AGGREGATION STUDIES, DESIGN AND SYNTHESIS OF AMYLOID AGGREGATION INHIBITORS FOR THE TREATMENT OF ALZHEIMER'S DISEASE

Summary of Ph. D. Thesis

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1. INTRODUCTION

Almost a century ago Alois Alzheimer first described the neuropathological characteristics of one of the most common neurodegenerative disorders that takes his name: the Alzheimer's disease (AD). His pioneer work opened the way to the investigation and better understanding of such diseases which became later defined as `protein conformational disorders´ associated with misfolding, aggregation and consequent accumulation of several proteins in the human brain.

AD is a progressive dementia of the elderly, leading to complete mental breakdown and death in maximum ten years. It is characterized by a progressive loss of memory as well as impairment of the cognitive functions. The number of the AD patients constantly increases with the increasing average age of the human population, but an efficient treatment for the disease is still lacking. It is not surprising therefore, that both the pathomechanism and the therapeutic efficacy of the different medications are extensively studied.

On the onset of the clinical symptoms, the brain has already been seriously damaged. The subsequent pathological hallmarks of AD are: (1) neuritic amyloid plaques located extracellularly in the brain parenchyma and around the walls of the cerebral vessels mostly consisting of 40-42 residue long peptides called β-amyloid (Aβ) peptides, (2) neurofibrillary tangles in the cytoplasm of neurons composed of hyperphosphorylated tau-proteins, and (3) loss of synapses and neuronal death.

It is still debated if there is a direct link between the Aβ peptides and the loss of cognitive functions in AD. The widely accepted `amyloid cascade hypothesis´ suggests that accumulation of Aβ peptides in the brain is the key event driving AD pathogenesis.

The main subject of my PhD-work was the investigation of the amyloid aggregation process and the role of short amyloid aggregation inhibitory sequences in the mechanism of neuroprotection against the toxic effect of the Aβ peptides. Therefore, we aimed to answer the following questions:

- How can the sample preparation influence the aggregation profile of the Aβ peptides? With the adaptation of TEM methodology, I intended to follow the fibrillization of Aβ peptides and monitor the effect of different treatments on this process.
- What is the reason for the observed ambivalent protective and toxic behaviour of the tetrapeptide Pr-IIGL$_a$ designed as a putative neuroprotective agent?
- We intended to design new, potential inhibitors against Aβ neurotoxicity based on the Pr-IIGL$_a$ sequence with the elimination of its inherent toxicity.

- What kind of chemical modifications improve the bioavailability of the biologically active LPYFD$_a$ pentapeptide without decreasing its bioactivity? The most effective peptidomimetics possessing enhanced enzyme resistance and advantageous metabolism had to be found in a lead optimization procedure.
- Through which mechanism can our short peptides exert their neuroprotective effect? The possible influence of the peptides on the fibrillization of Aβ *in vitro* can be visualized by TEM. These experiments help to decide whether the short peptides are able to act as real aggregation inhibitors.
- Since it is important to understand the mechanism of the biological activity *at the cellular level*, fluorescent derivatives of the Aβ peptides and the bioactive short peptide sequences have to be prepared. Therefore, we aimed to find an effective method whereby compounds selectively labeled with fluorophores at their N-terminus can be synthesized with acceptable yield and purity, and to examine the influence of the labeling on the formation of Aβ peptide aggregates with TEM.

2. MATERIALS AND METHODS

2.1. SOLID PHASE PEPTIDE SYNTHESIS

All the aggregation inhibitors were synthesized manually on MBHA resin with the use of standard Boc chemistry and DCC/HOBt coupling. N-terminal acylation of the peptides was performed either with the carboxylic acids pre-activated with DCC/HOBt, or with their acyl-chloride and dimethylaminopyridine.

For fluorescent labeling in liquid phase, a side-chain protected derivative, (Fmoc-N$^{\varepsilon}$-Lys^{28})Aβ[25-35]$_a$ was synthesized on MBHA resin with Boc-chemistry by an automated peptide synthesizer, while for solid-phase labeling of Aβ[25-35]$_a$ and Aβ[1-40]$_a$, both peptides were synthesized manually with the use of standard Fmoc-chemistry on a Rink-amide resin.

2.2. FLUORESCENT LABELING OF AMYLOID PEPTIDES AND THEIR DERIVATIVES

2.2.1 Fluorescent labeling in liquid phase

A general method was applied in each case: the crude peptide was dissolved in DMSO and reacted with FITC. After completing of the reaction, the reaction mixture was directly loaded onto a semipreparative HPLC column and purified. After labeling of Aβ[25-35]$_a$, the N$^{\varepsilon}$-Fmoc group of Lys^{28} was cleaved with 20% piperidine of the final volume for 30 min, and the mixture was neutralized with TFA prior to purification.

2.2.2 *Fluorescent labeling in solid phase*

Peptides were labeled on the resin either with 5(6)-CFLU or AMCA. The N^{α}-deprotected, resin-linked peptide was suspended in the solution of the reagents and shaken for a minimum of 4 hours. Met containing peptides were cleaved from the resin with a cocktail which contained 81% TFA, 5% phenol, 5% thiocresol, 3% H_2O, 2,5% DTT, 2% DMS and 1.5% TBAI, because this cocktail was capable to prevent the oxidation of the Met residue.

2.3 *TEM EXPERIMENTS*

2.3.1 *Sample preparations for TEM investigation*

In order to destroy the aggregates formed during the precipitation or the purification, Aβ peptides were dissolved in HFIP or DMSO and incubated for at least 24 hours at ambient temperature. Then the organic solvent was optionally removed *in vacuo,* followed by the dissolution in solvents depending on the required experimental conditions (commonly either in d.i. water or PBS at pH=7.4) to the final peptide concentration of 0.05-0.5 mg/ml. In some cases the organic solvent was not removed, but diluted with the aqueous medium to the required concentration. Samples were then ultrasonicated for 5 minutes and optionally seeded with a definite volume of pre-aggregated peptide solution (c=0.5 mg/ml, commonly in 1:50 volume ratio), followed by an incubation at 37 °C for the expected time-interval.

2.3.2 *TEM methodology*

Peptide samples were applied to 400 mesh carbon-coated copper grids, stained with 2% (w/v) uranyl acetate and studied with a Philips CM 10 transmission electron microscope operating at 100 kV. Images were taken by a Megaview II Soft Imaging System routinely at magnifications of ×25,000, ×46,000 and ×64,000.

2.4 *SHORT DESCRIPTION OF THE MTT VIABILITY ASSAY*

An improved and convenient MTT test was earlier developed in our laboratory. Differentiated SH-SY5Y neuroblastoma cells were cultured on a 96-well plate. The cells were treated with peptide solutions containing either pre-aggregated, fibrillar Aβ[1-42] alone (c = 10 μM) or a mixture of Aβ[1-42] (c = 10 μM) and one of the aggregation inhibitors (c = 50 μM). After incubation for 24 hours at 37 °C, MTT solution was added to the wells. The absorbance of the produced formazan was measured with a 96-well plate ELISA reader at 550 nm.

3. RESULTS

3.1 TEM STUDIES OF THE AGGREGATON OF Aβ PEPTIDES

Commercial and self-made batches of pure Aβ peptides contain pre-aggregates with unknown structure and weak solvation properties. Since the observed toxicity of a given sample is strongly dependent on the ratio of the different toxic aggregates formed during the aggregation process, standardization of the dissolution protocol is crucial. Based on the results of TEM investigations, we recommend the following method when a purely fibrillar sample is required for the investigations: (1) disaggregation by the application of a strong chaotropic solvent, such as HFIP, (2) removal of the solvent to the required extent, (3) repeated dissolution of the peptide in an aqueous media (dd. water, isotonic saline or PBS), (4) ultrasonication for 5 minutes, and (5) seeding of the sample with pre-formed fibrils in order to initiate and facilitate the nucleation-dependent aggregation of the Aβ peptide.

3.2 DESIGN AND SYNTHESIS OF AGGREGATION INHIBITORS BASED ON MODIFIED β-AMYLOID SEQUENCES: Pr-IIGL$_a$ AND LPYFD$_a$

The advantageous effect of the tetrapeptide Pr-IIGL$_a$ against the toxicity of Aβ[1-42] was first described by our group in 1997. Unfortunately, it behaved ambivalently in biological experiments: in some cases it was neuroprotective, while in the MTT test it showed moderate toxicity (37.8 ±0.5, self-toxicity result). One might ask whether it is possible that the ambivalent behaviour of Pr-IIGL$_a$ is a consequence of some kind of ordered aggregation process which results in the formation of a toxic species. Therefore, the aggregation of Pr-IIGL$_a$, was studied with TEM. The results prove that Pr-IIGL$_a$ displays a strong tendency to aggregate in aqueous solution by forming mature fibrils within 6 days. The propensity of Pr-IIGL$_a$ towards aggregation may explain its neurotoxicity observed in the MTT assay.

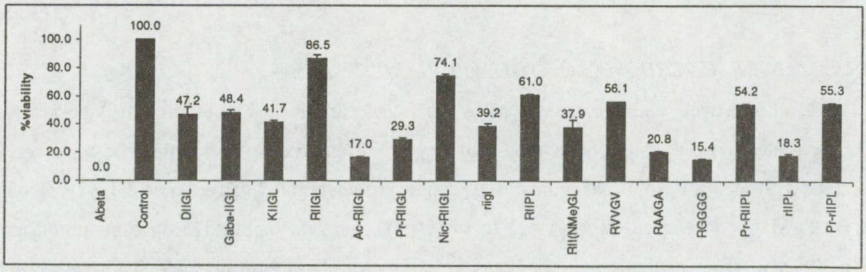

Fig. 1. Results of MTT assays of the peptides which did not show self-toxicity. The protecting efficiency of the peptides against Aβ[1-42] is depicted when applied together.

The design and synthesis of peptide derivatives based on the Pr-IIGL$_a$ peptide resulted in 31 new molecules. Concerning the neuroprotective effect of the syntesized short peptides, the MTT assays revealed that RIIGL$_a$ exerted an outstanding protecting efficacy (86.5±2.7%) (Fig. 1).

TEM fibrillization experiments revealed that RIIGL$_a$ did not aggregate when it was applied alone. Moreover, it could be considered as a real `amyloid aggregation inhibitor´ as it was able to significantly hinder the fibril formation of Aβ[1-42] in 6 days (Fig. 2).

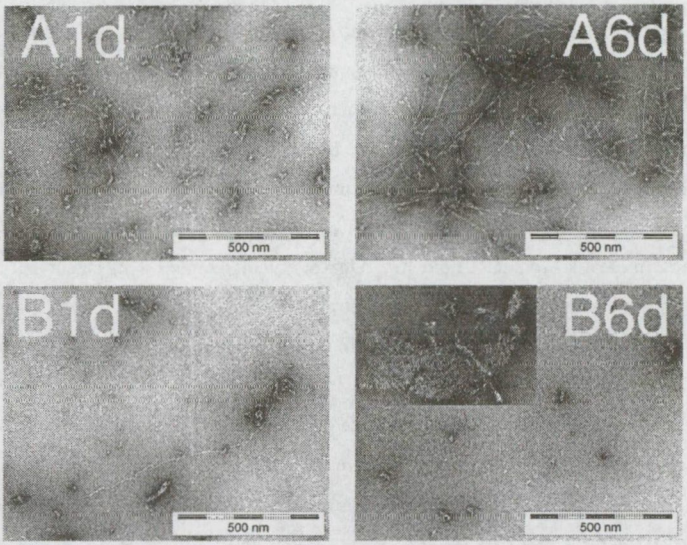

Fig. 2. Effect of RIIGL$_a$ on the aggregation of Aβ[1-42]. Aβ was incubated either alone (c = 100 μM) or together with RIIGL$_a$ (c = 500 μM) at 37 °C for 1 or 6 days (A1d and B1d or A6d and B6d, respectively).

The binding between the pentapeptide and the toxic amyloid aggregates proved to be stereospecific, as neither the *all-D* (39.2±1.6) nor the *all-D reverse* form (59.7±2.4) was able to reach the effectivity of RIIGL$_a$. On the basis of these results we may conclude that both the direction of the peptide bond and the application of L-amino acids seem to be essential for the biological activity. The guanidino group of Arg and the IIGL recognition sequence are also important factors, as both the replacement of Arg with other amino acids containing ionic groups in their side chain (Lys or Asp), and the systematic shortening of the Ile residues (RVVGV$_a$, RAAGA$_a$ and RGGGG$_a$) resulted in the loss of the original activity. Replacement of Gly by Pro altered the activity moderately (RIIPL$_a$: 61.0±0.6); interestingly, the (NMe)Gly-

substituted derivative possessed only poor activity (RII(NMe)GL_a: 37.9±5.8). Unfortunately, acylations of the amino terminus of the peptide and replacements of D-amino acid analogues caused a significant loss in the biological activity with the exception of Kyn-$RIIGL_a$ (65.4±1.1) and Nic-$RIIGL_a$ (74.1±1.4). The former showed slight self-toxicity which deteriorated the plausible effectiveness of the peptide.The latter, being non-toxic, could serve as a candidate in the lead-optimization process together with $RIIGL_a$. In this case, the role of the nicotinyl residue in the mechanism of the action must be elucidated.

With the modification of the Soto-peptide, LPFFD, we obtained the $LPYFD_a$ molecule which exerted a superior neuroprotective effect against Aβ[1-42] in MTT assay. Our efforts to increase the efficacy of $LPYFD_a$ by sequence modifications brought no success; in most cases, a significant loss of the biological activity was observed. The smallest decrease of the viability was observed in the case of the Leu→Ala change. This finding was successfully utilized in the subsequent lead-optimization process which resulted in 50 molecules, 45 of which constitute the basis of a joint patent of the Biogal-Teva Pharmaceutical Company and the Department of Medical Chemistry, University of Szeged. The patent entitled `Peptides and peptidomimetics for the therapeutical treatment of neurodegenerative diseases associated with abnormal protein folding to amyloid-like deposits´ was submitted as `Preliminary patent´ at the end of March, 2005.

TEM aggregation studies have revealed that $LPYFD_a$ does indeed alter the aggregation profile of Aβ[1-42], but the mechanism of its action can not be explained solely with an aggregation inhibiting effect (Fig. 3). On the contrary: new structures (large associates with an unknown structural order, possibly β-sheets combined with random coiled segments) were formed together with the usual aggregate forms (i.e. protofibrils and fibrils). Thus, $LPYFD_a$ can not be considered as a real AAI as it is not able to inhibit the aggregation of Aβ[1-42] completely, but rather, it changes the aggregation characteristics of this Aβ peptide.

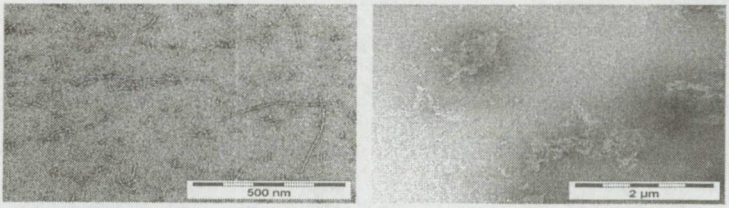

Fig. 3. Effect of $LPYFD_a$ on the aggregation of Aβ[1-42]. The images represent the aggregation of Aβ[1-42] co-incubated with $LPYFD_a$ after 5 days at two different magnifications.

3.3 SYNTHESIS OF PEPTIDES WITH FLUORESCENT GROUPS

3 peptides were labeled with FITC in liquid phase: Aβ[25-35]$_a$, LPYFD$_a$, and RIIGL$_a$, while 7 with a fluorescent group at the N-terminus in solid phase: 5(6)-CFLU-Aβ[25-35]$_a$, AMCA-Aβ[25-35]$_a$, 5(6)-CFLU-Aβ[1-40]$_a$, AMCA-Aβ[1-40]$_a$, 5(6)-CFLU-εAca-RIIGL$_a$ 5(6)-CFLU-LPFFD$_a$ and 5(6)-CFLU-LPYFD$_a$. In general, it can be concluded that the post-synthetic labeling of peptides with fluorescent chromophores lacks the easy separation of products from excess reactants, causing difficult purification. Therefore, fluorescent labeling in solid phase is a more reliable method for the preparation of molecules with fluorotags. In case of peptides which contained Met, the formation of Met-sulfoxide could be prevented during acidolysis by using different cleavage cocktails. The best results were achieved with the application of the cocktail which consisted of 81% TFA, 5% phenol, 5% thiocresol, 3% H_2O, 2.5% DTT, 2% DMS, and 1.5% TBAI.

The capability of the fluorescent Aβ peptides to form fibrillar aggregates under the usual experimental conditions was investigated by TEM (Fig. 4). Extensive fibril formation could be observed in all cases. Nevertheless, the fibrillization process of the labeled derivatives proceeded faster than that of the original sequences, possibly because of the aggregation promoting effect of the fluorescent aromatic ring systems.

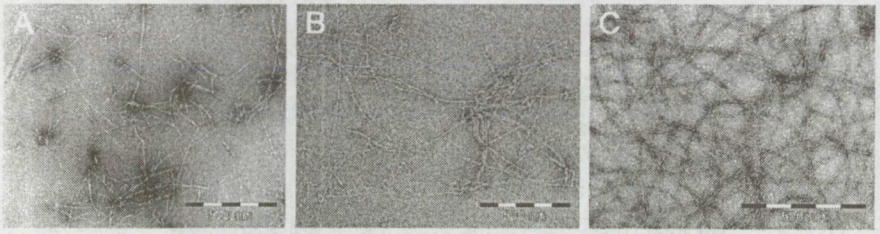

Figure 4. TEM image of (A) 5(6)-CFLU-Aβ[1-40]$_a$, (B) AMCA-Aβ[1-40]$_a$ and (C) 5(6)-CFLU-Aβ[25-35]$_a$ aggregated in d.i. water at 37 °C for 3 days.

4. CONCLUSIONS

The major results of this PhD research are the following:

- I succeeded in adopting the methodology of TEM for the visualization and characterization of the Aβ peptides synthesized and investigated in our laboratory. This is an important step forward, since previously we did not have any proper method to characterize the aggregation state of Aβ samples applied for biological purposes. Based on my results, I can now propose tailor-made treatments to obtain properly aggregated samples of Aβ peptide according to the demands of different experiments.
- With a series of TEM experiments I proved that the tetrapeptide Pr-IIGL$_a$ is capable of forming highly ordered, fibrillar aggregates in a relatively short time. The structure and the mechanism of the association of this peptide are currently under NMR investigation.
- I prepared 31 compounds by the systematic modification of the Pr-IIGL$_a$ sequence, which were tested in MTT assays. The pentapeptide RIIGL$_a$ exhibited the strongest protective effect against fibrillar Aβ[1-42]. Besides, it proved to be a real AAI in TEM fibrillization experiments.
- The lead optimization process of the LPYFD$_a$ pentapeptide involved the synthesis of 61 compounds, 45 of which formed the basis of a joint patent of the Biogal-Teva Pharmaceutical Company and the Department of Medical Chemistry, University of Szeged. Some of these compounds can be valuable candidates for therapeutic purposes.
- I demonstrated that the pentapeptide LPYFD$_a$ did not behave like a real AAI. It could definitely change the aggregation profile of Aβ[1-42] but did not impede the formation of ordered structures considerably.
- For the investigation of the mechanism of Aβ-mediated toxicity at the cellular level, I worked out effective methods to obtain peptides labeled fluorescently exclusively at the N-terminus. I prepared the fluorescent derivatives of two Aβ fragments (Aβ[25-35]$_a$ and Aβ[1-40]$_a$) and three pentapeptides (LPFFD, LPYFD$_a$ and RIIGL$_a$) either in liquid or in solid phase. Their application for fluorescent microscopic studies is currently in progress.

The results achieved during my PhD work are equally important for both basic and applied research. On the one hand, the TEM investigations and the availability of the fluorescently labeled compounds make it possible for us to get an insight into the actions of the Aβ peptides at the molecular level. On the other hand, the drug design and optimization studies resulted in therapeutic candidates which might serve as key medicaments in the combat against Alzheimer's disease in the future.

5. LIST OF PUBLICATIONS

Full papers directly related to the subject of the thesis

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2. **L. Fülöp**, M. Zarándi, Z. Datki, K. Soós, B. Penke: β-Amyloid derived pentapeptide RIIGL(a) inhibits Aβ1-42 aggregation and toxicity, Biochemical and Biophysical Research Communications, 2004, Vol. 324, pp. 64-69. Imp. f: 2.836
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5. Y. Verdier, E. Huszár, B. Penke, Z. Penke, G. Woffendin, M. Scigelova, **L. Fülöp**, M. Szűcs, K. Medzihradszky T. Janáky, Identification of synaptic plasma membrane proteins co-precipitated with fibrillar beta-amyloid peptide, Journal of Neurochemistry, 2005, in press, Imp. f: 4.825

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1. B. Penke, M. Zarándi, G. Tóth, Z. Datki, **L. Fülöp**, V. Szegedi, Z. Molnár, K. Soós, T. Farkas, Z. Penke, Y. Verdier, T. Janáky, G. Tóth, Peptides and peptidomimetics for the therapeutical treatment of neurodegenerative diseases associated with abnormal protein folding to amyloid-like deposits, Preliminary patent, submitted in January, 2005

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3. K. Soós, B. Penke, Z. Datki, **L. Fülöp**, M. Zarándi: An improved synthesis of beta-amyloid peptides for in vitro and in vivo experiments, Journal of Peptide Science, Supplement to Vol.10, Abstracts of the 28th EPS, Prague, 2004, pp. 136.
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LEMONDÓ NYILATKOZAT

Alulírott Szegedi Viktor és Dr. Kukovecz Ákosné Fülöp Lívia a következő nyilatkozatot teszik. Szegedi Viktor, mint az „Pentapeptides derived from AB1–42 protect neurons from the modulatory effect of AB fibrils—an in vitro and in vivo electrophysiological study" (Neurobiology of Disease, 2005, in press) című tudományos dolgozat első szerzője, a pentapeptidek tervezésével, szintézisével, valamint a transzmissziós elektron mikroszkópiás (TEM) vizsgálatokkal kapcsolatos eredményeket tudományos fokozat megszerzéséhez nem használta fel, és ezt a jövőben sem teszi, hanem átengedi azokat a dolgozat egyik társszerzője, Dr. Kukovecz Ákosné Fülöp Lívia részére, aki az említett eredményeket saját tudományos fokozatának (PhD) megszerzéséhez használja fel.

Szeged, 2005.január 24.

Szegedi Viktor
Első szerző

Dr. Kukovecz Ákosné Fülöp Lívia
Társszerző

LEMONDÓ NYILATKOZAT

Alulírott Dr. Yann Verdier és Dr. Kukovecz Ákosné Fülöp Lívia a következő nyilatkozatot teszik. Dr. Yann Verdier, mint az „Identification of synaptic plasma membrane proteins co-precipitated with fibrillar beta-amyloid peptide" (Journal of Neurochemistry, 2005, in press) című tudományos dolgozat első szerzője, a transzmissziós elektron mikroszkópiás (TEM) vizsgálatokkal kapcsolatos eredményeket tudományos fokozat megszerzéséhez nem használta fel, és ezt a jövőben sem teszi, hanem átengedi azokat a dolgozat egyik társszerzője, Dr. Kukovecz Ákosné Fülöp Lívia részére, aki az említett eredményeket saját tudományos fokozatának (PhD) megszerzéséhez használja fel.

Szeged, 2005 március 24.

Dr. Yann Verdier
Első szerző

Dr. Kukovecz Ákosné Fülöp Lívia
Társszerző

LEMONDÓ NYILATKOZAT

Alulírott Datki Zsolt László és Dr. Kukovecz Ákosné Fülöp Livia a következő nyilatkozatot teszik. Datki Zsolt László, mint az „In vitro model of neurotoxicity of Aβ 1-42 and neuroprotection by a pentapeptide: irreversible events during the first hour" (Neurobiology of Disease, 2004, Vol. 17/3, pp. 507-515) című tudományos dolgozat első szerzője a transzmissziós elektron mikroszkópiás (TEM) vizsgálatokkal kapcsolatos eredményeket tudományos fokozat megszerzéséhez nem használta fel, és ezt a jövőben sem teszi, hanem átengedi azokat a dolgozat egyik társszerzője, Dr. Kukovecz Ákosné Fülöp Lívia részére, aki az említett eredményeket saját tudományos fokozatának (PhD) megszerzéséhez használja fel.

Szeged, 2004. november 9.

Datki Zsolt László
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