilitates the formation of a covalent bond between the ubiquitin monomer from the E2 enzyme and the target protein. Activated ubiquitin molecules are sequentially added to the first ubiquitin proteins to form a polyubiquitin chain. Proteins tagged with chains of four or more ubiquitins are recognized by the 26S proteasome for degradation. It is the E3 ligase that confers specificity to the process by selectively binding to a protein target. Ubiquitin monomers are liberated after proteasome degradation or are actively removed by the ubiquitin carboxyl-terminal hydrolases (Fig. 3) (Layfield, 2003).

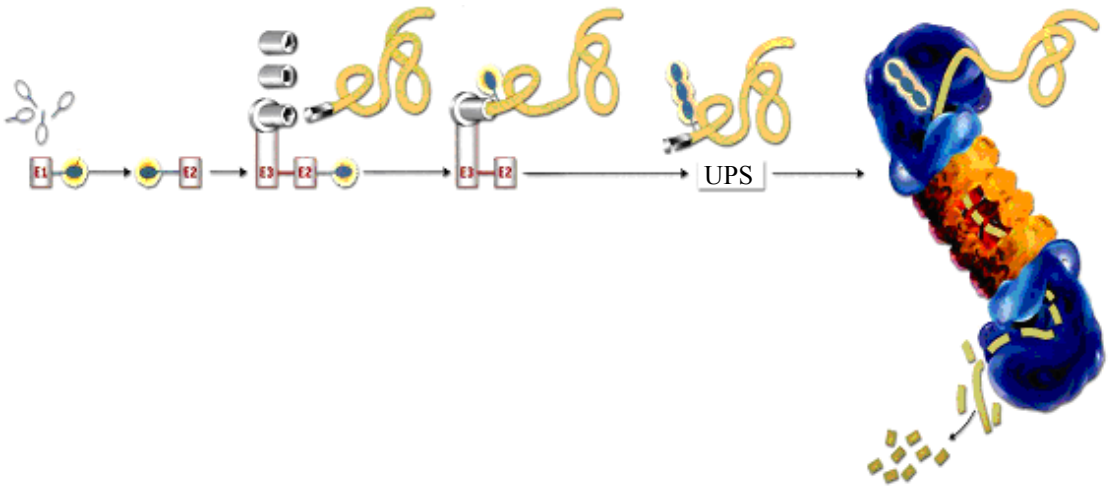


Figure 3: Overview of the ubiquitin–proteasome system (UPS). The protein ubiquitination pathway. Ubiquitin (Ub) is activated by E1 and transferred to the E2 enzyme and is, finally, conjugated to substrate proteins with a specific E3 ligase. Further polyubiquitination is required to target proteins for degradation.

Growing evidence suggest that alterations in the UPS function may be involved in AD pathogenesis. This view is supported by evidence showing that in AD brains ubiquitin accumulates in both plaques and tangles. It has also been shown that these structures contain ubiquitin-B mutant protein (UBB+1), a mutant ubiquitin carrying a 19-amino acid C-terminal extension generated by a transcriptional dinucleotide deletion. Notably, UBB+1 has been shown to block ubiquitin-dependent proteolysis in neuronal cells, to cause neuritic beading of mitochondria in associating with neuronal differentiation and it has been suggested to be a mediator of βA-induced neurotoxicity. The AD brain is also characterized by the accumulation of oxidized proteins, which may further exacerbate the decrease in proteasome activity. These data strongly argue that dysfunctional UPS function may be involved in AD pathogenesis (Oddo, 2008).

1.2.4 ROLE OF GLIAL CELLS IN AD

Glial cells are not only essential for maintaining a healthy well-functioning brain, but they also protect and aid the brain in the functional recovery

from injuries. The activation of glial cells in the CNS is the first defence mechanism against pathological abnormalities that occur in neurodegenerative diseases. Different signals can mediate and influence the fate of glial cell activation. A dysfunction of glial cell receptors, which alters the glial cells sense of their environment, can lead to the development of neurological diseases. Emerging evidences suggest that changes in the dividing process of glial cells during aging might lead to their abnormality and may provide clues to the pathogenesis of devastating diseases, such as AD. Nevertheless, because there is such diversity among each glia family (astrocyte, microglia and oligodendrocyte), they are still a mystery that holds the key to their modification. However, glial cell plasticity and diversity may allow the maturation of new functional cells, which can help the neuronal repair process (Farfara, 2008). In particular, besides a role mainly fagocitic, microglia activation plays multiple roles in modulating the stress response of neuronal cells and eventually the neuronal death and survival. Microglia can secrete various trophic factors, such as transforming growth factor beta and neurotrophins that could potentially increase the survival of damage neurons. It has been suggest that a proper regulation of microglial response to injury will arrest neuronal damage and promote neuronal survival, whereas a failure in the regulation will lead to chronic neurodegeneration. This suggest that the ability of microglia to induce or prevent neuronal death depends on reciprocal cellular interactions as well as on signals arising from injured neurons (Streit, 1999).

1.2.5 APOPTOSIS IN AD

The final step in AD is characterized by βA-induced apoptosis, a mechanism of cellular death activated by mitochondrial pathway or by the interaction with specific neuronal cell-surface receptors.

Mammalian apoptosis is regulated by the Bcl-2 family of proteins, the adaptor protein Apaf-1 (apoptotis protease-activating factor 1) and the cysteine protease caspase family. Neurons share the same basic apoptosis programme with all other cell types. However, different types of neurons, and neurons at different developmental stages, express different combinations of Bcl-2 and caspase family members, which is one way of providing the specificity of regulation. The Bcl-2 family of proteins has a crucial role in intracellular apoptotic signal transduction. This gene family includes both anti-apoptotic and pro-apoptotic proteins that contain one or more Bcl-2 homology domains. The major anti-apoptotic members of the Bcl-2 family, Bcl-2 and Bcl-xL, are localized to the mitochondrial outer membrane and to the endoplasmic reticulum and perinuclear membrane. Bax is a pro-apoptotic member of the Bcl-2 family that is widely expressed in the nervous system. During apoptosis, cytosolic Bax is translocated to the mitochondrial membrane where it induces cytochrome c release. Once in the cytosol, cytochrome c is a co-activator of Apaf-1 in the cleavage of procaspase-9 and execution of apoptosis through the mitochondrial pathway. Exposure of cells to βA peptide has been shown to result in mitochondrial perturbation and subsequent caspase activation. In addition, βA induces cytochrome c release via direct mitochondrial membrane permeabilization, which appears to be associated with profound changes in membrane lipid and protein structure. Thus, βA peptide also modulate protein levels of Bcl-2 family members, resulting in up-regulation of pro-apoptotic Bax and increased expression of anti-apoptotic Bcl-2.

A central role for βA protein is also supported by the effects of genetic mutations that cause familial Alzheimer's disease, all of which predispose to amyloid deposition, and by the observation that βA can be neurotoxic *in vitro* and *in vivo*. The toxicity of abnormal structural forms of βA provides a unifying theme with other age-related neurodegenerative disorders

characterized by the appearance of pathological protein structures. The mechanism of βA neurotoxicity and its precise cellular locus of action are unsettled, but it has been shown that βA can induce oxidative stress and elevate intracellular Ca^{2+} concentration. βA might induce apoptosis by interacting with neuronal receptors, including the receptor for advanced glycation endproducts (RAGE), which can mediate free-radical production, the p75 neurotrophin receptor, which can induce neuronal cell death, and the amyloid precursor protein, which can also induce neuronal cell death. These various βA-receptor interactions might activate several different cell-death-signalling pathways (Fig. 4). For example, βA can activate a set of immediate early genes similar to those induced by trophic factor withdrawal, and can activate caspases. Furthermore, neurons deficient in caspase-2 and caspase-12 have decreased vulnerability to βA toxicity, suggesting that selective caspase inhibition might be a potential therapeutic approach in Alzheimer's disease. Several recent studies suggest that presenilins might be λ-secretases, proteases that participate in the generation of βA, although this remains to be established definitively. Presenilin mutations can also increase neuronal vulnerability to apoptosis. Activation of microglial cells is a prominent feature of the inflammatory response in the brain in Alzheimer's disease that is likely to contribute to neuronal cell death. Microglial activation is associated with amyloid plaques and can be induced experimentally by βA. βA-induced microglial activation results in the secretion of TNF-α and other toxic factors that can induce neuronal apoptosis. Similar microglial-based mechanisms have been implicated in other neurodegerative disorders. Thus, pathological neuronal cell death might be a direct consequence of toxic insults such as βA, or an indirect consequence of a complex interaction between neurons, microglia and toxic factors.

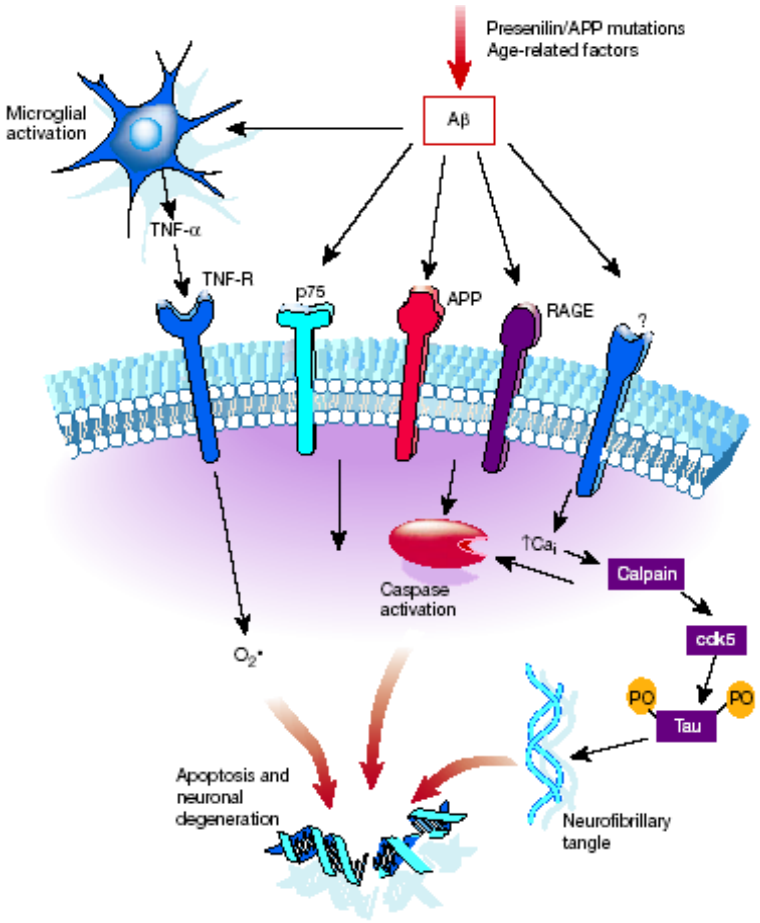


Figure 4: Cellular pathways of βA protein neurotoxicity in AD.

1.3 THERAPEUTIC INTERVENTIONS

With the advent of the new research on molecular mechanisms of AD pathogenesis, the promising prospects of development of drugs for the treatment of AD seem closer than ever. There probably is not one single cause, but several factors are important to describe the etiology of disease. Therefore, combination of compounds which act at more than one target site could be useful for the treatment (Parihar, 2004). Knowledge of the neurotransmitter disturbances in Alzheimer's disease has led to the development of drugs with symptomatic e. ects, which are approved in many countries. Research advances in the molecular pathogenesis of Alzheimer's disease have also led to new drug candidates with disease-modifying potential, which have now come to testing in clinical trials.

Epidemiological data have suggested additional drug candidates, some of which have been investigated in randomised trials.

1.3.1 SYMPTOMATIC TREATMENTS

Acetylcholinesterase inhibitors: The cholinergic hypothesis in Alzheimer's disease states that degeneration of cholinergic neurons in the basal forebrain nuclei causes disturbances in presynaptic cholinergic terminals in the hippocampus and neocortex, which is important for memory disturbances and other cognitive symptoms. One therapeutic approach to enhance cholinergic neurotransmission is to increase the availability of acetylcholine by inhibiting acetylcholinesterase, the enzyme that degrades acetylcholine in the synaptic cleft. The acetylcholinesterase inhibitors donepezil, rivastigmine, and galantamine are approved for clinical use in Alzheimer's disease. Benefits of acetylcholinesterase inhibitors are also seen for functional and behavioural symptoms. There is no evidence that these drugs differ in efficacy. They are not expected to change the natural course of Alzheimer's disease, but only to temporarily mitigate some of the symptoms.

Memantine: Glutamate is the major excitatory neurotransmitter in the brain. Under normal conditions, glutamate and the N-methyl-D-aspartate (NMDA) receptor have important roles for learning and memory processes. Under abnormal conditions, such as in Alzheimer's disease, increased glutamatergic activity can lead to sustained low-level activation of NMDA receptors, which may impair neuronal function. Memantine is a non-competitive NMDA-receptor antagonist that is believed to protect neurons from glutamate-mediated excitotoxicity without preventing the physiological NMDA-receptor activation needed for cognitive functioning. Memantine show modest positive effects on cognitive and behavioural

symptoms, and improved ability to perform activities. Additionally, combination therapy with donepezil and memantine show positive effects on symptoms relative to donepezil alone.

Treatment of behavioural signs: Behavioural signs, such as aggression, psychomotor agitation, and psychosis (hallucinations and delusions), are very common in patients with Alzheimer's disease, especially in the late stages of the disease. Such symptoms not only affect quality of life for patients and caregivers, but also contribute to care burden and economic cost. Atypical antipsychotic drugs produce fewer extrapyramidal side-effects than do conventional neuroleptics, and are thus preferred for the management of psychosis or agitation. Risperidone and olanzapine reduced the rate of aggression, agitation, and psychosis. Alternative treatments include anticonvulsants, such as divalproate and carbamazepine, and short-acting benzodiazepines, such as lorazepam and oxazepam. Additionally, the cholinergic deficits can contribute to the development of behavioural symptoms, and treatment with acetylcholinesterase inhibitors also shows improvements in behavioural symptoms.

1.3.2 DRUG CANDIDATES WITH POTENTIAL DISEASE-MODIFYING EFFECTS

The major focus has been to inhibit brain βA production and aggregation, and to increase βA clearance from the brain.

Secretase modulators:

Because βA is so closely associated with AD, the proteases that generate this peptide β- and γ-secretase are the top targets for therapeutic development. The finding that BACE1 knockout mice have abolished βA production without any clinical phenotype made BACE1 inhibitors an

attractive therapeutic strategy. β-secretase inhibitors have been developed to reduce brain βA concentrations in Alzheimer's disease transgenic mice. γ-Secretase is considerably more complicated than β-secretase. A single protein is responsible for β-secretase activity, whereas γ-secretase seems to be a complex of integral membrane proteins that includes presenilin. In particular, γ-secretase is composed of at least four integral membrane proteins (Fig. 5). The active site apparently resides in presenilin, an eight-transmembrane protein that contains two crucial aspartate residues (Asp) that are required for γ-secretase activity. Presenilin is cleaved into two pieces (see arrow for cleavage site) that remain associated as heterodimers. Each piece contributes one of the key aspartate residues. Nicastrin, as well as the newly identified APH1 and PEN2 proteins, associate with presenilin heterodimers and are required for protease activity. CHO on Nicastrin symbolizes glycosylation: a highly glycosylated form of Nicastrin is associated with γ-secretase activity.

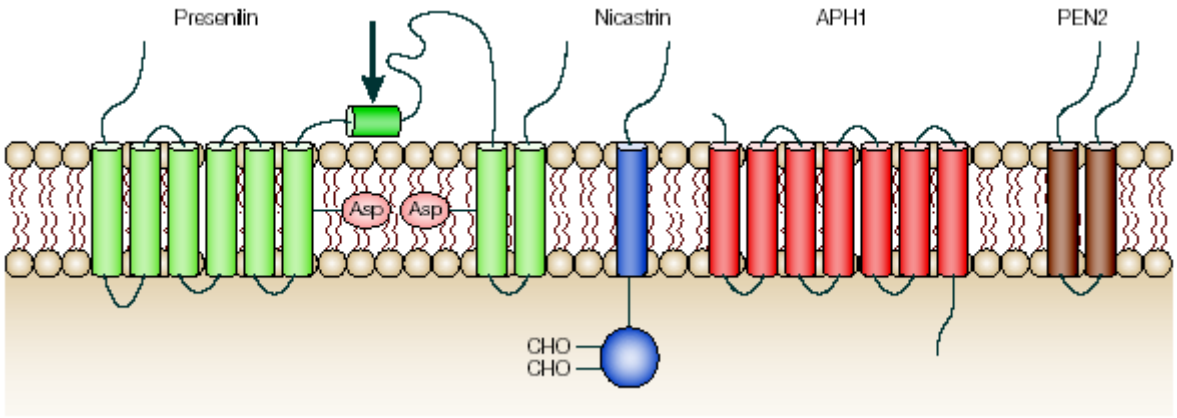


Figure 5: Components of the γ-secretase complex.

Despite the complexity of γ-secretase, identifying cellpermeable inhibitors has been easier for this proteas than for β-secretase. The probable reason is that the active site of γ-secretase is hydrophobic, which is reflected by the fact that the enzyme recognizes and cleaves transmembrane domains. So,

inhibitors of γ-secretase should also be hydrophobic to allow interaction with the enzyme, and this characteristic aids membrane permeability. Some of more potent inhibitors of γ-secretase are *transition state mimics, benzodiazepine, sulphonamides, dipeptide* and *benzocaprolactam.* For γ-secretase there is a concern about adverse effects because it cleaves other substrates such as Notch, might be crucial to normal physiology. Nevertheless, γ -secretase inhibitors have been developed that do not affect Notch signalling, and have shown good tolerability in phase I studies. Drugs that stimulate α-secretase can shift APP processing towards the nonamyloidogenic pathway, thus reducing βA production. Bryostatin, a protein kinase C activator currently tested in clinical trials as an anticancer drug, substantially enhances α-secretase processing of APP and reduces brain $\beta A_{1\text{-}42}$ concentrations in Alzheimer's disease transgenic mice.

βA immunotherapy:

Another general strategy for preventing βA build-up is to enhance the clearance of this peptide. A novel approach is immunization, either actively with the βA peptide itself or passively with anti-βA antibodies. In 1999 has been reported the effects of immunizing with $\beta A_{1\text{-}42}$ in transgenic mice harbouring an AD-causing mutant human APP, which normally develop βA-containing plaques within 3-6 months. Immunization at a young age (six weeks) prevented the formation of Aβ-containing plaques and subsequent AD-related neuropathological changes. Even the immunization of older mice (11 months old) led to a substantial reduction of plaque burden and pathology, raising the hope that this approach might not only be an effective means of prevention but could also possibly reverse some of the devastation in those already afflicted. Active immunization has been shown to reduce cognitive dysfunction in transgenic mice.

Similar results were obtained by use of passive immunisation with antibodies against βA. The effect might be mediated by anti-βA antibodies that bind to βA plaques and induce βA clearance by microglia, or alternatively bind soluble βA in the periphery, thereby driving an βA efflux from the brain. A related study by researchers showed that administration of anti-βA antibodies to transgenic mice leads to a rapid and large increase in peripheral βA levels and can reverse memory deficits. In this case, the antibodies apparently did not bind to brain βA deposits, indicating that the sequestration of plasma βA causes a marked reduction in βA deposition in the brain. Despite some discrepancies between the two studies, the overall results are promising, because they indicate that active immunization might not be necessary. Such findings are of immediate practical importance, as early clinical trials for active immunization with $βA_{1-42}$ caused serious central nervous system (CNS) inflammation in a small, but unacceptable, fraction of the subjects. Passive immunization might not be the only solution to this problem; other strategies include conjugating βA or βA epitopes to carrier proteins.

βA fibrillisation inhibitors:

Small peptides that interfere with βA-βA or βA-ApoE interactions can prevent the conformational change of βA to β-sheet structure and subsequent fibrillisation. Two such peptides have been shown to reduce βA fibrillisation in vitro and brain βA load in Alzheimer's disease transgenic mice, without inducing an immune response. Glycosaminoglycans bind βA and can promote its aggregation. The drug candidate NC-531 (Alzhemed) is a glycosaminoglycan mimetic designed to interfere with the association between glycosaminoglycans and βA, but the paucity of publications precludes a more detailed review. A phase III clinical trial is ongoing. Copper and zinc ions can induce βA aggregation and toxic effects. The

metal chelator clioquinol (PBT-1) reduces brain βA deposition in Alzheimer's disease transgenic mice. A small phase II trial showed marginal cognitive improvements with clioquinol, but because of toxic impurities (a di-iodo form of clioquinol) during production, further clinical trials have been halted. A new drug, PBT-2, which does not contain iodine, is currently undergoing clinical trials.

Anti-tau drugs:

In addition to amyloid deposits, the AD brain is also characterized by intraneuronal tangles that contain a filamentous form of the microtubule-associated protein tau. Hyperphosphorylation of tau leads to the formation of paired helical filaments of this protein. These neuronal tau filaments develop after the initial deposition of amyloid, indicating that amyloidosis is an initial pathogenic event, with tau being a downstream mediator of neuronal toxicity. Modulating tau phosphorylation is therefore also considered to be a reasonable therapeutic strategy; particularly targeting the responsible kinases, such as cyclin-dependent kinase 5 (CDK5) and glycogen-synthase kinase-3β (GSK-3β), for inhibition. However, since tau phosphorylation is regulated by the balance between multiple kinases and phosphates, inhibition of a single kinase might be insufficient to normalise tau phosphorylation and the development of agents that selectively block these kinases is still in the early stages.

1.3.3 DRUG CANDIDATES BASED ON EPIDEMIOLOGY

Epidemiological studies have served as the theoretical basis for several treatment approaches.

Anti-infiammatory drugs:

Plaques in Alzheimer's disease are accompanied by local infiammatory characteristics, but whether the infiammation contributes to neurotoxic effects or represents a secondary reaction to βA deposition is unclear. Most epidemiological studies suggest that the risk of the disease is reduced in patients treated with non-steroidal anti-infiammatory drugs (NSAIDs). Several, but not all, NSAIDs reduce brain βA burden in Alzheimer's disease transgenic mice, which may be mediated by either inhibition of cyclo-oxygenase (COX), or by a direct effect on γ-secretase, thereby reducing βA generation independently of COX inhibition. Clinical trials on anti-infiammatory drugs, including prednisone, hydroxychloroquine, and the selective COX-2 inhibitors celecoxib and rofecoxib, showed no effects on cognition in Alzheimer's disease. The first large-scale clinical trial on both non-selective and COX-2 selective NSAIDs in the disease was also disappointing. One explanation is that these drugs might be protective only if given during mid-life, but will not reverse the degenerative process in patients with established pathology. A primary prevention trial of NSAIDs has been started to test whether they can be protective in patients with MCI.

Cholesterol-lowering drugs:

The first link between cholesterol and Alzheimer's disease was suggested in a study reporting that rabbits fed with very high-cholesterol diet develop intracellular βA accumulation. However, when feeding Alzheimer's disease transgenic mice a high-cholesterol diet, both reduced and increased brain βA load has been reported. Retrospective case-control studies suggesting that treatment with cholesterol-lowering drugs (statins) reduce the incidence of the disease have received much attention. Subsequent studies on Alzheimer's disease transgenic mice also suggested that cholesterol-lowering drugs diminish brain βA load. However, more recent prospective

cohort studies have not shown any association between statin use and reduced risk of the disease. Furthermore, treatment trials in patients with the disease have not reported any change in plasma or CSF βA_{1-42} and a 12-month placebo controlled double-blind study on atorvastatin showed only borderline cognitive improvement.

Oestrogens:

Epidemiological studies have reported an association between reduced risk of dementia and postmenopausal oestrogen supplementation. Animal studies also suggest that oestrogens could have several beneficial effects on neuronal function. However, large randomised controlled clinical trials of oestrogens have not shown a reduced risk of the disease.

Antioxidants:

Large observational studies suggest that dietary intake of antioxidants, such as vitamin E, could reduce the risk of the disease. One randomised controlled clinical trial of vitamin E supplementation in the disease showed only a marginal effect on time to institutionalisation and need of care. However, another well-designed randomised controlled trial showed no effect of vitamin E supplementation on the rate of progression to Alzheimer's disease in MCI.

The fact that AD involves progressive cognitive deterioration, formation of neurofibrillary tangles, senile plaques and synapse and selective neuronal loss that progress slowly and leaves end stage patient's bed ridden. Deposition of βA has been presented as major culprit of AD pathogenesis. The ultimate goal is to block the formation of βA oligomers and fibrils to inhibit the fibrilization and fibrilization-dependent neurotoxicity. Using this kind of approach, however need detailed study of molecular nature of

secretases and also mechanism as to how do it interact with presenilin. Does it stop the cognitive decline in AD patients and what toxicity will β- and γ-secretases inhibitor have, present a major academic challenge and thus central to the future developments of a rational molecular therapy for AD. To date, it remains unknown in which percentage of patients hereditary factors are the cause of the disease. However, it is widely accepted that, apart from the above-mentioned mechanism of AD pathology, a substantial part of the disease is of multifactorial origin, with oxidative stress, mitochondrial dysfunction, inflammation and environmental factors. For this reason, the treatment will also be complex and might require different interventions for treatment. Being multifactorial pathogenicity of the disease, it is our opinion that a combined molecular, genetic and biochemical approaches should be employed for the development of drugs that can delay the onset of AD. The knowledge derived from these approaches could be extended from animal models to therapy of symptomatic AD patients.

1.4 PURINES IN THE CENTRAL AND THE PERIPHERAL NERVOUS SYSTEM

1.4.1 HISTORICAL OVERVIEW

Purine bases and their pyrimidine counterparts are the building blocks of the nucleic acids that form deoxyribonucleic acid (DNA) and ribonucleic acid (RNA). ATP is considered the universal cellular energy currency; as well as other purine nucleotides and nucleosides, it is involved in biochemical pathways and energy transfer within the cell. Cyclic nucleotides such as adenosine 3':5'-cylic monophosphate (cAMP) and guanosine 3':5'-cylic monophosphate (cGMP) act as important intracellular second messenger molecules during signal transduction. Although purines

have been traditionally viewed as having only intracellular roles, about 80 years ago Drury and Szent-Gyorgyi (1929) discovered that extracellular adenosine is a hormone released by the heart during ischemia. Over the last 30 years, however, the roles of adenosine and ATP as neurotransmitters and neuromodulators in the central, peripheral and enteric nervous systems have been elucidated (Burnstock, 1991). Specific receptors for ATP (Abbracchio, 1994) and adenosine (Fredholm, 1988) have now been identified. Outside the nervous system, adenosine and ATP were found to act not only as neurotransmitters, but also as hormones with "trophic" roles. Trophic substances affect the development, structure (or) and maintenance of a target cell or tissue over a characteristically longer time course than the milliseconds to seconds involved in neurotransmission. Trophic effects include the plastic changes involved in memory and learning, collateral sprouting of nerve processes, neuroprotection against noxious stimuli and even regulation of cells number through induction of apoptosis. In addition to their well-documented functions as neurotransmitters and modulators in the central, peripheral and enteric nervous systems (Burnstock, 1991), work from several laboratories has indicated a direct trophic role for extracellular purines in the development and maintenance of the nervous system and its response to disease or injury (Neary, 1996). For example, purine nucleotides may regulate neurite outgrowth (Gysbers, 1992; 1996), the proliferation of glial cells (Abbracchio, 1994; Pettifer, 1994; Kim, 1991; Neary, 1996) and of brain capillary endothelial cells (Rathbone, 1999). Purines have also been shown to regulate neurotrophin and pleiotrophin synthesis and release (Middlemiss, 1995; Ciccarelli, 1997), and to play a role in the activation of microglia and in glial scarring (Neary, 1996). Potentially, purine nucleotides and nucleosides may act as trophic agents on the cells of the nervous system in several ways. They may target glial cells directly,

inducing functional changes which modulate neuronal differentiation and they may stimulate the synthesis and the release of trophic factors from neuronal and non neuronal cells. Purines may enhance the effects of growth factors on their target cells and they may interact directly with neurons or neuronal precursors, eliciting neuritogenesis, biochemical differentiation, or the maintenance of existing neuritis or enhancing neuronal survival. Also purine nucleotides and nucleosides may act as trophic agents through a combination of these actions.

1.4.2 SOURCE OF PURINES IN THE CENTRAL AND PERIPHERAL NERVOUS SYSTEMS

If extracellular purines play physiological and pathological roles, they must be present at effective extracellular concentrations. The extracellular concentrations of purines depend on several factors which include the amount of purines released, the local extracellular volume, uptake mechanisms which internalize extracellular purines and the presence of extracellular enzymes which metabolize purines. Potential sources of extracellular purines in the nervous system include: cellular sources, from damaged, necrotic or lysed cells and release from neurons, glia, endothelial cells and platelets. Neurones and glia release purine nucleotides and nucleosides at rest and upon stimulation even if the molecular mechanisms of their release is greatly different from each others. ATP and GTP co-localize in synaptic vesicles of adrenergic, cholinergic and purinergic neurons and are released following depolarization by exocytosis. In astrocytes, ATP are probably released through some P-glycoprotein channels. Both glia and neurons have nucleoside transport systems which play an important role in regulating the extracellular concentrations of purine nucleosides. Once released extracellularly, purine nucleotides and nucleosides undergo a complex extracellular metabolism by many cell

surface located enzymes which have the same function of the corresponding intracellular enzymes. In fact, intracellularly, enzymatic mechanisms provide for the salvage of purine nucleosides and bases, thus assuring the continuous synthesis of ribo- and deoxyribo-nucleotidases and cholesterol precursors. But, extracellular nucleosides may undergo further metabolism. Adenosine deaminase (Ada), which converts adenosine (Ado) to inosine (Ino), is predominantly a cytosolic enzyme, which helps to maintain a strict control of Ado levels, thus avoiding the cytotoxic actions of Ado and especially deoxyadenosine in peripheral tissues as well as in brain. Intracellular Ino, or hypoxanthine (Hypo) derived from Ino by the activity of purine nucleoside phosphorylase (PNP), may be reutilized for salvage of inosine 5'monophosphate synthesis. This is a crucial point for metabolic recycling of both adenine- and guanine-based intracellular purine nucleotides (Harkness, 1988). Ada is also associated with the external surface of the cells in the CNS (Ciruela, 1996). Ada activity is most prominent in glia, where its activity is at least five times that in peripheral ciliary ganglion neurons and nine-fold that in central neurons (Ceballos, 1994). Ada has also been found on synaptosomes and synaptic vesicles, which also contain other ectoenzymes such as 5' nucleotidase and ecto-adenosine triphosphatase. The combined effect of ecto-5'-nucleotidase, nucleoside transport and Ada may serve to regulate the effective local concentration of Ado and therefore the extent of adenosine receptor activation. Indeed, binding of Ada to the A1 receptor enhances the affinity of the A1 adenosine receptor for its ligands, suggesting Ada enhances coupling between the A1 receptor and heterotrimeric G-proteins, thus increasing the ligand-induced second messenger production (Ciruela, 1996). Thus Ada, in addition to metabolizing Ado, may also facilitate signal transduction via the A1 receptor. Specific mention must be made of the metabolic fate of guanine (Gua) (Fig 6). Guanosine (Guo) and Gua are

readily interconvertible by PNP while Gua is deaminated by guanine deaminase (guanase), in an essentially irreversible reaction. The activity of guanase shows marked regional variations, up to 50 - fold, in various parts of the CNS. Guanase activity is high in the olfactory bulb and in the hippocampus but very low in the pons, medulla and cerebellum. Large regional variations in the activity of a brain enzyme often implies that it is associated with neurotransmitter metabolism. This has led several investigators to suggest that Guo may be a neurotransmitter in the CNS (McGeer, 1987).

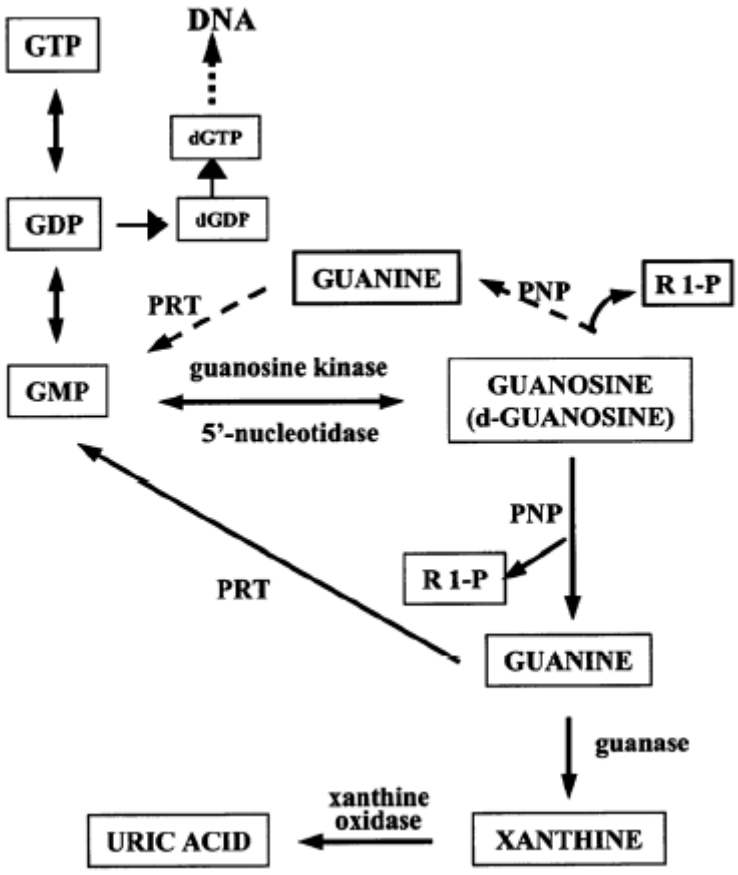


Figure 6: Intracellular metabolism of guanine-based purines.

1.4.3 GUANOSINE

Guo participates in structural and regulatory components of cells, but also exists extracellularly (Ciccarelli, 1999). In brain, extracellular guanine-based purines are primarily released from glial cells, likely mostly as nucleotides that are metabolized by ectonucleotidases to extracellular Guo (eGuo) (Ciccarelli, 2001). Stimulation of astrocyte cultures by, for example, combined oxygen-glucose deprivation or field electrical stimulation markedly increases eGuo (Ciccarelli, 1999), and *in vivo* focal brain injury elevates eGuo for up to 1 week. eGuo exerts numerous neurotrophic effects including: proliferation of glial cells, neurite outgrowth (Gysbers, 1996) synthesis and release of purines and trophic factors, such as nerve growth factor (NGF), from several cell types (Middlemiss, 1995; Di Iorio, 2001; Ciccarelli, 2000; Gysbers, 1992); and anti-apoptotic effects (Di Iorio, 2002; Di Iorio, 2004). It is suggested that the effects of Guo are mediated through putative G-protein linked cell-surface receptors (Traversa, 2002; Caciagli, 2000). Although the localization of guanine deaminase described above raises the possibility that eGuo and eGua may also play a role in synaptic transmission, most effects of eGuo thus far delineated in the CNS appear to be 'trophic', regulating cell growth, differentiation and survival. eGuo stimulates the proliferation of astrocytes *in vitro* (Kim, 1991), apparently due at least in part to the presence of very small quantities of contaminating microglia in the cultures, since the effects of Guo were increased in proportion to the number of microglia present (Ciccarelli, 2000). Guo apparently stimulated the release of interleukin-1 (Il-1) by microglia, which stimulates astrocyte proliferation. However, eGuo also stimulates proliferation of other cell lines that do not contain microglia, so it likely also directly affects proliferation of some cell types. In addition to its effects on microglia, eGuo also enhances the synthesis of NGF and basic fibroblast growth

factor (bFGF) mRNA in cultured mouse astrocytes (Middlemiss, 1995; Gysbers, 1992), and NGF and transforming growth factor β1 (TGFβ1) from rat astrocytes (Di Iorio, 2001). Not only does eGuo promote synthesis and release of peptide trophic factors but also eGuo stimulates release of adenine-based purines including adenosine. Exposure of astrocytes to 300 μM eGuo for 1 hour increased extracellular levels of endogenous adenosine in culture medium by 1,5 fold (Ciccarelli, 2000). This may contribute to the trophic effects of eGuo. A further biological effect of Guo that has been studied extensively is its ability to stimulate outgrowth of nerve processes (neurites). eGuo promotes neurite outgrowth in PC12 cells and also synergistically enhances NGF-dependent neurite outgrowth (Gysbers, 1996; 1992). PC12 cells treated with 300 μM Guo for 48 hours demonstrated neurite extensions in 6% of PC12 cells, significantly more than control conditions (Gysbers, 1996). The presence of 40 ng/ml of 2,5S NGF resulted in 20-35% of PC12 cells with neurite extensions, and the co-presence of 300 μM Guo with 40 ng/ml of 2,5S NGF resulted in 40-65% of PC12 cells with neurite extensions following 48 h (Paletzki, 2002). Moreover, primary cultures of fetal mouse neurons responded with neurite outgrowth to much lower concentrations of eGuo - 1 to 100 μM (Rathbone, 1998). Finally, eGuo has been shown to have neuroprotective effects *in vitro* and *in vivo*. Anti-apoptotic effects of eGuo have also been observed in astrocytes; cultures exposed to eGUO 1 hour before staurosporine had significantly fewer apoptotic cells than those exposed to staurosporine alone - 23% and 54% respectively (Di Iorio, 2004). Apoptosis induced by βA in SH-SY5Y human neuroblastoma cells was also reduced by eGuo (Pettifer, 2004).

Although these trophic effects of eGuo are well established, the way in which eGuo produces its effects is only now being elucidated. Initially, the

mitogenic effects of eGuo on astrocytes appeared be mediated in part through adenosine receptors, since the effects were at least partly inhibited by theophylline, an adenosine receptor antagonist (Kim, 1991). In a variety of primary and tumor cell lines from chicks, mice and humans the effects of eGuo were inhibited by 1,3-dipropyl-7-methylxanthine (DMPX), an adenosine A2 receptor antagonist, but not by 1,3,-dipropyl-8-(2-amino-4-chorophenyl)xanthine (PACPX), an A1 antagonist. And the ability of eGuo to stimulate [^{3}H]thymidine incorporation into cultures of rat fetal astrocytes was also partially inhibited by A1 and $A2_B$ receptor antagonists (Ciccarelli, 2000). Although the effects of eGuo on neurite outgrowth were also found to have a component that could be explained by eGuo-induced increases in extracellular adenosine in the medium (Gysbers, 1996), eGuo had a distinct extracellular effect unrelated to adenosine. Indeed, the neuritogenic effects of Guo were not inhibited by nitrobenzylthioinosine (NBTI) or dipyridamole, nucleoside transport inhibitors, suggesting Guo exerts its effects at the cell-surface (Gysbers, 1996; 1992). It has been found that Guo-induced effects on astrocytes are partially inhibited by purinergic receptor antagonists; however, Guo does not bind adenine purinoceptors with high affinity (Muller, 1993) and Guo did not act through an A1/A2 receptor, suggesting it may exert neuritogenic effects through its own cell-surface receptor (Gysbers, 1996; 1992). These considerations prompted a successful search for distinct Guo binding sites on cultured astrocytes and in whole rat brains (Di Iorio, 2001; Traversa, 2002). These have characteristics of G-protein coupled receptors. Subsequently it is apparent that eGuo activates intracellular signalling pathways that are apparently related to presumptive Guo receptors. Thus, for example, the anti-apoptotic effects of eGuo on βA-induced apoptosis in SH-SY5Y cells were inhibited by pretreatment with LY294002, a PI3K inhibitor, and PD98059, an MEK inhibitor. Guo further increased phosphorylation of Akt/PKB, which was

also abolished by LY294002 and PD98059 treatment (Pettifer, 2004). Similarly, in astrocytes exposed to the apoptosis-inducing agent staurosporine, eGuo reduces apoptosis and this effect is antagonised by pre-treatment with pertussis toxin (PTX). Differently, in this case, the eGuo effect was antagonized by SB202190 (an inhibitor of p38 MAPK pathway). eGuo also inhibited effects of glycogen synthase kinase-3β (GSK-3β), a pro-apoptotic enzyme a downstream target of the PI3K/Akt/PKB pathway (Di Iorio, 2004). Furthermore, Guo promoted upregulation of mRNA and protein expression of Bcl-2, an antiapoptotic protein and another downstream target of the PI3K/Akt/PKB pathway (Di Iorio, 2004). Thus eGuo appears to exert effects through both the p38 MAPK and the PI3K/Akt/PKB pathways. Signalling pathways involved in promotion of neurite outgrowth by eGuo are similar to those implicated in its anti-apoptotic effects. Guo treatment has been shown to promote increases in intracellular cAMP in astrocytes (Rathbone, 1991) and PC12 cells (Gysbers, 1996). Increases in cAMP were inhibited by SQ22536, an inhibitor of adenylate cyclase; however, neurotrophic effects of Guo were only partly inhibited (Gysbers, 1996). These findings suggest that Guo-induced effects are mediated by both cAMP-dependent and -independent mechanisms (Rathbone, 1991). Guo activated cAMP-dependent mechanisms are suggested to activate the MAPK cascade and potentially other protein kinases (Rathbone, 1991). Guo-induced effects are abolished by PTX, wortmannin and PD98059, MAPK inhibitors, suggesting Guo acts on a Gi-protein coupled cell-surface receptor to activate the MAPK pathway. Within the MAPK pathway, Guo has been found to activate and stimulate phosphorylation of MAP kinases ERK-1 and -2 in cultured astrocytes that may act to promote NGF synthesis and release (Di Iorio, 2001; Gysbers, 1992; Rathbone, 1998). The MAPK cascade has been identified as the major pathway by which NGF induces growth and

differentiation in PC12 cells (Rathbone, 1999) and may therefore represent a convergent mechanism for NGF and Guo. The biological significance of eGuo *in vivo* is only just beginning to be understood and the field is fertile for further exploration. Increases in eGuo are likely a natural response to injury, since eGuo is elevated for prolonged periods after experimental brain injury. But the new data indicating that it may also affect quiescent progenitor cells in the nervous system raise the possibility that the effects of eGuo potentially extend to more chronic situations as well as to acute injury. Moreover, manipulation of eGuo concentration may be of potential therapeutic value in certain pathological conditions. Studies of the metabolism of eGuo will of great importance. For example, it may elucidate the functional interrelationship of the extracellular guanine-based purine nucleotides, Guo and Gua. Extracellular guanine nucleotides also have trophic effects on cell proliferation (Rathbone, 1992; Kim, 1991), as well as differentiation of neurons (Gysbers, 1996; Rathbone, 1998) and myoblasts (Pietrangelo, 2002). Therefore their extracellular conversion to Guo may be one level at which their biological effects can be regulated. Moreover, the presence of high concentrations of guanine deaminase in certain discrete populations of neurons (Paletzki, 2002) imply that Guo and Gua have important extracellular roles in brain that extend beyond the trophic effects that have been elucidated to date. It is attempting to unravel the roles of these intriguing non-adenine-based purines. Better comprehension of their biological effects, their metabolism and the mechanisms through which their effects are mediated may prove useful not only in understanding physiological and pathological processes in the CNS, but also in targeting interventions for several pathological conditions.

2. AIM OF THE STUDY

AD is the most common age-related neurodegenerative disorder, and one of the most devastating diagnosis that patients and their families can receive. The clinical symptoms result from the deterioration of selective cognitive domains, particularly those related to memory (Oddo, 2008). AD is characterized by progressive memory loss and deficit of cognitive skills. The pathological hallmarks of AD include selective damage to synapses and neurons, neurofibrillary tangles, activated glia and presence of senile plaques (Selkoe 2001). βA is the major constituent of senile or amyloid plaques found in the brains of AD patients. βA is derived from the processing of the amyloid precursor protein, and it is thought to play a critical role in the onset or progression of AD (Ramalho, 2004). Pathological, biochemical and genetic evidences favour the 'amyloid hypothesis' of AD, although understanding this evidence requires an appreciation of how the βA peptide is produced (Wolfe, 2002). Different studies suggest that βA plays a central role in the pathogenesis of neuronal dysfunction in AD. In fact, amyloid oligomers, which represent intermediates in the fibril formation process, could be primarily responsible for amyloid pathogenesis, rather than the mature fibrils that accumulate as large aggregates (Hardy, 2002; Kayed, 2003). Growing evidences suggest that alterations in the UPS function may be involved in AD pathogenesis. For instance, amyloid oligomers can disrupt ion homeostasis inside and outside the cell and can interfere with the UPS. The soluble oligomers might serve as "all-purpose" β strands that can interact with transiently unfolded or nascent proteins, where interior β sheet edges are exposed. The proteins, trapped in misfolded states through this interaction, become substrates for ubiquitination, targeting them for proteasomal degradation.

This “misfolding trap” mechanism could potentially act in concert with other deleterious amyloid interactions, with proteasomes and chaperone proteins for example, ultimately overloading the UPS (Gruschus, 2008). This view is supported by evidence showing that in AD brains ubiquitin accumulates in both plaques and tangles. The accumulation of βA and tau makes AD a proteins-misfolding disease, or proteopathy, and suggests that alterations in protein quality control mechanisms may be directly or indirectly involved in the disease pathogenesis (Oddo, 2008).

Understanding the molecular pathways by which the various pathological alterations compromise neuronal function and integrity and lead to clinical symptoms has been a long-standing goal of AD research.

Purines and purine nucleotides are essential constituents of all living cells. ATP is used as an energy source for nearly all cellular activity, whereas adenine is a component of nucleic acids. Perhaps as a result of their ubiquitous nature, purines have also evolved as important molecules for both intracellular and extracellular signaling, roles that are distinct from their activity related to energy metabolism and the genetic transmission of information. Extracellular brain concentrations of purines - such as adenosine, Guo and inosine - are markedly elevated by a diverse array of pathological stimuli. Many of the effects of purines that are observed to a minor extent under normal conditions are greatly augmented during pathological events and are neuroprotective in that context (Ciccarelli, 1999; Hasko, 2004; Dunwiddie, 2001).

The aim of the study was to investigate the protective role afforded by the guanine-based purine nucleoside, Guo, in cellular cultures exposed to βA_{25-35} and βA_{1-42}, specific neurotoxins able to reproduce Alzheimer’s disease in SHSY5Y cells, a human neuroblastoma cell line. Different aspects involved in βA-induced toxicity were studied. Firstly, mitochondrial dysfunction and translocation of phosphatidylserine (PS), a

marker of apoptosis, were evaluated using MTT and Annexin-V assay, respectively. As a following step, we investigated the basic mechanism of neuroprotective effects of Guo against βA peptide-induced toxicity and we supposed that neuroprotection of Guo can be ascribed to its ability to modulate proteasome activity levels, using lactacystin, a specific inhibitor of proteasome. To rule out the possibility that Guo increases proteasome activity, the chymotrypsin-like activity was assessed employing the fluorogenic substrate Z-LLL-AMC. In parallel, we evaluated the modulation of ubiquitinated protein levels at similar experimental conditions adopted. We then estimated an involvement of anti and pro-apoptotic proteins, such as Bcl-2, Bad and Bax by western blot analysis.

3. EXPERIMENTAL PROCEDURES

3.1 MATERIALS

3.1.1 TREATMENTS

The **Guo** (Fig. 7) was purchased from Sigma-Chemical Co. (St Louis, Missouri-USA). All other reagents were of the highest grade of purity commercially available. The stock solution (40 mM) was prepared according to the followings proportions: 1/10 of Guo stock (0,4 M in NaOH 1 N); 6/10 distilled water; 3/10 HCl 0,1 N. Then, the working solutions were prepared after dilution of the stock solution.

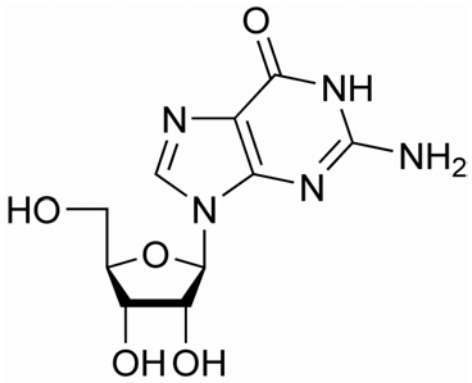


Figure 7: Chemical structure of Guo.

βA$_{1-42}$-peptide was purchased from Sigma-Chemical Co., while **βA$_{25-35}$**-peptide from Bachem (Bubendorf-Switzerland).

H-Asp-Ala-Glu-Phe-Arg-His-Asp-Ser-Gly-Tyr-Glu-Val-His-His-Gln-Lys-Leu-Val-Phe-Phe-Ala-Glu-Asp-Val-Gly-Ser-Asn-Lys-Gly-Ala-Ile-Ile-Gly-Leu-Met-Val-Gly-Gly-Val-Val-Ile-Ala-OH

↓

H-Gly-Ser-Asn-Lys-Gly-Ala-Ile-Ile-Gly-Leu-Met-OH

Figure 8: Sequences of βA$_{1-42}$ and βA$_{25-35}$.

βA-peptide preparation

βA_{1-42} peptides were first dissolved in hexafluoroisopropanol to 1 mg/ml, sonicated, incubated at room temperature for 24 h and lyophilized. The resulting unaggregated βA_{1-42} film was dissolved with dimethylsulfoxide and stored at -20 °C until use. The βA_{1-42} aggregation to oligomeric and fibrillar forms were prepared as described previously by Maezawa et al. (2006) and Dahlgren et al. (2002), respectively. The morphology of oligomeric and fibrillar βA_{1-42} forms obtained was checked by transmission electron microscopy (TEM).

3.1.2 CELL CULTURE

Human neuronal-like SH-SY5Y cells were purchased from Interlab Cell Line Collection (ICLC Genova-Italy) and were routinely grown at 37°C in a humidified incubator with 5% CO2 in Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum, 2 mM glutamine, 50 U/ml penicillin and 50 µg/ml streptomycin (Lonza Group Ltd.-Switzerland). To determine the neuroprotective effects of Guo, SH-SY5Y cells were seeded in 96-well plates at $3x10^4$ cells/well, whereas to evaluate apoptosis and necrosis, the cells were seeded in 60-mm cultures dishes at $1,5x10^6$ cells/dish. To evaluate proteasome activity and protein levels by western blot analysis after treatment with Guo, cells were seeded in 100-mm cultures dishes at $4x10^6$ cells/dish. All experiments were performed after 24 hours of incubation at 37°C in 5% CO2

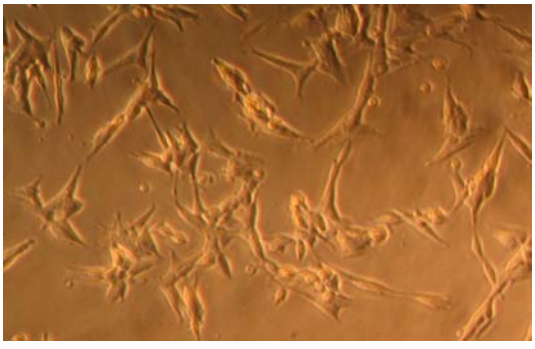


Figure 9: SH-SY5Y neuroblastoma cells observed at optic microscope (10x).

3.2 METHODS

3.2.1 TRANSMISSION ELECTRON MICROSCOPY (TEM)

To analyze the morphology of aggregated βA_{1-42} forms, the TEM was used as previously reported by Gupta et al. (2007). Briefly, aggregated $\beta A_{1–42}$ solution (40 μM) was absorbed onto formvar-carbon coated grids (200 mesh size) for 40 minutes and stained with 2% aqueous phosphotungstic acid solution before viewing with a Philips CM10 transmission electron microscope at 80 kV.

3.2.2 CELL VIABILITY ASSAY

The neuronal viability in terms of mitochondrial metabolic function was evaluated by the reduction of MTT to formazan. The assay is based on the cleavage of the yellow tetrazolium salt [3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium] (Sigma-Chemical Co.) to blue formazan crystals by metabolically active cells. Cultured cells were treated with βA_{25-35} and Guo at different concentrations and for different exposure times. Briefly, after removal of the treatment, SH-SY5Y cells were washed with phosphate buffered saline (PBS) and incubated with MTT (5 mg/ml) in PBS for 2 hours at 37°C in 5% CO_2. After further washing, the formazan crystals were dissolved with isopropanol and measured (570 nm, ref. 690 nm) with a spectrophotometer. The neuronal viability was expressed as a percentage of control cells.

3.2.3 INTRACELLULAR REACTIVE OXYGEN SPECIES FORMATION (ROS)

Cultured cells were treated with Guo at different concentrations and for different exposure times. Formation of intracellular ROS was determined using the non-fluorescent cell permeant compound 2',7'-dichlorodihydrofluorescein diacetate (DCFH-DA), as previously reported (Rosenkranz, 1992). Following de-esterification by endogenous esterases its polar metabolite, which diffuses out of the cell far more slowly than its parent compound, can be oxidized by peroxides producing the fluorescent compound 2',7'-dichlorofluorescein (DCF). The fluorescence of the cells from each well was measured using 485 nm excitation and 535 nm emission with a microplate spectrofluorometer (VICTOR3 V^{TM} Multilabel Counter, Perkin Elmer, Wellesley, MA, USA). The values were expressed as the percentage increase of intracellular oxidative species evoked by exposure to Guo and calculated by the formula [100 x (F_t – F_{nt})/F_{nt}], where F_t = fluorescence of treated cells and F_{nt} = fluorescence of non treated cells.

3.2.4 APOPTOSIS AND NECROSIS ASSAY

To determine the neuronal apoptosis and necrosis, the Annexin-V-FLUOS Staining Kit (Roche Diagnostics, Mannheim, Germany) was used according to the manufacturer's instructions. Cells were treated with Guo in presence of βA-peptide and Lactacystin (Sigma-Chemical Co.). The annexin-V binds phosphatidylserine exposed on the outside of the intact membrane of apoptotic cells whereas propidium iodide (PI) penetrates the broken membrane of necrotic cells and binds to DNA (Fig 10). The number of stained cells was counted by fluorescence microscopy and the values are expressed as percentages of annexin-V or PI-positive cells.

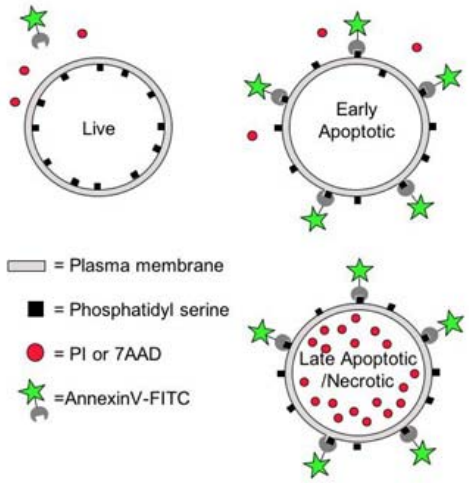


Figure 10: Schematic representation of the Annexin-V assay.

3.2.5 PROTEASOME ACTIVITY

After treatment of SH-SY5Y with Guo (25-75 μM) for 0-6 hours and/or inhibitors such as Lactacystin, LY294002 and PD98059 (Sigma-Chemical Co.), cells were washed with PBS, harvested, suspended in 50 mM Tris/HCl, and sonicated for 5 sec to lyse the cells. Protein concentrations were determined using the Bradford method (Bio-Rad, Milano-Italy). Proteasome activity was assayed by incubating 30 μg of protein with the proteasome substrate Z-LLL-AMC (carbobenzoxy-Leu-Leu-Leu-4-methylcoumarinyl-7-amide) (Merck Chemicals Ltd-UK) (20 μM) in a total volume of 100 μl of assay buffer (0.38 mM EDTA and 50 mM Tris/HCl, pH 7.5) at 37 °C for 4 hours. As a negative control, 30 μg of a cell lysate protein sample was emploied. AMC (4-methylcoumarinyl-7-amide) liberated from the substrate by proteasome activity was determined by measuring fluorescence (excitation, 380 nm; emission, 450 nm; Wallac-Victor^2), and reading from an AMC standard curve.

3.2.6 WESTERN BLOTTING

Western blot analysis was used to detect Ubiquitin, Bad, Bax and Bcl-2 protein levels (SantaCruz Biotechnology Inc-USA). Cultured cells were

treated with 75 μM of Guo at different exposure times. At the end of the drug treatment times, cells were washed twice with ice-cold PBS and then harvested at 4°C in a lysis buffer (25 mM Tris buffer, pH 7.4, containing 150 mM NaCl, 100 μM sodium orthovanadate, 1.5 mM $MgCl_2$, 1.0 mM EDTA, 1.0 mM EGTA, 1% NP40, 10% glycerol, 1 mM PMSF, 5 μg/ml leupeptin, 5 μg/ml aprotinin). After 20 min on ice cells were centrifuges at 14,000 rpm for 10 min at 4°C. Supernatant aliquots were used for determination of protein concentrations by Bradford method. Protein were diluted in Laemmli-SDS sample buffer and boiled for 5 minutes. Equal amounts of protein were loaded into each lane of 12% SDS-PAGE gel and resolved at 80 V constant. Gels were transferred onto nitrocellulose membrane (Sigma-Chemical Co.) at 100 V constant for 60 minutes at room temperature, and membranes were blocked in blocking buffer (PBS, 0.1% Tween-20 with 5% w/v non fat dry milk) for 2 h. Blots were incubated overnight at 4°C with specific primary antibodies. All primary antibodies were diluted 1:1000 in primary antibody dilution buffer (PBS, 0.1% Tween-20 with 2,5% w/v non fat dry milk). After washing three times for 15 min each with wash buffer (PBS, 0.1% Tween-20) membranes were exposed to a secondary antibody diluted 1:2,500 for 1 h at room temperature. Immunocomplexes were visualized using the enhancing chemiluminescence ECL kit, (GE-Healthcare, Milano-Italy) detection system. Densitometric analysis were performed for the quantification of the immunoblots using the Quantity One 1-D Analysis Software (Bio-Rad).

3.2.7 STATISTICAL ANALYSIS

Data are reported as mean ± SD of at least 3 independent experiments. Statistical analysis was performed using one-way ANOVA and the Dunnett post hoc test was used. Differences were considered significant at $p<0.05$. Analyses were performed using PRISM 3 software on a Windows platform.

4. RESULTS

4.1 GUANOSINE CITOTOXICITY

In order to study Guo citotoxicity in SH-SY5Y cells, mitochondrial activity loss was evaluated by MTT assay, ROS formation by a fluorimetric detection with DCF-DA and the translocation of phosphatidylserine (PS), a marker of apoptosis, using Annexin-V assay.

SH-SY5Y cells were treated with Guo at a concentration range of 12,5-400 μM for 24 hours. Treatment of SH-SY5Y cells with Guo for 24 hours caused loss of mitochondrial activity only at concentrations higher than 100 μM. Starting from 200 μM, we found a 20% loss of mitochondrial activity. Concentrations lower than 100 μM did not compromise cell viability after 24 hours of exposure (Fig.11a).

In the same experimental conditions, we evaluated free radical formation induced by 45 mins exposure of SH-SY5Y cells to Guo. Results demonstrated that similar concentrations of Guo induced free radical formation in a dose-dependent manner. In particular, neurons showed a significantly increase in ROS production at a concentration of 100 μM, with a maximum effect observed at 400 μM (40%) (Fig. 11b). Cell death was evaluated by Annexin/Propidium Iodide staining kit. Treatment of SH-SY5Y cells with Guo for 24 hours induced a strong and a dose-dependent increase of necrotic cells, whereas no increases of apoptotic cell percentage were seen (Fig. 11c).

The concentration of 75 μM has been chosen to study the neuroprotective activity of Guo against βA peptide-induced toxicity in SH-SY5Y cells.

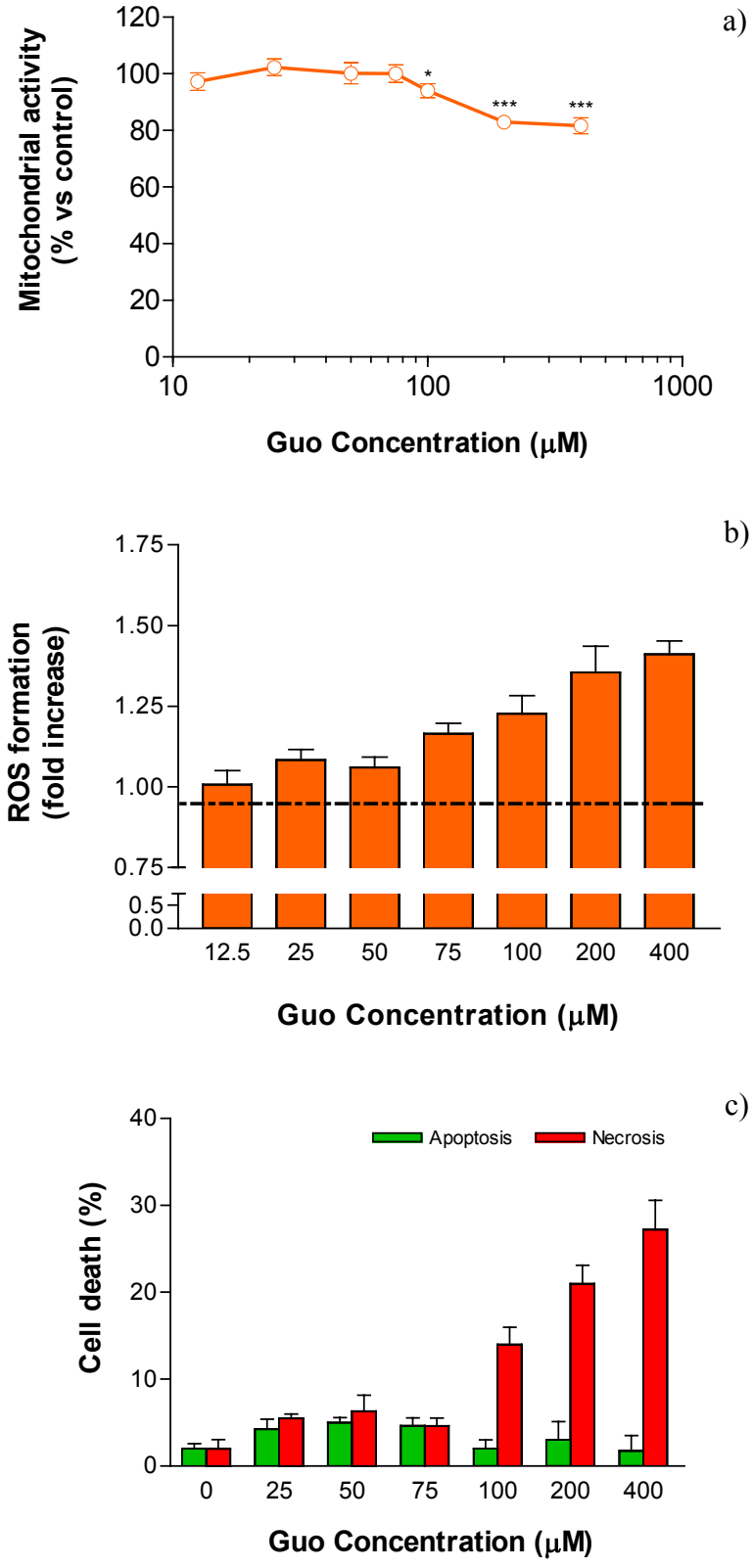


Figure 11: Effects of Guo in SH-SY5Y cells on (a) mitochondrial activity; (b) ROS formation; (c) apoptotic and necrotic cells formation. (*p<0.05; ***p<0.001 *vs* untreated samples; ANOVA with Dunnett post hoc test).

4.2 β-AMYLOID TOXICITY

In order to study βA-induced toxicity in neurons, cell viability reduction was evaluated by MTT assay. We first evaluated the neuronal damage induced by treatment with βA_{25-35} fragment. βA_{25-35} peptide is often used for modeling different aspects of AD. The 11-amino acid fragment is a C-terminal peptide of βA_{1-42} located in its hydrophobic functional domain. MTT reduction method was used to determine cell viability and to provide information on mitochondrial function.

The dose-dependent effect of βA_{25-35} on cell viability was assessed after 24 hours of exposure. SH-SY5Y cells were treated with βA_{25-35} at a concentration range of 0,01-100 μM. Treatment of neuronal cells induced a similar decrease of mitochondrial activity starting at 1 μM. Concentrations lower than 1 μM did not compromise cell viability (Fig 12a). According to the cell viability results, the EC_{50} value was approximately 1 μM; so this concentration was used for the following experiments.

We also evaluated the time-dependent cell viability reduction after treatment for 1-24 hours. βA_{25-35} treatment reduced cell viability at all the exposure times explored, starting from 1 hour (Fig. 12b).

To determine whether βA_{25-35}-induced cell death was attributed to apoptosis, the cells were exposed to 1 μM of peptide for 1-24 hours. Figure 12c showed the percentage of apoptotic and necrotic cells after treatment with βA_{25-35}. According to Annexin/propidium iodide double-dying results, the apoptotic cell death increased after 3 hours of treatment and the effect was almost the same until 24 hours of treatment. Instead, necrotic cell death increased after only 1 hour of βA_{25-35} treatment.

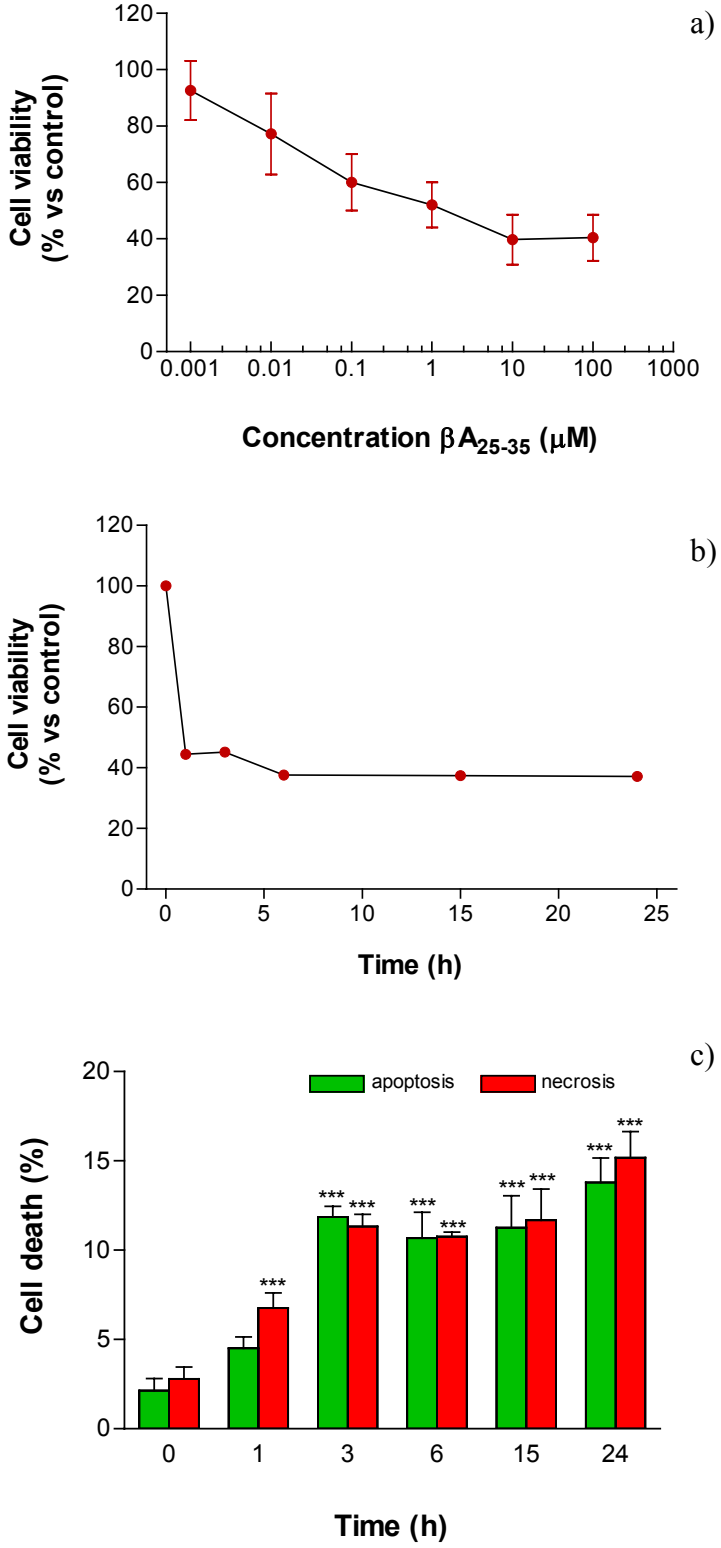
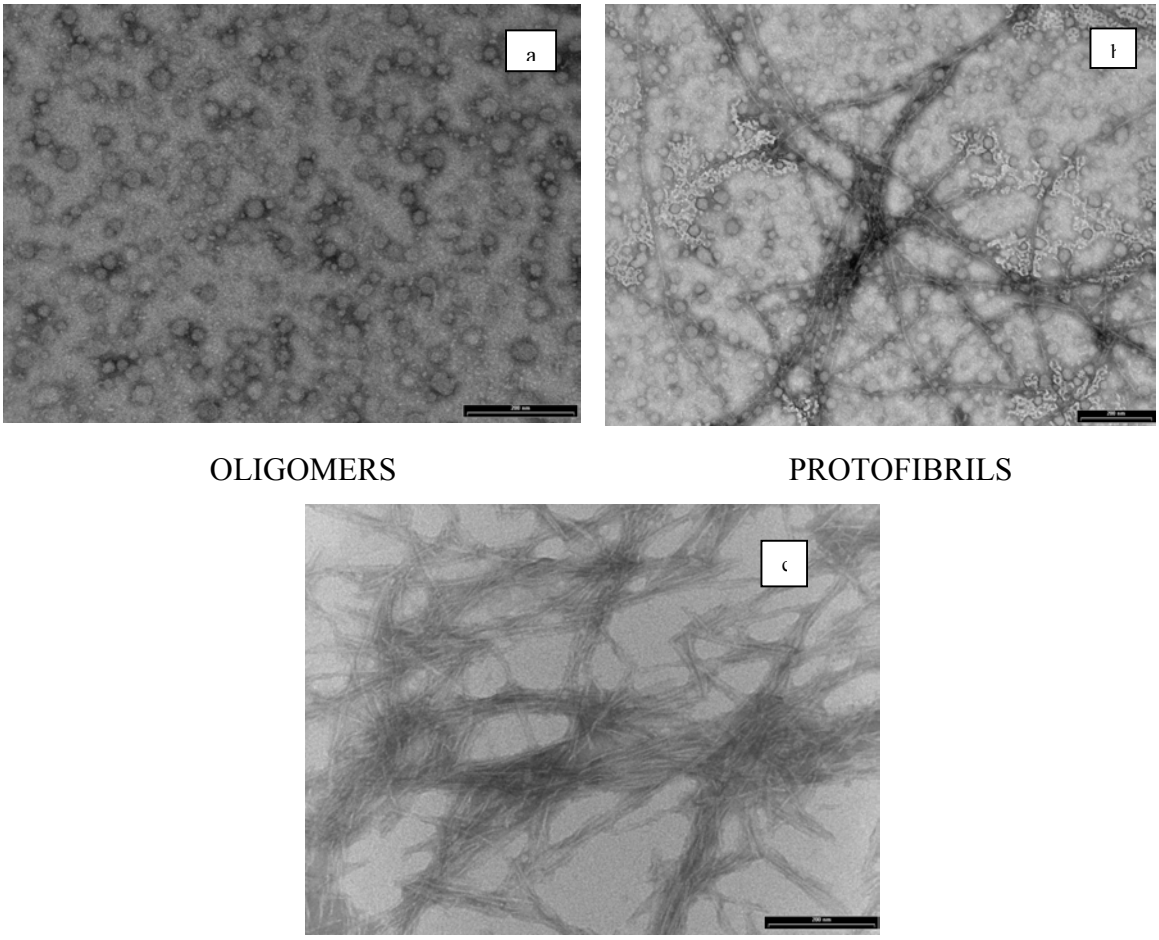


Figure 12: Effects of βA_{25-35}-toxicity in SH-SY5Y cells on (a) dose-dependent cell viability after 24 h; (b) time-dependent cell viability after treatment with 1 μM; (c) time-dependent apoptotic and necrotic cells formation after treatment with 1 μM. (***p<0.001 *vs* untreated samples; ANOVA with Dunnett post hoc test).

βA can exist in multiple assembly states - monomers, oligomers, protofibrils and fibrils - and it is the ability of this peptide to form fibrils and other intermediate states that impart the unique pathophysiological characteristics that define AD. Oligomeric and protofibrillar species are considered potent blockers of long-term potentiation, a form of synaptic plasticity. Based on this evidence, we then evaluated the kinetics of βA_{1-42} aggregation to oligomeric and fibrillar forms by transmission electron microscopy (TEM). As shown in Fig. 13a, the aggregation process started after 24 hours of incubation at 37°C (oligomers) and after 48 hours in the same conditions oligomers and protofibrils coexisted in the preparation (fig. 13b). The aggregation process was complete after 5 days when the presence of fibrils was evident (fig. 13c).



OLIGOMERS PROTOFIBRILS

FIBRILS

Figure 13: Aggregation kinetics of βA_{1-42} peptide [40 μM] in physiologic conditions.

We also estimated the effects of treatment with oligomeric and fibrillar forms of βA_{1-42} (1 μM). The apoptotic and necrotic cell death was induced by βA_{1-42} treatment for 1-24 hours. According to Annexin/propidium iodide double-dying results, the oligomeric form showed an increase of the apoptotic cell death after 3 hours of treatment, with a maximum peak observed at 15 hours. Necrotic cell death increased after 15 hours of oligomeric βA_{1-42}-treatment (Fig. 14a). Instead, the fibrillar form showed an increase of the apoptotic cell death after only 1 hour of treatment and the necrotic cell death increased after 6 hours (Fig. 14b).

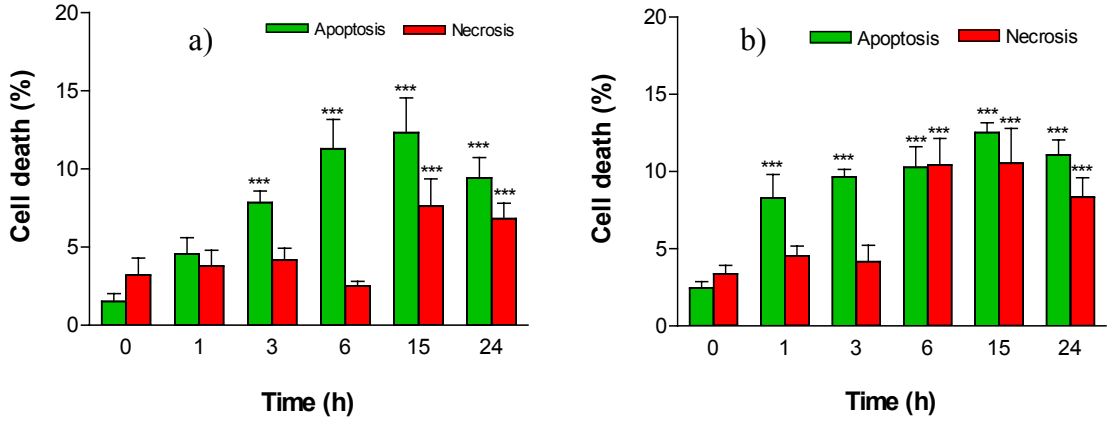


Figure 14: Apoptotic and necrotic cell death in SH-SY5Y cells after several times of exposure with (a) βA_{1-42} oligomeric form and (b) βA_{1-42} fibrillar form.

4.3 PROTECTIVE EFFECTS OF GUANOSINE

The protective effects of Guo against βA_{25-35}-induced neurotoxicity, was investigated in terms of cell viability loss in SH-SY5Y cells by using MTT assay. In particular, SH-SY5Y cells were treated with Guo during the βA_{25-35} short-term exposure. SH-SY5Y cells treatment for 3 hours with

Guo prevented the decrease in neuronal viability induced by βA_{25-35} (1 μM). The toxicity induced by βA_{25-35} was significantly reduced by cotreatment with 50 and 75 μM of Guo (Fig. 15a). To confirm these effects we also evaluated apoptosis and necrosis, at long term exposure, using Annexin-V Fluos Staining Kit. SH-SY5Y cell treatment for 24 hours with Guo (75 μM) after βA_{25-35} exposure showed an inhibitory effect on βA_{25-35}-induced apoptosis but not necrosis (Fig. 15b).

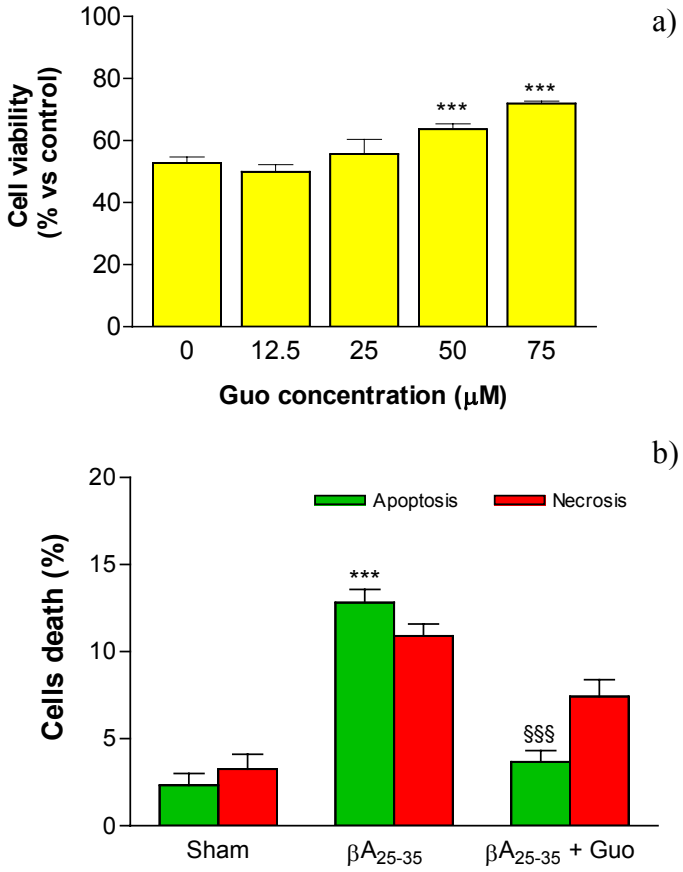


Figure 15: Protective effects of Guo on βA_{25-35}-induced cell death in SH-SY5Y cells: (a) neuronal viability after cotreatment for 3h; (b) apoptosis and necrosis after cotreatment for 24h. (***p<0.001 *vs* untreated samples; §§§ p<0.001 *vs* treated with Aβ alone. ANOVA with Dunnett post hoc test).

Subsequently, we determined whether the anti-apoptotic effects of Guo were also observed with oligomeric and fibrillar form of βA_{1-42} (1 μM). As shown in Figure 16, SH-SY5Y cell treatment with Guo (75 μM) after both oligomeric and fibrillar βA_{1-42}-exposure showed an inhibitory effect on βA_{1-42}-induced apoptosis, but not on necrosis.

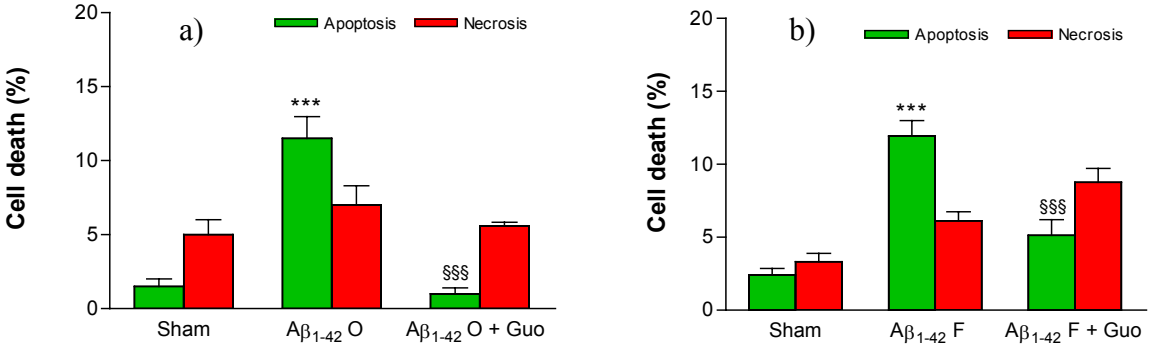


Figure 16: Cell death in SH-SY5Y cells treated with 1 μM of (a) oligomeric and (b) fibrillar form of βA_{1-42} in presence of Guo 75 μM for 24 h. (***p<0.001 *vs* untreated samples; §§§ p<0.001 *vs* treated with Aβ alone. ANOVA with Dunnett post hoc test).

4.4 INVOLVEMENT OF PROTEASOME IN GUO NEUROPROTECTION

To investigate whether neuroprotection of Guo can be ascribed to its ability to modulate proteasome activity levels, we used lactacystin, a specific and irreversible inhibitor of proteasome. We co-administered Guo (25-75 μM) and lactacystin (2,5 μM) with βA peptides (1 μM) (all the peptides in study), to SH-SY5Y. We used non apoptotic concentrations of the all compounds. As shown in fig. 17, the antiapoptotic effects observed with Guo were completely abolished by lactacystin.

To rule out the possibility that this effects resulted from an increase in proteasome activity induced by Guo, the chymotrypsin-like activity was assessed employing the fluorogenic proteasome substrate Z-LLL-AMC. The treatment of SH-SY5Y with Guo (75 μM for 0-6 h) induced a strong

time and dose-dependent increase of proteasome activity, with a peak at 2 hours treatment with 75 µM Guo. In similar experimental conditions, western blot analysis didn't show any increase of ubiquitinated protein levels.

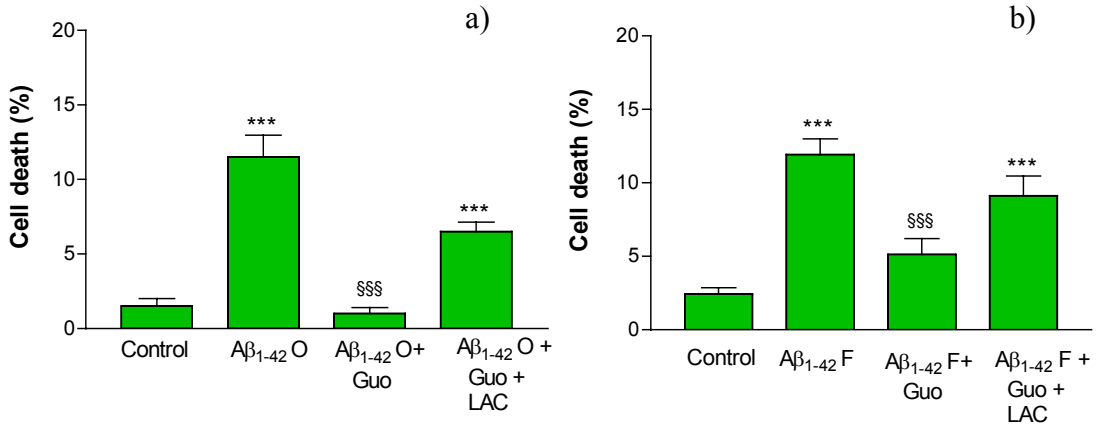


Figure 17: Apoptosis in SH-SY5Y cells treated with 1 µM of (a) monomeric βA_{25-35}, (b) oligomeric and (c) fibrillar form of βA_{1-42} in presence of Guo 75 µM and Lactacystin 2,5 µM for 24 h. (***p<0.001 *vs* untreated samples; §§§ p<0.001 *vs* treated with Aβ alone. ANOVA with Dunnett post hoc test).

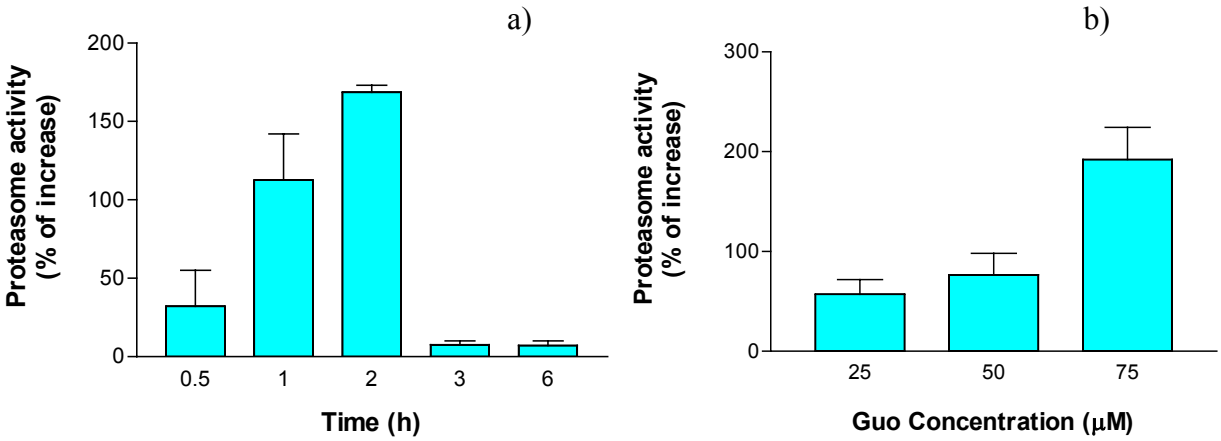


Figure 18: Proteolytic activities of proteasome in SH-SY5Y cells after treatment (a) for several times with Guo (75 µM) and (b) for 2 h with Guo (25-75 µM).

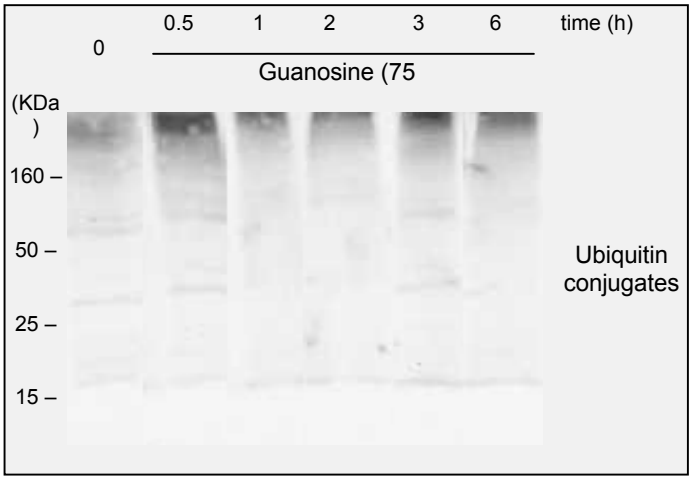


Figure 19: Ubiquitinated protein levels after treatment with Guo (75 μM) in SH-SY5Y cells for several times.

It is known that Guo is able to activate the PI3-Kinase pathway in rat cultured astrocytes as well as in mouse microglia (Di Iorio, 2004; D'Alimonte, 2007) and to determine a marked ERK1/2 and p38 phosphorylation in mouse microglial cells (D'Alimonte, 2007). So, in order to evaluate signal transduction pathways involved in Guo-induced up-regulation of proteasome activity, SH-SY5Y cells were treated with Guo (75 μM) in presence of specific inhibitors of the proteasome (Lactacystin), of PI3K/Akt/GSK3β (LY294002) and of ERK1/2/MAPK (PD98059) survival pathways. As shown in fig. 20, the co-treatment of neurons with Guo and the specific inhibitors did not increase the proteasome activity. According to our results, Guo neuroprotection was completely abolished by the specific inhibitor of proteasome. Guo-neuroprotection was also inhibited by LY294002 and PD98059, which could suggest that Guo acts on the proteasome through the activation of the survival pathways.

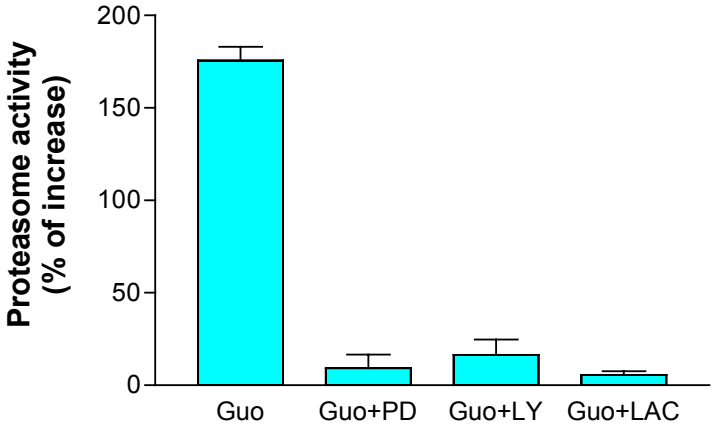


Figure 20: Proteolytic activities of proteasome in SH-SY5Y cells after treatment for 2 h with Guo (75 µM) and specific inhibitors: PD98059 (5µM); LY294002 (10 µM); Lactacystin (2,5 µM).

4.5 GUANOSINE AND PROTEIN EXPRESSION LEVELS

We then evaluated the capability of Guo to modulate anti- and pro-apoptotic proteins such as Bcl-2, Bad and Bax by western blot analysis. The time course (0-6 hours) of the protein activation induced by Guo (75 µM) was evaluated in SH-SY5Ycells. In particular, the analysis of anti-apoptotic Bcl-2 and pro-apoptotic Bad showed a decrease after 30 mins and 2 hours of treatment with Guo. Instead, Bax expression decreased after 30 mins, with a minimum peak after 2 hours. With longer treatments values were not substantially modified (Fig. 21).

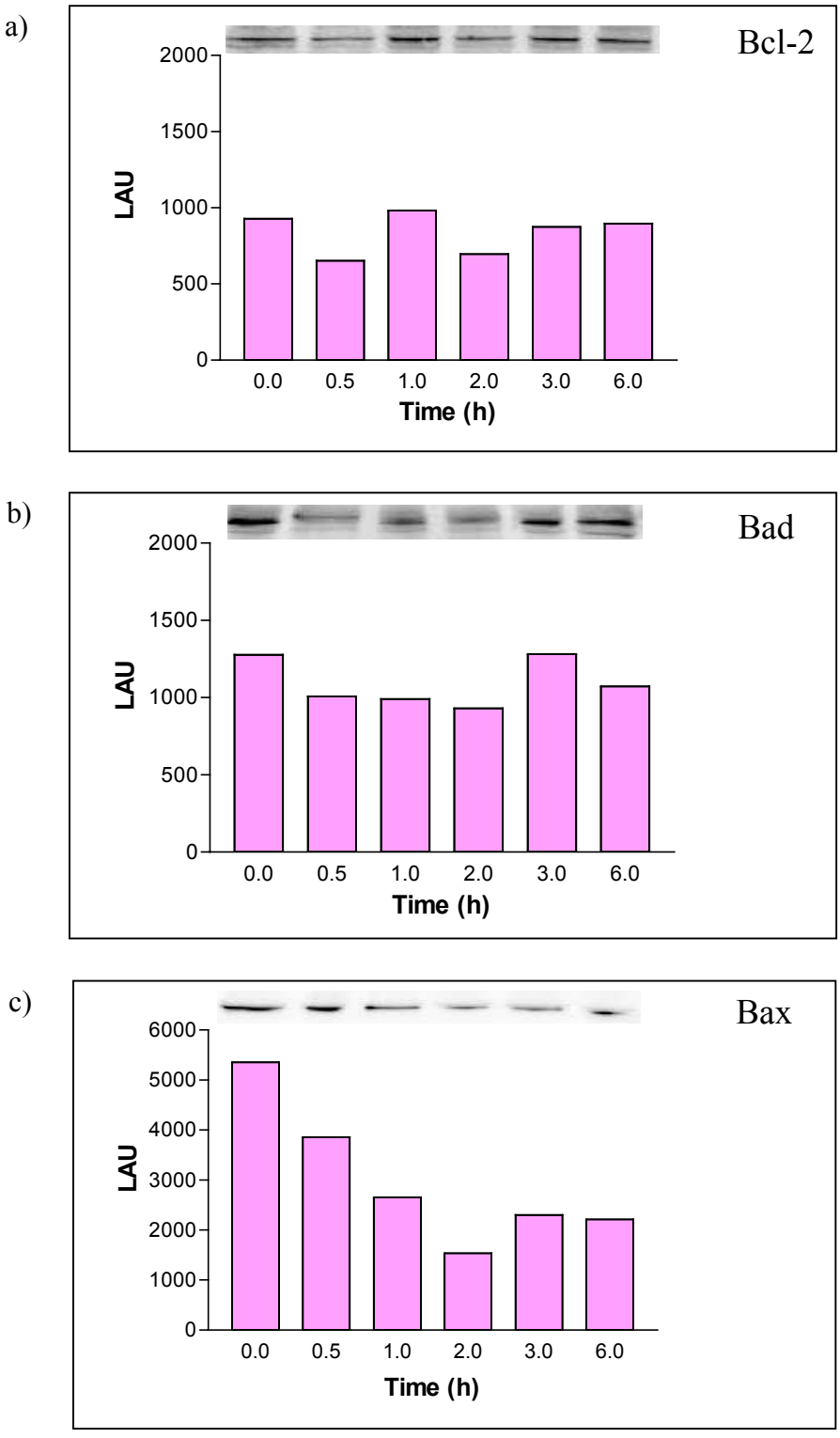


Figure 21: The protein expression of anti- and pro-apoptotic of Bcl-2 family in SH-SY5Y after treatment with Guo 75 μM for 0-6 h: (a) Bcl-2 protein; (b) Bad protein; (c) Bax protein.

5. DISCUSSION

In AD the progressive nature of neurodegeneration suggests an age-dependent process that ultimately leads to degeneration of synaptic afferent systems, dendritic and neuronal damage, and the formation of abnormal protein aggregates throughout the brain. Diverse lines of evidence indicate that βA plays a central role in the pathogenesis of neuronal dysfunction in AD. Recent studies suggest that the relatively weak correlation between plaque burden and the severity of cognitive impairments might be explained by the activity of multiple different βA assembly forms and probably memory impairment could be mediated by soluble oligomers. In fact, amyloid oligomers, which represent intermediates in the fibril formation process could be primarily responsible for amyloid pathogenesis, rather than the mature fibrils that accumulate as large aggregates. The membrane integrity disruption by amyloid oligomers and the concomitant increase in intracellular calcium may be the proximate initiator of several pathogenic pathways, including reactive oxygen species (ROS) production, altered signaling pathways and mitochondrial dysfunction. Mitochondrial dysfunction may also feed back to upstream pathways that regulate the level of mis-folded proteins. The final step in AD is characterized by βA-induced apoptosis, a mechanism of cellular death activated by mitochondrial pathway or by the interaction with specific neuronal cell-surface receptors. The Bcl-2 family of proteins has a crucial role in intracellular apoptotic signal transduction. However, it is widely accepted that, apart from the above-mentioned mechanisms, AD is a complex multifactorial disorder. Guo exerts numerous neurotrophic effects including: proliferation of glial cells (Rathbone, 1992; Kim, 1991), neurite outgrowth (Gysbers, 1996), synthesis and release of purines and trophic

factors, such as nerve growth factor (NGF), from several cell types (Middlemiss, 1995; Rathbone, 1992; Di Iorio, 2001; Ciccarelli, 2000; Gysbers, 1994), and anti-apoptotic effects (Di Iorio, 2002; Di Iorio, 2004). It is suggested that the effects of GUO are mediated through putative G-protein linked cell-surface receptors (Traversa, 2002; Caciagli, 2000).

In the present study, it was interesting to evaluate the possible effect of Guo on toxicity induced by βA-peptide in human neuroblastoma cell line, SH-SY5Y cells. Guo has been reported to protect SH-SY5Y cells and rat primary astrocytes against apoptosis induced by βA-peptide and staurosporine, respectively (Pettifer, 2004; Di Iorio, 2004). The analisys of cell viability by MTT assay in presence of βA_{25-35}, the neurotoxic core of βA-protein, was evaluated in a dose- and time-dependent manner. The dose-dependent treatment with βA_{25-35} for 24 hours induced a similar decrease of cell viability, starting at 1 μM. Whereas, the time-dependent treatment reduce cell viability at all the exposure times explored, starting from 1 hour. Besides, the annexin/propidium iodide results confirmed that apoptotic cell formation increased after 3 hours and necrotic cell formation after only 1 hour of βA_{25-35} treatment. We also evaluated the effects of treatment with oligomeric and fibrillar forms of βA_{1-42}. According to Annexin/propidium iodide results, the oligomeric form showed an increase of the apoptotic cell death after 3 hours of treatment and the necrotic cell death increased after 15 hours of oligomeric βA_{1-42}-treatment. Instead, the fibrillar form showed an increase of the apoptotic cell death after only 1 hour of treatment and the necrotic cell death increased after 6 hours. We after employed the nucleoside Guo to evaluate the possible neuroprotection against toxicity induced by βA-protein. Exogenous Guo (75 μM), added to SH-SY5Y cells exposed to βA_{25-35} for 3 hours, showed a protective effect by significantly reducing cell death (about 60%). Also, this inhibitory

effects resulted to be evident after Annexin-V staining (about 64%). The anti-apoptotic effects of Guo were observed with oligomeric and fibrillar form of βA_{1-42}. Growing evidence suggest that alterations in the UPS function may be involved in AD pathogenesis. The possibility that the protective effect of Guo was due to an involvement of the UPS has been demonstrated by using a specific inhibitors of proteasome, lactacystin. The co-treatment with lactacystin abolished completely the neuroprotective effects of Guo. To rule out the possibility that Guo-neuroprotection effects resulted from an increase in proteasome activity, the chymotrypsin-like activity of the proteasome has been valued in SH-SY5Y cell lysates, by assaying cleavage of Z-Leu-Leu-Leu-AMC. Results proved the increase in proteasome activity in a dose-dependent manner with a peak after 2 hours of treatment. Moreover, to exclude an alteration in the proteasome activity, we also demonstrated that no increase of ubiquitinated protein levels could be observed by western blot analysis.

The MAP kinase/ERK1/2 signaling pathway is reported to play a critical role in the regulation of cell growth and differentiation (Crews, 1992; Alessi, 1994). In particular, it is known that it is essential for the normal development and functional plasticity of the CNS. It is also demonstrated that PI3K/Akt pathway plays an important role in a variety of biological processes including cell survival, proliferation, cell growth and glycogen metabolism (Franke, 1997; Vivanco, 2002; Hajduch, 2001). In recent studies it has been reported that Guo is able to activate the PI3-Kinase pathway in rat cultured astrocytes, as well as in mouse microglia (Di Iorio, 2004; D'Alimonte, 2007); it also determines a marked ERK1/2 and p38 phosphorylation in mouse microglial cells (D'Alimonte, 2007). Based on this evidence, we evaluated the effects of some inhibitors of the proteasome, PI3K/Akt/GSK3β and ERK1/2/MAPK on the Guo-induced up-regulation of proteasome activity. Lactacystin, LY294002 and PD98059

abolished the Guo-neuroprotection. This suggests that Guo might be able to act on the proteasome through the activation of the survival pathways.
It is well known that another important downstream target of PI3K/Akt is the antiapoptotic protein Bcl-2. The Bcl-2 family consists of a number of evolutionarily conserved proteins that regulate apoptosis through the control of mitochondrial membrane permeability and release of cytochrome c. Released cytochrome c binds Apaf-1 (apoptotic protease activating factor 1) and forms an activation complex with caspase-9. In Bcl-2 family it is possible to distinguish two groups of proteins upon function and sequence homology: anti- and pro-apoptotic proteins. In the present research, we evaluated the intracellular levels of Bcl-2, Bad and Bax in SH-SY5Y after Guo-exposure. Among these proteins, Bax expression resulted down-regulated (about 70%) after Guo-treatment, in according to Kalfon et al (2006). Thus, the mechanism of neuroprotection induced by Guo could involve a rapid degradation of pro-apoptotic proteins by the proteasome.

It is attempting to unravel the roles of these intriguing non-adenine-based purines. Better comprehension of their biological effects, their metabolism and the mechanisms through which their effects are mediated could be prove useful not only in understanding physiological and pathological processes in the CNS, but also in targeting interventions for several pathological conditions. Being multifactorial pathogenicity of the disease, it is our opinion that a combined molecular, genetic and biochemical approaches should be employed for the development of drugs that can delay the onset of AD. The knowledge derived from these approaches could be extended from animal models to therapy of AD patients.

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7. PUBLICATIONS

PAPER:

Andrea Tarozzi, **Adriana Merlicco,** Fabiana Morroni, Francesca Franco, Giorgio Cantelli-Forti, Gabriella Teti, Mirella Falconi and Patrizia Hrelia. Cyanidin 3-O-glucopyranoside protects and rescue SH-SY5Y cells against amyloid-beta peptide-induced toxicity. *NeuroReport*, 2008; 19 (15): 1483-1486.

ABSTRACTS:

Tarozzi A., Morroni F., Marchesi A., Angeloni C., Hrelia S., **Merlicco A**., Cantelli Forti G., Hrelia P. Protective effects of sulforaphane against oxidative stress-induced apoptosis in human neuronal cells. *Oxidants and Antioxidants in Biology*. Alba, 7-10 September 2005; pag. 215.

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Fabiana Morroni, **Adriana Merlicco**, Andrea Tarozzi, Patrizia Hrelia, Giorgio Cantelli-Forti. La Cianidina 3-O-Glucopiranoside protegge le cellule neuronali in coltura dalla tossicità indotta dal peptide beta-amiloide. *XV Congresso Nazionale, Società Italiana di Tossicologia.* Verona, 19-22 Gennaio 2009; pag 219.

8. APPENDIX

The research object of the present thesis has been developed in compliance to the laws safety in environments of job (D.L.vo 626/94 and following). The experimental procedures are been performed in according to the Procedures Operational Standards (SOPs) of the laboratories of Genetic Toxicology of the Department of Pharmacology, University of Studies in Bologna as foreseen by the OECD-GLPs for the evaluation of the chemical mixtures.

- OECD, 1982. Good Laboratory Practice in the Texting of Chemicals.
- OECD, 1988. Final Report of the Working Group on Mutual Recognition of Compliance with Good Laboratory Practice.
- CEE, 1986. Board directive of 18/12/86, concern the reapproaching of the legislative, regulation and administrative dispositions related to the application of the principles of good routines of laboratory and to the control of their application for the tests on the chemical substances (87/18/CEE).
- Official gazette n. 198 of 27/8/86. DM 26/06/86: application of the principles of good practices of laboratory on the chemical substances and criterions for the release of the authorizations foreseen by the DPR n. 927/81, art. 6.