# *β-sheet breakers in the fibrillogenesis and aggregation of amyloid: an update on chemical mechanisms and potential applications.*

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# New β-sheet breakers related to the 17-21 fragment of $A\beta_{1-40}$

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***Introduction***

Since in the Alzheimer disease (AD) the neurotoxicity was associated to the preliminary acquisition by amyloid peptides (Aβ) of a beta sheet conformation [1,2] which in turn leads to different forms of toxic aggregates, the inhibition of the aggregation could be a realistic therapy for this pathology. β-sheet breakers (BSB) are short synthetic peptides able to interact with Aβ without becoming part of the β-sheet structure, destabilizing the Aβ conformer, thus precluding reciprocal stacking. In this study a series of analogues of the peptide Ac-LPFFD-$NH_2$ (*i*Aβ5p), reported by Soto as a BSB model [3-5], were synthesized and tested as fibrillogenesis inhibitors.

## *Results*

Firstly we found that the substitution of the *N*-acetyl group of *i*Aβ5p with an *N,N*-dimethyl-taurine (DM-Tau) gave a sulphonamide junction-containing analogue, which showed a higher BSB activity on $A\beta_{1-40}$ and an increased resistance to proteolysis, compared to *i*Aβ5p itself [6]. On this basis, we synthesized and tested the activity of three new analogues in which a taurine, an *N,N*-dimethyl-β-alanine and a β-alanine substituted the DM-Tau into the original sequence of the previous esapeptide. These new compounds demonstrated an *in vivo* protective effect of the taurine-containing analogues and confirmed their capability to hinder fibrillogenesis *in vitro* [7].

Successively, we pay our attention on the synthesis of new peptides containing the same *N*-terminal modifications along with changes at their central region by substituting one or both phenylanines of *i*Aβ5p and of its DM-Tau-containing derivative with an α,β-dehydro-phenylalanine (ΔPhe).

***Conclusions***

These results underlined the usefulness of the modification at the *N*-terminal position of the peptide derivatives related to *i*Aβ5p and pointed a cytoprotective activity of the sulfonamide junction against the amyloid toxicity *in vivo*. Studies on the ΔPhe-containing peptides are now in progress.

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# Pathways and interactions in amyloid β-peptide aggregation

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## Introduction

Due to its involvement in Alzheimer's disease, the understanding of the mechanisms underlying the aggregation of the amyloid β-peptide (Aβ) ) is of extreme importance. In fact, depending on external parameters such as pH, temperature, concentration, ionic strength, stirring, etc., Aβ peptide may undergo a variety of different aggregation processes, leading to aggregates of different size, morphology and toxicity level[1]. These processes have been characterized under various conditions. Moreover, possible mechanisms of inhibition of the aggregation, for the Aβ peptide as well as for other proteins, have been studied by using a-casein as a stabilizing agent.

## Methods

The experimental approach is based on the application of different complementary techniques. Light scattering, both static and dynamic, is used to gain information on the size and the structure of particles in solution. The nature and the properties of the aggregates are investigated by means of circular dichroism, Atomic Force Microscopy, and fluorescence on external dyes such as Thioflavin T and ANS.

## Results

At low pH, Aβ peptide undergoes a colloidal coagulation, leading to very large aggregates, composed of smaller amyloid structures[2]. At physiological pH, the nature of the obtained aggregates (amyloid or amorphous) strongly depends on the ionic strength of the solvent and on stirring[3].

The milk protein α-casein was found to be a potent inhibitor of the amyloid aggregation of the Aβ peptide, acting by means of monomers and/or small oligomers sequestration[4]. The study of the effects of α-casein on the amyloid aggregation on a larger protein (Concanavalin A), revealed another possible mechanism of action of α-casein[5], which can have analogies with the mechanisms of the inhibition observed in the case of Aβ peptide with β-breakers.

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# Thioflavin T triggers β amyloid peptide (1-40) fibrils formation.

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## *Introduction*

A general characteristic of aggregation is the multiple interaction and cross-feedback among distinct mechanisms occurring at different hierarchical levels. The comprehension of the different species interconversion during aggregation is very important since emerging evidences indicate intermediate oligomeric aggregates as primary toxic species. In this context, Aβ amyloid peptide provides a challenging model for studying aggregation phenomena both for the complexity of its association process and for the direct implications in Alzheimer's Disease. Aggregates growth conditions strongly affect the final morphology, the fibrillar molecular structure as well as the aggregation pathway which is characterized by the occurrence of multiple transient species.

## *Methods*

The fluorescent dye Thioflavin T (ThT) is widely used to detect amyloid deposits and it is often used in situ to study aggregation kinetics, under the hypothesis that its presence does not affect the aggregation processes under study. Here we present an experimental study on Aβ(1-40) peptide fibrillation kinetics at pH 7.4. In the observed conditions, Aβ(1-40) undergoes aggregation only if Thioflavin T is present in solution. This phenomenon was analyzed as a function of temperature, ThT and peptide concentrations in order to explore the underlying fibrillation mechanism. Light scattering, ThT fluorescence emission, two photon excitation fluorescence microscopy, were used in a kinetic fashion to highlight different sides and critical phases of the aggregation pathway. Circular Dichroism and FTIR measurements are used to characterize secondary structure of the aggregates.

## *Results*

The selected approach gives detailed information on the time evolution of Aβ(1-40) fibrillation process highlighting structural changes at molecular level, different aggregate species growth and their morphologies. Our data show that Aβ(1-40) fibrillation process occurs only in the presence of ThT and that the observed aggregation involves at least three different aggregation mechanisms acting in competition. In the first step, small oligomers, which bind ThT, are formed via non nucleated polymerization mechanism and represent an activated state for following fibrils growth. This process appear to be a rate limiting step for two distinct fibril nucleation mechanisms probably affected by an high degree of spatial heterogeneity.

## *Conclusions*

We demonstrated that in the selected experimental conditions ThT triggers the Aβ(1−40) fibrillation process (D'Amico et. al 2012). Sterical and chemical properties of ThT molecule may modulate the peptide conformation, with similar mechanisms to the ones that usually drive the binding of this dye to already formed amyloids. So, the presence of ThT in solution may change the thermodynamic equilibrium trapping specificmore ordered conformations prone to supramolecular assembly.

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# Quantitative Structure–Activity Relationships of peptides: basic notions.

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## Introduction

Since the birth of modern chemistry, investigators have always been eager to understand the structural and physical chemical basis of the biological activity of chemicals, one of the main aims being their "domestication". A brilliant illustration of how the concepts and practice of Structure-Activity Relationships (SAR) had a strong acceleration in toxicology in the mid 1980's is provided by E.J. Ariens [1]. One approach is the qualitative one, that takes into account the significance of particular groups in the molecule for particular aspects, part processes, in the biological action. Examples are groups described as pharmacophores or toxicophores or Structural Alerts (SA). This can be called a coarse-grain approach. The other approach (fine-tuned) is the formalized Quantitative Structure-Activity Relationships (QSAR) approach.

The foundation of QSAR came almost fifty years ago, when Corwin Hansch found the way to bring together two areas of science which had seemed far apart for many years: physical organic chemistry, and the study of Chemical ↔ Life Interaction [2,3]. This model worked for an enormous number of biological problems, and its success is demonstrated clearly by its widespread diffusion. In the years subsequent to the 1960's, the need to solve new problems, together with the contributions of many other investigators, generated hundreds of variations of the Hansch approach, as well as approaches that are formally completely new. For example, new descriptors generated through direct mathematical modeling of chemical structures were introduced [4,5,6 7,8], and the range of mathematical models used to link chemistry and biology has expanded accordingly [9,10,11,12,13,14].

## Conclusions

The QSAR science still maintains a fundamental unity, founded on the systematic use of mathematical models and on the multivariate point of view. At present, the QSAR science is one of the basic tools of modern drug and pesticide design, and has an increasing role in environmental sciences [15,16,17,18,19,20,21]. A great aspect of the QSARs –especially when applied within individual chemical classes- is that they point to the chemical determinants of the biological activity of the compounds; thus they can contribute to the rationalization of the biological activity mechanisms [3].
Its nature of general methodology permits the application and extension to all types of biological activity, including peptides and proteins [22,23].

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# Multi Agent System (MAS) based simulation of β –breakers' function.

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## *Introduction*

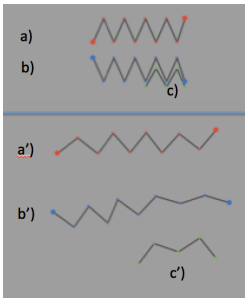
Mathematical models (MMs) are abstract models using mathematics to describe and possibly predict the behaviour of physical and biological systems. By appropriate assumptions MMs reveal precious to gain insight into the dynamics of systems whose differential equations are not amenable to analytical solutions. Models based on Multi Agent Systems (MASs) consider clusters of computational units (agents) interacting among each other and with the environment on the basis of simple rules. The time-dependent changes in MAS features can be taken as a reliable simulation of the complex, phenomena involving social, economic, and even molecular events.

## *Methods*

Any MAS simulator ultimately derives from the seminal work of John Conway on cellular automata [1], namely autonomous, self-organizing and self-steering computational elements. In the present contribution we took_advantage, in the family of the Artificial Intelligence 'Logo' languages, of the NETLOGO [2] programming environment endowed – among a number of desirable features - with a friendly graphical user interface. We used NETLOGO to simulate: a) the action of β-breakers on the β -sheet structure of single fibrils at a microscopic, molecular level, and b) the emergence of cooperative effects in switching from one conformation to another at a macroscopic, population level.

## *Results*

A crucial role in fibrillogenesis was assigned to the II structure of palindromic sequences in prion peptides [3] where by far the *β-sheet* prevails. Hence, such palindromic sequences should be included in any model accounting for the properties of *β-breakers.* Fig. 1 shows how MAS simulators reproduce the effect of the repulsive force taking place between single palindromic sequences and β-breakers.




Even the massive accumulation of insoluble amyloid aggregates can be associated to the switching from the coil to the β-sheet conformation at the level of single fibrils, and simulated accordingly. In the bidimensional representation of Fig. 2 the two conformations are represented by the black or white color assigned to nodes in a network where distances (links) between each pair of nodes have been omitted for the sake of clarity. For each single node, switching from one to the other state depends on the state of its neighbours, and this is shown to produce, at the global population level, different final states from the same initial configuration.

***Figure1***. *Simulating the effect of a β-breaker on the II structure of the VAAAAAAAAV decapeptide.*

In the top and bottom panels the II structure of the decapeptides is reported before (a, b) and after (a', b') running 100 iteration steps, respectively. In each step the initial relative position of decapeptide and *β-breaker* residues (c, c') is modified by an algorithm trying to get the residues as far as possible from each other, subject to the limitation imposed by the links (peptide bonds). Notice that in both panels changes in the a, a' decapeptides are minimized by the bigger distance from the β-breakers (c, c').

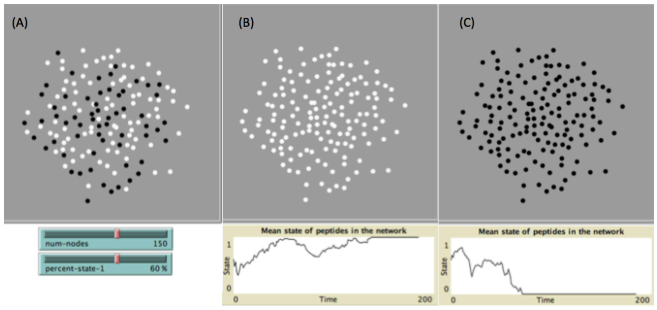



***Figure 2***. *Simulating the β–sheet ↔ coil cooperative switch in a fibrils population.*

The left panel shows the randomly distributed conformations (black or white color) within a population of amyloid fibrils. In the representation of the system as a dynamic network, at each time step the conformation of each node (fibril) is made the same as that of the first neighbours. The abundance of the two conformational states is reported in the bottom section of each panel. For the given initial configuration (panel A), the initial prevalence (60%) of one state does not originate the same final state after 200 time steps (panel B, C), pointing to the randomic nature of the aggregation process.

## *Conclusions*

Although the structural modifications induced by β-breakers on amyloid fibrils can be successfully reproduced by standard molecular dynamics [4], a MAS based simulation study is made worth of pursuing by the following considerations: i) The implementation even of sophisticated mechanistic models is relatively straightforward. Thus, the influence of a large number of variables on the very complex amyloid aggregation process can be qualitatively explored in a short time. ii) For a given model, the statistical significance of the simulations can be increased at will by up-scaling the size of the agents' population. iii) The highly flexible time and space parceling in the iteration steps, allows to explore a wide range of time windows and spatial arrangements.

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# Quantitative Structure-Activity Relationships (QSAR) for peptides and proteins

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## *Introduction*

The basic assumption of medicinal chemistry is that the most similar two chemicals are, the most similar their biological activity is supposed to be (1). Even if there exist many exceptions to this rule we can consider the relation between chemical and biological activity similarities a reliable and useful work hypothesis as demonstrated by its widespread use in applied science (2). Establishing a Quantitative Structure Activity Relation (QSAR) implies a thorough definition of what chemical similarity is, that in the case of small organic molecules is approached by the use of multidimensional statistics: the considered molecules are the statistical units (rows) of a data set (training set) having as columns (variables) some carefully selected chemico-physical descriptors of the considered molecules. These variables correspond to the set of 'regressors' (or X variables) , while one or more Y variable are added as well in order to describe the biological activity of the same compounds. The goal is to find a statistically relevant correlation linking X and Y variables that will be in turn verified by its ability to predict the biological activity of another independent set of molecules (test set). The same approach can in principle (and as a matter of fact it was) used for peptides and proteins, the basic point to keep in mind is the need of a careful consideration of the 'order' properties of biological polymers that must be embedded in the data representation. Here we will briefly discuss the two main methods to take into consideration this point in protein and/or peptide QSAR.

## *Methods*

There are two basic methods in order to keep the 'native order' of polymers alive in a QSAR-like approach a direct and an indirect (or holistic) one. The direct method ends up into an Xs (regressors) matrix having as rows the different peptides (proteins) and as columns the relevant chemico-physical descriptors at the corresponding positions along the sequence. The following tables depict the essence of this kind of approach: the analyzed sequences are initially represented like this:

Table 1a

| Peptide | Pos.1 | Pos.2 | Pos.3 | Pos.4 | Pos.5 | Pos.6 | Pos.7 |
|---|---|---|---|---|---|---|---|
| P1 | A | R | A | N | N | D | C |
| P2 | A | H | I | N | N | W | C |
| P3 | R | R | A | D | N | D | M |

The variables correspond to the different locations along the sequence with their corresponding residues, the above matrix is then transformed as in Table 1b by the substitution of symbols with corresponding chemico-physical descriptors of the corresponding residue.

Here we will report as descriptor Miyazawa-Jernigan (MJ) (3) hydrophobicity score, but we can in principle add many other descriptors by adding new columns (keeping invariant the original peptide order) to the matrix.

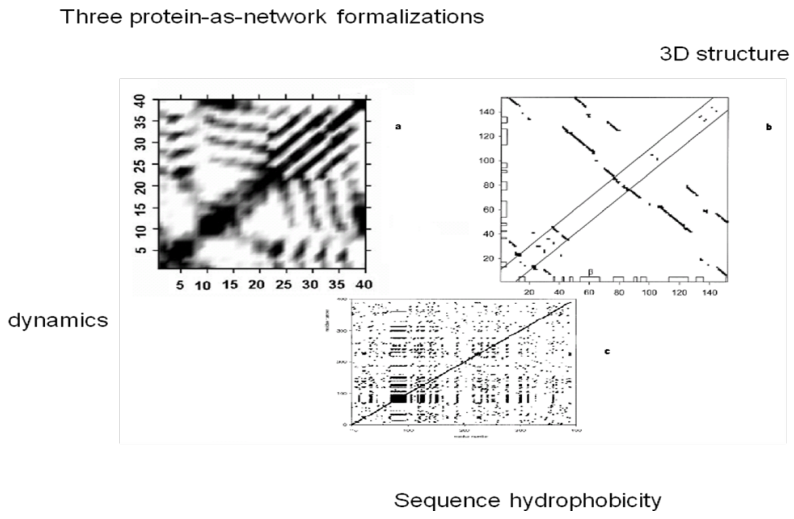
Table 1b

| Peptide | Pos.1 | Pos.2 | Pos.3 | Pos.4 | Pos.5 | Pos.6 | Pos.7 |
|---|---|---|---|---|---|---|---|
| P1 | 1.6 | -12.3 | 1.6 | -4.8 | -4.8 | -9.2 | 2 |
| P2 | 1.6 | -3 | 3.1 | -4.8 | -4.8 | 1.9 | 2 |
| P3 | -12.3 | -12.3 | 1.6 | -9.2 | -4.8 | -9.2 | 9.36 |

Before entering the supervised learning phase the original data set is made amenable to a sound modelling phase by the ordinary multidimensional statistical methods (4). What is important to note is that the direct (beside the need to have equal length sequences that can be circumvented by autocorrelation techniques like in (5)) makes a strong assumption about the existence of a 'specific role' typical of each position and invariant across the data set. When this is actually the case, this approach is extremely powerful given it produces a very detailed information that is immediately operative in terms of suggestions for the synthesis of new molecules expected to have a relevant biological activity without the need of any further theoretical assumptions.

When the above constraints are not met and we are facing very different systems (7) in terms of both size and shape, we must change our strategy accordingly trying and select some invariant descriptors from protein structure and/or sequence that, being relative to the protein molecule as a whole, can be applied to very different systems keeping alive their general meaning. There exist plenty of these global descriptors (8,9,10) , relying on the consideration of proteins as graphs having

as nodes the aminoacid residues and as edges the scoring of a significant interaction between the nodes (11). In the case of protein dynamics the interaction between graph nodes is the correlation between the trajectories of the correspondent aminoacids. In the case of primary structures the relevant interaction corresponds to a pairwise chemico-physical similarity between the residue pairs (11) , in the case of 3D structure their small distance in space (8). In all given protein graphs are described by an NxN (with N=number of residues) adjacency matrix whose invariants correspond to the global descriptors. Fig.1 reports three different adjacency matrix correspondent to the above three protein views:




The network invariants correspond to different descriptors of the graphs based only on their wiring structure (and thus independent on the kind of interaction they are focused on) such as general connectivity, characteristic length (or average shortest path, the average length of the shortest path linking the different couples of nodes) and so forth that represent the regressors for predicting protein physiological features.

## *Conclusions*

All in all, we can safely state that the merging between multidimensional statistics and sensible quantitative descriptors of proteins at different level of detail can be a very powerful method to approach specific problems like the synthesis of efficient beta-breakers peptides in a rational and quantitative way.

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## Looking "inside" breakers and Aβ-peptide interaction: a computational approach

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We present preliminary results of classical Molecular Dynamics (MD) simulations performed with the main goal of reproducing and thus possibly interpret the salient features of findings (1) of selected experiments, purposely devised for comparing the anti-aggregating activity of a group of specific beta-sheet-breakers (BSBs) peptides. The strength of our MD approach resides in the possibility provided, at least in principle, by numerical simulations of blowing up even very small portions of the investigated system and looking at what happens at the atomic level. Four model systems have been subjected to MD simulations. They are built by dissolving in water either the $A\beta_{1-40}$ alone (from now on called ***abeta*** model) or in the presence of 10 copies of the following three BSB peptides: LPFFD, chosen to mimic the 17-21 sequence of the $A\beta_{1-40}$ peptide (***lpffd*** model); taurine-LPFFD, ***tau-lpffd***; and LPFFN, ***lpffn***. After a standard equilibration procedure, a 80 ns NpT MD simulation at 300 K is started. The starting $A\beta_{1-40}$ structure is taken from PDB (ID: I1YT) while the BSB's are all taken in an all-trans configuration. Structural differences among the four models are already visible after the equilibration procedure, and they become much greater at the end of the actual MD simulation. In particular, it seems that in the ***abeta*** and ***lpffn*** systems the $A\beta_{1-40}$ peptide has the tendency of conserving longer α -helix portions (2). Looking at ***lpffd*** model, the $A\beta_{1-40}$ peptide looks more disordered than in the ***abeta*** one, but another important feature emerges: in the course the MD evolution, β-sheet strands are formed, between $A\beta_{1-40}$ and either one or two BSBs. The same feature is also visible in the ***tau-lpffd*** model. In the ***lpffn*** case, instead, the β-sheet structure, that appears already after 10 ns MD, is now formed between two breakers and does not involve the $A\beta_{1-40}$ peptide. What is interesting to note is that the conserved α-helix $A\beta_{1-40}$ portion is located in the region that happens to be nearest to the two BSBs that form the β-sheet. In summary, three main preliminary results are worth recalling and retaining for the moment: i) The Aβ peptide loses most of its α-helix structure already as it is solvated; ii) there seem to be no preferred locations along the Aβ peptide for BSB docking; iii) β-sheet structures get formed between the Aβ peptide and one BSB or between pairs of LPFFN breakers. For the immediate future, we are planning to add in solution at least a second Aβ peptide, by either having a system with two Aβ peptides in water free to interact, or by starting from an aggregated two-Aβ-peptide structure with the aim of studying the ability of BSBs of "undoing" the aggregate. We would like also to test the effect of adding metals (like Cu or Zn ions) to the systems studied, as they are known to affect Aβ aggregation propensity.

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# DNA methylation: a unifying hypothesis on Alzheimer's disease and Down syndrome?

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## Introduction

Folate metabolism, also referred to as one-carbon metabolism, is required for either DNA synthesis or methylation. Methylation of cytosine represents one of the most important epigenetic mechanisms for gene regulation and tipically occurs in a CpG dinucleotide context. Promoter hypermethylation is commonly associated with gene silencing and promoter demethylation with gene expression. DNA methylation also forms the basis of chromatin structure, and participates in chromosome recombination and segregation. Increasing evidence suggests that impairments of gene-specific DNA methylation might be involved in the pathogenesis of Alzheimer's disease (AD) (1). Moreover, virtually all individuals with Down syndrome (DS) develop AD by the fourth decade of life, due to the presence of three copies of the *APP* gene that maps to chromosome 21, and it has been suggested that maternal impairments in DNA methylation of centromeric regions might favour chromosome 21 malsegregation and predispose the mother to the birth of a child with DS (2). We are currently investigating the contribution of genetic polymorphisms of genes involved in folate metabolism and DNA methylation reactions to the risk of both AD and DS.

## Methods

We are screening a large cohort of almost 400 AD subjects and 300 healthy matched controls for the presence of common polymorphisms of genes involved in folate metabolism in order to correlate them to both AD risk and to circulating levels of folate, homocysteine and vitamin B12 levels. Similarly we are screening a cohort of over 100 mothers of DS individuals and 100 matched control mothers to evaluate the contribution of folate gene polymorphisms to the maternal risk for having a birth with DS, as well as to chromosome damage and malsegregation events.

## Results

We observed significantly increased homocysteine and decreased folate levels in AD patients with respect to controls (3). Both *MTHFR* C677T and *MTRR* A66G polymorphisms have been associated with increased AD risk and with folate and/or vitamin B12 levels in our cohort (3). Promoter polymorphisms of the *DNMT3B* were not associated with increased AD risk (4), and ongoing studies suggest interaction of *RFC1* and *TYMS* polymorphisms with homocysteine levels. Concerning DS risk, we observed complex interactions among *MTHFR* and *RFC1, MTHFR* and *MTR*, and *MTHFR* and *TYMS* polymorphisms with the maternal risk for birth of a child with DS (5,6). Moreover, we observed an increased frequency of chromosome damage and malsegregation events in peripheral lymphocytes of MDS with respect to control mothers, and an association of *MTHFR* polymorphisms with the chromosome damage (6,7). More recently, we observed association of *DNMT3B* promoter polymorphisms and maternal risk of birth of a child with DS (8). Meta-analyses of the literature are ongoing to further address the contribution of one-carbon metabolism to the maternal risk of birth of a DS child.

## Conclusions

The results obtained by us in recent years point to a possible contribution of polymorphisms of gene participating in folate metabolism to both circulating levels of folate, homocysteine and vitamin B12, as well as to chromosome damage and malsegregation events, thereby contributing to the risk of late-onset AD and to the maternal risk of birth of a child with DS. Interestingly, a five-fold increased AD risk was observed in mothers of DS individuals with respect to control women (9), and chromosome 21 malsegregation was observed not only in MDS blood cells, but also in blood, buccal cells and brains of AD subjects (10-12). *In vitro* studies showed association between folate deficiency and chromosome 21 aneuploidy (13). Further studies are necessary to clarify whether the observed occurrence of chromosome 21 malsegregation in AD cells is caused by folate deficiency in AD subjects, likely as a consequence of impaired chromosome 21 methylation, and contributes to disease development, or if it is rather a consequence of the disease progression.

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# Flavonoids and cognition: potential therapeutic role in Alzheimer's disease

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As the elderly population expands, the prevalence of both Alzheimer's disease (AD) and Parkinson's disease (PD) is likely to augment. Although the exact cause is not yet finally known, it has been postulated that the behavioural and neuronal declines associated with these age-related neurodegenerative disorders are triggered by multi-factorial events including neuroinflammation, glutamatergic excitotoxicity, increases in iron and/or depletion of endogenous antioxidants (1-3). Accumulating evidence suggests that diet and lifestyle can play an important role in delaying the onset or halting the progression of neurodegenerative diseases and improving cognitive function (4). With regards to diet, flavonoids have been associated with a reduced risk of developing dementia, an improved cognitive performance in normal ageing and an improved cognitive evolution (5-6). However, recent evidence is suggestive that carrier of the *APOε4* genotype may influence the beneficial effect of flavonoids in relation to dementia and AD (7-8).While many of the mechanisms underpinning their beneficial effects remains to be elucidated, it has become clear that they in part involve decreases in oxidative/inflammatory stress signaling, increases in protective signaling, and may also involve hormetic effects to protect neurons against oxidative and inflammatory stressors. Nethertheless, the therapeutic and pharmacological potential of these natural compounds, still remains to be translated in humans in clinical conditions. The challenge ahead therefore, is to proceed cautiously until rigorous randomized controlled clinical trials have been undertaken to determine empirically whether flavonoids and/or their metabolites have efficacy in individuals affected by dementia and other neurodegenerative conditions.

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# One-carbon metabolism and DNA methylation regulate amyloid-β production

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## *Introduction*

The greatest risk factor for Late Onset Alzheimer's Disease (LOAD) is aging, which is influenced by physiological and environmental stimuli. Epigenetic modifications can mediate environmental insults that a person encounters during his life. Therefore, epigenetic mechanisms can be considered as a link between environment and its effect on the genome. Advances in epigenetic research stressed the involvement of epigenetics in different neurodegenerative diseases, including LOAD, the most representative among neurodegenerative diseases. The role of B vitamins and high homocysteine (HCY) levels in the onset and progress of LOAD is a controversial topic. Although low B vitamins and high HCY levels were, at different degrees, associated with the disease, much work is still necessary to clarify the causal or consequential relationship and the underlying molecular mechanisms of this association.

## *Methods*

In the last years, our laboratory was aimed at studying the role of DNA methylation in AD; to this scope, we analyzed methylation of genes associated to the disease in a murine AD model. TgCRND8 mice (carrying a double Indiana/Swedish mutated APP transgene) were grown either with control or vitamin B deficient diet, with or without oral supplementation of S-adenosylmethionine 400 mg/day. We measured: i) methylation metabolites by HPLC; ii) activity of DNA methylases/demethylases; iii) PSEN1 promoter methylation; iv) PSEN1 and BACE expression by Real-Time PCR and western blotting; v) amyloid deposition by ELISA tests and immunohistochemistry; vi) cognitive status by water maze.

## *Results*

We found that PSEN1 gene, involved in amyloidogenesis, is regulated by methylation in hyperhomocysteinemic mice (a condition exacerbating AD-like features) through the inhibition of DNA methylases and promotion of DNA demethylation. S-adenosylmethionine is able to revert (or prevent) the observed exacerbation of Alzheimer-like features induced by B vitamin deficiency in TgCRND8 mice: SAM/SAH ratio decrease, DNA methylation unbalance, PSEN1 promoter hypomethylation, PSEN1 and BACE over-expression, amyloid processing and deposition, cognitive impairment.

## *Conclusions*

Our results demonstrate the existence of specific mechanisms by which epigenetics, in particular DNA methylation, affects the progress of neurodegeneration. These findings confirm and extend our previous observation on the role of one-carbon metabolism and of methylation reactions in particular, and highlight the molecular mechanism connecting this metabolism with AD onset and progression. Possible intervention on one-carbon metabolism, and the use of SAM in particular, for the treatment of AD finds an evident rationale on the basis of these results. Since the magnitude of increased/decreased amyloid deposition is apparently greater than if simply due to the PSEN1 regulation, we hypothesize than other regulation (e.g. the effect of methylation on amyloid aggregation) could be involved.

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# Cellular response to oxidative stress induced by Amyloid β-peptide: in vitro and in vivo studies

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## *Introduction*

Neurodegenerative disorders, in particular Alzheimer's disease (AD), are characterized by severe oxidative stress. The major cause of free radicals overproduction in AD seems to be related to the overproduction of Amyloid beta (Aβ), which could function as a pro-oxidant generating oxygen free radicals via a direct or an indirect mechanism: in the direct one, Aβ binds to transition metals ions, acquiring an oxidase activity leading to hydrogen peroxide production, whereas in the indirect mechanism neurons or microglia stimulated by Aβ produce oxygen free radicals by activation of NADPH oxidase.
Free radical injury may be responsible for neuronal loss by inducing DNA damage that in turn activates poly(ADP-ribose)polymerase enzyme (PARP-1) which catalyzes the covalent addition of the ADP-ribose moiety of $NAD^+$ and the subsequent elongation of the polymer mainly to nuclear proteins. A causal relationship between PARP and AD is demonstrated by the finding that nuclear proteins are extensively poly(ADP-ribosylated) in the hippocampus of AD affected individuals and that the NonAmyloid-beta Component (NAC) of senile plaques activates PARP.
In the present study we focused on the comprehension of the molecular mechanisms that lead to PARP-1 activation by Aβ and to the downstream ways activated by PARP-1.

## *Methods*

Our experiments were performed both in a cellular model (i.e. SH-SY5Y neuroblastoma derived cells) treated with the $A\beta_{25-35}$ fragment, kindly provided by Dr. C. Giordano (CNR centre of Biomolecular Chemistry, Sapienza University), and in an animal model of AD (i.e. in TgCRND8 transgenic mice, an early onset model of the disease) kindly provided by Prof. S. Scarpa and Dr. A. Fuso (Dept. of Surgery P. Valdoni, Sapienza University).

## *Results*

Our data show that challenge of SH-SY5Y cells with $A\beta_{25-35}$ significantly increased PARP-1 activity following ROS generation and DNA damage. We also evaluated the capability of betasheet breaker peptides (BSBPs) to counteract cellular responses to $A\beta_{25-35}$-induced damage. Our results demonstrate that BSBPs containing a taurine moiety at the N-terminal were particularly efficient in inhibiting PARP-1 activation and in counteracting $A\beta_{25-35}$-induced cell toxicity.
$A\beta_{25-35}$ also activated NFkB via PARP-1, and induced a significant increase in p53 protein levels and a parallel decrease of the anti-apoptotic Bcl-2 protein, both of which were reversed by cell pretreatment with MC2050, a new PARP-1 inhibitor.
In vivo experiments confirmed an involvement of PARP-1 in neurodegeneration. Indeed, PARP-1 activity in brains specimens of TgCRND8 transgenic mice was found to be significantly increased in the hippocampus and the same trend observed for p53 and Bcl-2 in treated cells was confirmed in this brain area.

## *Conclusions*

The overall data suggest that PARP-1 has a prominent role in the molecular mechanisms induced by Aβ that lead to cell death and neurodegeneration, through activation of NF-kB signalling and the modulation of p53 and Bcl-2. Our data also support the evidence that BSBPs or PARP-1 inhibitors may represent attractive therapeutic agents to counteract neurodegeneration in AD.

# Modulation of Tau phosphorylation by GSK3β and PP2A

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## *Introduction*

One of the histopathological hallmarks of Alzheimer's disease (AD) is the intraneuronal neurofibrillary tangles that are composed of abnormally hyperphosphorylated tau protein. Generally, the level of tau phosphorylation is regulated by the equilibrium between the activities of its protein kinases and phosphatases. Although several protein kinases are known to act on tau, glycogen synthase kinase 3*β* (GSK3*β*) is the major kinase that phosphorylates tau in the brain. Recent studies show that overexpression of active Gsk3b results in AD (1, 2). The major phosphatase that dephosphorylates tau is protein phosphatase 2A (PP2A) (3). Different groups showed that the highly conserved carboxyl-terminal sequence of PP2A C subunit is the site of a reversible methyl esterification reaction that control the formation of $AB_aC$ heterotrimers and then the activity (4).

## *Methods*

Both SK-N-BE neuroblastoma cell line and TgCRND8 mice were used. *In vitro,* we used culture media without folate, B12 and B6, whereas *in vivo,* mice were fed with a diet deficient of the same vitamins. Postmortem human entorhinal cortex of healthy control subject and AD patients categorized as stage VI of Braak and Braak were used to study GSK3β gene promoter. The methylation status of GSK3β promoter was analyzed by bisulphite method by standard procedures (5) with modifications previously described (6). We analyzed expression levels of kinase and phosphatase with quantitative Real-Time PCR, western blot and activity assay.

## *Results*

We found that alteration of homocysteine (Hcy) metabolism by B vitamin deficiency causes PP2Aca and GSK3b genes overexpression both in neuroblastoma cells and in mice brain. Western blot analysis showed a increase of the Leu309-demethylated PP2Ac and a decrease of methylated PP2Ac in cells and in mice brain in B vitamin condition. As a consequence of lowered PP2AC-terminal Leu309 methylation, we found a decrease of PP2A activity and exogenous S-adenosylmethionine (SAM) was able to restore the activity of major tau phospatase. Although we observed a decrease of inactive form of GSK3β (p-GSK3β Ser9) levels both in cells and in mice brain, no modulation on GSK3b activity was observed. Tau phosphorylation at Ser396 and at Ser202/Thr205, increased in B vitamin deficiency, was reverted to control like value by SAM supplementation. The analysis of a putative CpG islands in human GSK3β promoter in postmortem AD brain showed a general hypomethylation of this region without differences between control subject and AD patients as well as GSK3β mRNA expression. Instead, a prominent increase of inactive form of GSK3β in AD patients was found.

## *Conclusions*

The aim of this work was to investigate if DNA or protein methylation could affect equilibrium between the major kinase and phosphates involved in tau hyperphosphorylation (GSK3*β* and PP2A). While both genes were up-regulated by inhibiting methylation reactions, only PP2A activity was influenced by Hcy metabolism alteration (7). PP2A activity decreased as a consequence of lowered PP2AC-terminal Leu309 methylation and exogenous SAM was able to restore the activity of major tau phospatase (8). Therefore, we suppose that tau hyperphosphorylation appears to result from a decreased PP2A activity towards tau rather than increased kinase activity.

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# Effect of S-adenosylmethionine and Superoxide-dismutase on amyloid deposition in TgCRND8 mice.

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***Introduction***

Recent hypotheses stress a central role of one-carbon metabolism both in amyloidogenesis and tau alterations, the two major hallmarks of Alzheimer's Disease (AD), due to changes in methylation and oxidation reactions. Previous data obtained in our laboratory, using a nutritional B-vitamin deficiency model, already showed that two genes involved in amyloid production (PSEN1 and BACE1) were modulated by one-carbon metabolism alterations. In particular, PSEN1 was modulated by DNA methylation whereas the mechanisms responsible for BACE1 modulation are probably dealing with oxidation. Consequently to modulation of these two genes, production of amyloid peptides was increased. We also demonstrated that S-adenosylmethionine (SAM) supplementation was able to contrast the AD-like features induced by B vitamin deficiency. Here we studied the effect a combination of SAM and SOD (Superoxide-dismutase), in the same model.

***Methods***

TgCRND8 mice (carrying a double Indiana/Swedish mutated APP transgene) were grown either with control or vitamin B deficient diet, with or without oral supplementation of SAM + SOD (SAM 200 µg + SOD 2.5 per day) or SAM (400 µg) and SOD (5 U) alone. We measured: i) methylation metabolites by HPLC; ii) oxidative stress by lipid peroxidation assay; iii) PSEN1 and BACE expression by Real-Time PCR and western blotting; iv) amyloid deposition by ELISA tests and immunohistochemistry.

***Results***

SAM and SOD were able to revert (or prevent) the observed exacerbation of Alzheimer-like features induced by B vitamin deficiency in TgCRND8 mice: SAM/SAH ratio decrease, lipid peroxidation, PSEN1 and BACE over-expression, increase in amyloid processing and deposition. SAM and SOD combination shows a synergic effect versus the use of the two single molecules.

***Conclusions***

These findings evidence the beneficial effects of the combined administration of SAM and SOD in AD transgenic mice. Interestingly, SAM + SOD were able to contrast also the amyloid deposition normally observed in these mice (without the induction exerted by B vitamin deficiency). Although the precise mechanism of action of SOD remains to be elucidated, these preclinical data stress the importance of a clinical trial with SAM + SOD in order to evaluate its efficacy in AD treatment or prevention, alone or as co-adjuvant of current therapies.

**Disclosure:** This work was partially granted by Gnosis s.p.a. A. Fuso and S. Scarpa are co-owner of a patent on the use of SOD and SAM in AD.

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# Protein aggregation and aggregate removal strategies ( A minireview )

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## *Introduction*

During lifetime a number of proteins escape the intracellular proteostatic control and accumulate. Accumulated, not in time degraded proteins tend to aggregate and built up various forms of intracellular protein aggregates [1,2]. Noteworthy such protein accumulation is a long lasting process and, therefore, the amount of aggregated proteins increase with aging [2,3]. Under certain, often unknown, conditions protein aggregation is enhanced, so that often in a defined set of cells protein aggregates accumulate in a relatively early stage of life. Such an early protein aggregation is accompanying several diseases [3]. Whether the protein accumulation/aggregation is the cause for the disease or just accompanying the pathophysiology, is the subject of intensive investigation. However, there are a number of reasons for such metabolic changes leading to protein aggregation, including genetic ones as in Huntington disease or the familiar form of Amyotrophic Lateral Sclerosis, or more likely environmental or metabolic ones as in Alzheimers or Parkinsons disease [4].
However, it is widely accepted that the reduction of protein aggregation or growth rate will have a positive effect on the outcome of the patients or slow down the progression of these diseases.

## *Intracellular proteostatic control*

There are several major intracellular protein homeostasis systems. This includes in general the protein chaperoning system to ensure the normal protein folding during protein synthesis and to prevent the aggregation of proteins in an unfolded state and of course the intracellular proteolytic systems [4,5,6]. In general there are two major proteolytic systems in a mammalian cell: the proteasomal system and the lysosomal-autophagic system. However, in mitochondria for example additional systems exist [4,7]. The proteasomal and the autophagic system seem to be connected via a targeting system, which includes at least the ubiquitination system. The proteasomal system in general is responsible for the degradation of short lived, regulatory proteins [4,7], while the autophagy is responsible for the degradation of long-lived proteins and cellular organelles. Interestingly, there is convincing evidence that the proteasomal system is also responsible for the degradation of oxidatively damaged proteins [8,9,10]. However, once oxidized proteins are aggregated the proteasome is unable to degrade them [1, 11]; in fact the activity of the proteasomal system is rather negatively influenced by the presence of protein aggregates [11, 12].
Importantly, the autophagic-lysosomal system is able to deal with aggregated proteins and take them up into the autophagosome via a process called macroautophagy or in this case aggrephagy [13,14]. These protein aggregates are then transferred into lysosomes, where proteolytic enzymes (the cathepsins) are present [15].

## *Oxidized cross-linked protein aggregates*

As pointed out above, proteins might accumulate and aggregate due to metabolic processes or in the pathophysiology of the diseases. It is assumed that the aggregation of proteins takes place at least at the beginning or to a certain extend due to non-covalent interactions. It is

thinkable, that such a process takes place due to insufficient degradation of malfolded newly synthesised proteins. The major driving force for the accumulation of such proteins is perhaps surface hydrophobicity [1, 16]. Such unfolded proteins accumulate in structures referred to as aggresomes [17] and are afterwards via aggrephagy transferred into lysosomes and degraded. However, if such aggresomes stay in an intracellular environment for a long time, it is likely that proteins become covalently cross-linked due to the interaction with carbohydrate- or lipid-driven metabolic cross-linkers. The same is true for oxidatively damaged proteins. Such proteins are bearing highly reactive surface motives, including carbohydrates or hydroperoxides, which are able to cross link with other proteins [1,18]. With other words such protein aggregates tend to attract new proteins and grow with time [1,18]. For such covalently cross-linked proteins the degradation is in mammalian cells impossible. The proteasomal system is for sure not able to degrade protein aggregates [1,10], moreover it is inhibited by formed aggregates [11,12]. One way to avoid the dramatic inhibition of the proteasome by protein aggregates is the uptake in the autosomal-lysosomal compartment. However, cross-linked proteins are also not completely degraded within lysosomes, but accumulate there and form lipofuscin [19,20]. So, more that 90% of the intracellular lipofuscin is accumulated in the lysosomal compartment [20].

## *Possible strategies to reduce protein aggregates*

Obviously, two possible strategies for the reduction of the protein aggregate amount exist: the reduced formation or the dissolving of the protein aggregates. Both processes are theoretically possible, but are in practice problematic. The prevention of protein aggregate formation might be achieved via a modulation of the metabolism, which is difficult since in aging and several diseases the protein aggregation takes years or even decades. A permanent stimulation of proteostatic control systems might be favourable. Positive results were achieved in genetically modified experimental models [21]. Since such an approach for humans is rather impossible, the dissolving of protein aggregates is perhaps the way to go. One way here would be the 'breaking' of the covalent cross-links of protein aggregates. This might result in soluble break-down products, which can be either excluded from the intracellular environment or be degraded by intracellular proteolytic systems. Another way to reduce the protein aggregation rate is to prevent in a rather advanced stage of aggregate formation the growing of the aggregate. It is thinkable, that due to pharmacological intervention the reactive surface motives of protein aggregates become neutralized and the rate of aggregate growths is slowed down.

It is unclear today which way of prevention or slowing down the dramatic protein aggregation in aging and several diseases will be successful. However, achieving such a goal will be of utmost importance for the prevention and treatment of several diseases.

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