Memantine and Adamantine KLVFF Conjugates: Synthesis, Aβ interaction and Effects on Recognition Memory in Mice.

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INTRODUCTION

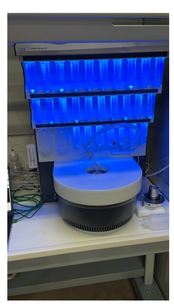
Adamantane derivatives, such as memantine (Mem) and amantadine (Ada), are clinically active drugs with distinct mechanisms of action and therapeutic applications. While Ada is primarily used as an antiviral and anti-Parkinson drug and is not reported to have significant pro-cognitive effects, Mem has been demonstrated to be effective in various clinical conditions characterized by cognitive deficits, including Alzheimer's disease (AD). In our mind, preserving Aβ monomers from being recruited into oligomers may represent a viable therapeutic approach in AD. We designed and characterized two peptide conjugates of the KLVFF fragment bearing a memantinyl (Mem-Succ-KLVFF; Mem=3,5-dimethyladamantan-1-amine,) or an amantadinyl (Ada-Succ-KLVFF; Ada=Adamantan-1-amine) moiety, linked to the KLVFF sequence through a succinyl spacer. Their ability to counteract in vitro Aβ fibrillogensis and in vivo enhancement of memory performance was investigated

METHODS

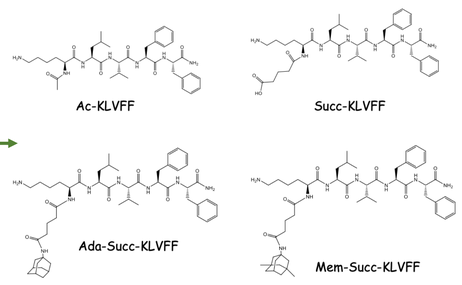
Here we report the synthesis and structural characterization of two KLVFF peptide conjugates with the adamantane derivative drugs memantine or adamantine. Time course Circular Dichroism (CD) were carried out to investigated on the ability of the conjugated peptides to interfere with the $A\beta_{42}$ fibril formation. Complementary Th-T fluorescence and western blot experiments provided further information about the capacity of the conjugated peptides to impact the Aβ's fibrillogenic process. analyses. The interaction of these conjugates with β-CyD was also considered in view of their administration to CD1 mice as inclusion complexes to carry out Novel Object Recognition (NOR) tests. The whole of the results are discussed in terms of Structure Activity Relationships (SAR) based on the in vivo observation in comparison with the evoked cellular pathways connected with memory as well as HR-ESI-MS analyses carried out on the brain homogenates of the treated mice.

SYNTHESIS AND MOLECULAR CHARACTERIZATION OF THE CONJUGATES

MW-Assisted SPPS



Novabiochem TGR resin



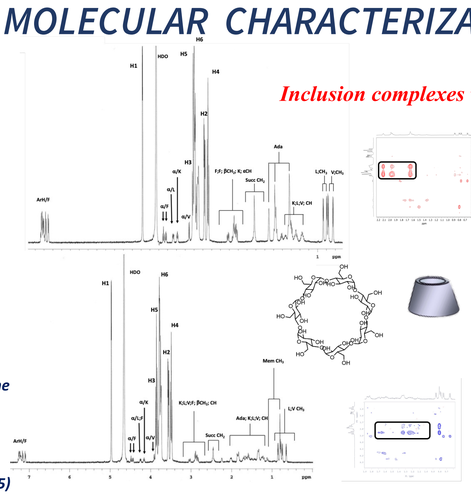
Coupling: TBTU / HOBT / DIEA activation method. MW power 25W, coupling reaction temperature 75 °C, coupling reaction time 300 sec

Deprotection: 20% piperidine in DMF. MW power 25Watt, temperature 75 °C, reaction time 180 sec.

Mem/Ada Conjugation: manually on peptidiyl-resin using DIC/Oxima and free-base Mem (1:4) or HATU, DIEA and Ada•HCl (1:5)

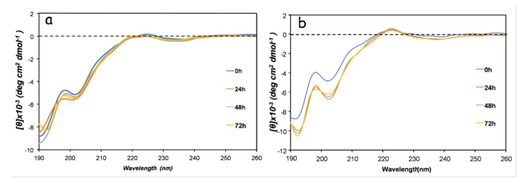
Cleavage: TFA/TIS/H_2O (95/2.5/2.5; v/v/v), 1h RT

Inclusion complexes with β–cyclodextrins

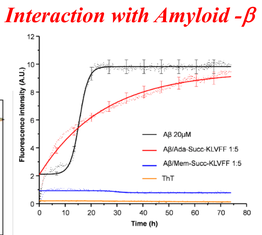


^1H NMR spectrum of Ada-Succ-KLVFF (A) and Mem-Succ-KLVFF (B) peptides in the presence of β-CyD.

Interaction with Amyloid -β



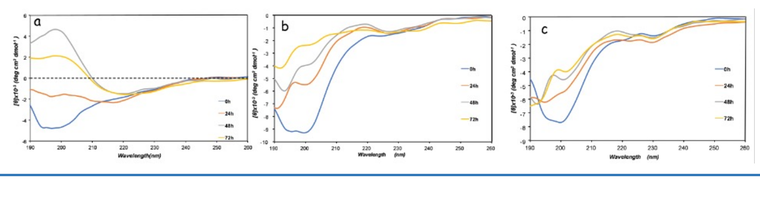
The CD spectra of Ada-Succ-KLVFF (a) and Mem-Succ-KLVFF (b) at different intervals of time as reported in the graphics. Spectra were recorded at 25 °C, in 10 mM phosphate buffer pH 7.4 and at 25 mM sample concentration



Time course of Th-T fluorescence assay of the $A\beta_{42}$/Ada-Succ-KLVFF and $A\beta_{42}$/Mem-Succ-KLVFF mixtures in a 1/5 ratio. $A\beta_{42}$ aggregation curve is reported for comparison

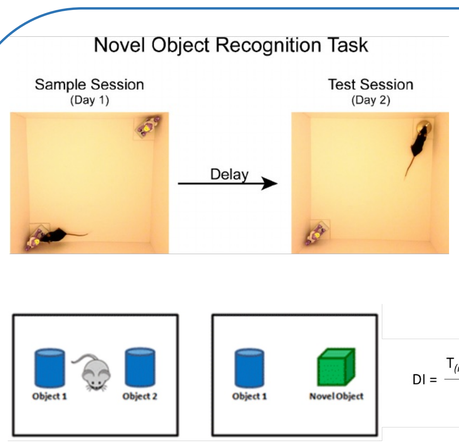
Table 1. Kinetic parameters of Aβ (20 μM) and conjugates at 1:5 molar ratio (100 μM)

Parameters	Aβ	Aβ/Ada-Succ-KLVFF	Aβ/Mem-Succ-KLVFF
F_{max} (a.u.)	9,82 ± 0,02	9,49 ± 0,1	0,79 ± 0,01
$k(h^{-1})$	1,76 ± 0,05	24,86 ± 3,2	2,28 ± 1,2
$t_{1/2}(h^{-1})$	15,49 ± 0,02	N.A.	29,35 ± 1,4
t_{lag} (h^{-1})	11,97 ± 0,04	N.A.	24,79 ± 1,0

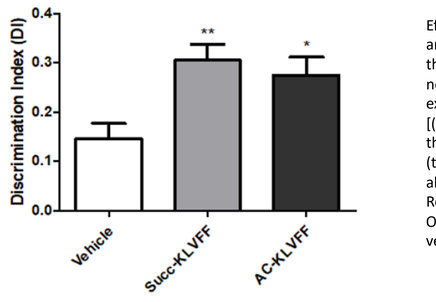


The CD spectra of $A\beta_{42}$ (a); $A\beta_{42}$/Ada-Succ-KLVFF 1:5 molar ratio(b); $A\beta_{42}$/Mem-Succ-KLVFF 1:5 molar ratio (c). Spectra were acquired at different intervals of time, at 25 °C, in 10 mM phosphate buffer pH 7.4.

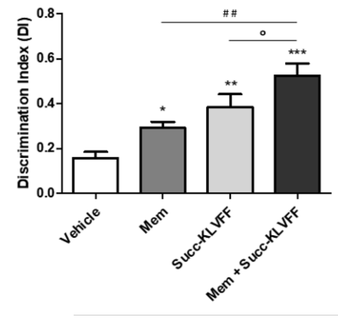
IN VIVO STUDIES: NOVEL OBJECT RECOGNITION TASK



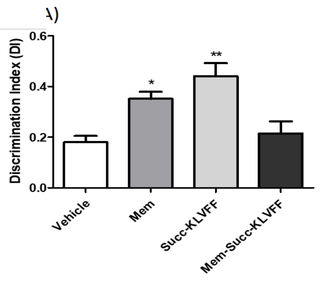
The test was performed according to Ennaceur and Delacour (1988) with slight modifications. To reduce the stress associated with the novel environment, mice received one habituation session 24 hours before testing. During this session, each mouse was placed in a behavioral box for 10 minutes without objects (habituation). Twenty-four hours later each mouse received the treatment and was placed in the housing cage. After 15 minutes the mouse was returned to the behavioral box. In this first trial, each mouse was exposed to two identical objects (familiarization) and allowed to explore the identical objects for 10 minutes before being returned to the housing cage. Three hours later, in the testing trial, one of the two objects was replaced by a new one and the mouse was allowed to explore the novel and the familiar object for 10 minutes. Each trial was analyzed by a researcher blind to the treatment. Exploration of the object was characterized by sniffing and touching the object. The time spent exploring the familiar and novel object was measured and expressed in seconds for each mouse. The results are expressed as discrimination index (DI) calculated as follows: DI = [(time spent exploring the novel object) – (time spent exploring the familiar object)]/[(time spent exploring the novel object) + (time spent exploring the familiar object)]. The DI represents the ability to discriminate the novel object from the familiar one.

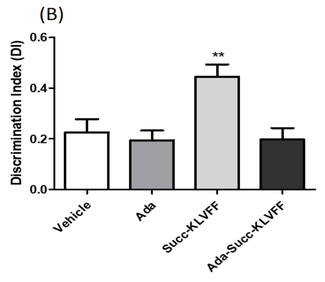


Effects of Succ-KLVFF and Ac-KLVFF in the NOR task. Succ-KLVFF and Ac-KLVFF (27.9 mM, 1ml/kg, ip), injected 15 minutes before the training session significantly increased the exploration of the novel object compared to the vehicle group. Results are expressed as discrimination index (DI) calculated as follows: DI = [(time spent exploring the novel object) – (time spent exploring the familiar object)]/[(time spent exploring the novel object) + (time spent exploring the familiar object)]. DI represents the ability to discriminate a novel object from the familiar one. Results are the MEAN ± SEM of 12-15 mice per group.
One-way ANOVA, followed by Tukey's test. *p<0.05 ; **p<0.01; vs vehicle group.



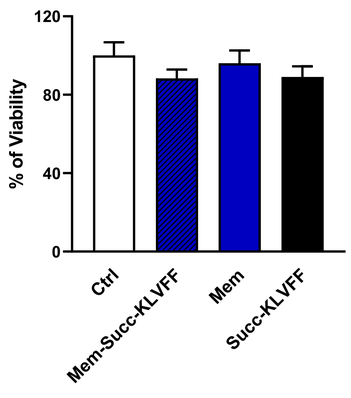
Effects of the combination treatment with Mem and Succ-KLVFF in the NOR task. The combined treatment with Mem (27.9 mM, 1 ml/kg, ip) and Succ-KLVFF (27.9 mM, 1ml/kg, ip) injected 15 minutes before the training session significantly increased the exploration of the novel object compared to the vehicle group and showed a synergic effect when compared to MEM or Succ-KLVFF alone. Results are expressed as discrimination index (DI) calculated as follows: DI = [(time spent exploring the novel object) – (time spent exploring the familiar object)]/[(time spent exploring the novel object) + (time spent exploring the familiar object)]. DI represents the ability to discriminate a novel object from the familiar one. Results are the MEAN ± SEM of 12-15 mice per group. One-way ANOVA, followed by Tukey's test. *p<0.05; **p<0.01; vs vehicle group.



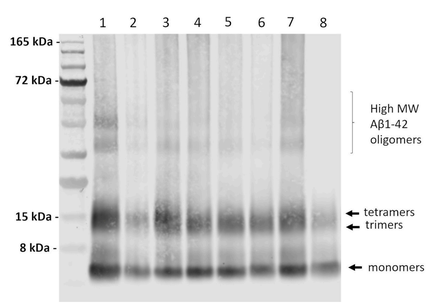


Effects of Mem (A) or Ada (B) alone or conjugated with Succ-KLVFF in the NOR task. Mem (27.9 mM, 1ml/kg, ip) and Succ-KLVFF (A: 27.9 mM, 1ml/kg, ip; B: 330 mM, 1 ml/kg, ip) alone 15 minutes before the training session significantly increased the exploration of the novel object compared to the vehicle group. Neither Mem-Succ-KLVFF (27.9 mM, 1ml/kg, ip) nor Ada-Succ-KLVFF (330 mM, 1 ml/kg, ip) or Ada (330 mM, 1 ml/kg, ip) affected the DI compared to the vehicle group. Results are expressed as discrimination index (DI) calculated as follows: DI = [(time spent exploring the novel object) – (time spent exploring the familiar object)]/[(time spent exploring the novel object) + (time spent exploring the familiar object)]. DI represents the ability to discriminate a novel object from the familiar one. Results are the MEAN ± SEM of 12-15 mice per group. One-way ANOVA, followed by Tukey's test. *p<0.05; **p<0.01; vs vehicle group.

IN VITRO STUDIES: MTT AND WESTERN BLOT

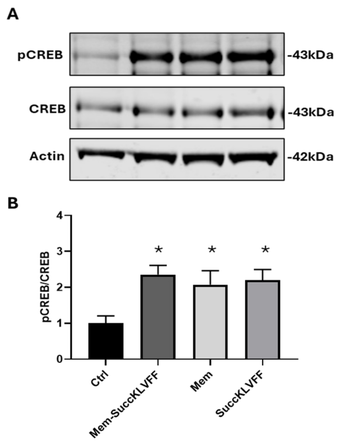


MTT Assay of fully differentiated SH-SY5Y treated for 48 hours with Mem-Succ-KLVFF, Mem, and Succ-KLVFF at the concentration of 5 μM. Bars represent means ±SEM of three independent experiments with n=3 each



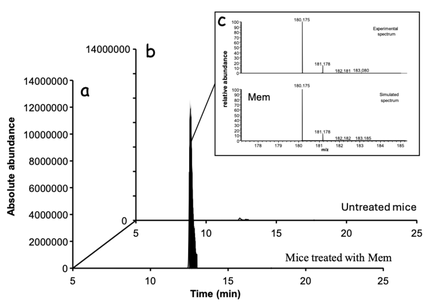
1. oAβ1-42/Mem-Succ-KLVFF (1:1)
2. oAβ1-42/Mem-Succ-KLVFF (1:5)
3. oAβ1-42/Mem (1:1)
4. oAβ1-42/Mem (1:5)
5. oAβ1-42/Succ-KLVFF (1:1)
6. oAβ1-42/Succ-KLVFF (1:5)
7. oAβ1-42
8. mAβ1-42

Representative western blot of Aβ oligomers (oAβ) prepared in the presence or absence of different molar ratios (1:1, 1:5) of Mem-Succ-KLVFF, Memantine (Mem) and Succ-KLVFF. Samples were separated into a 4-12% Bis-Tris SDS-PAGE gel and blotted with anti-Aβ N-terminal1-16 mouse monoclonal antibody 6E10 (1:800).

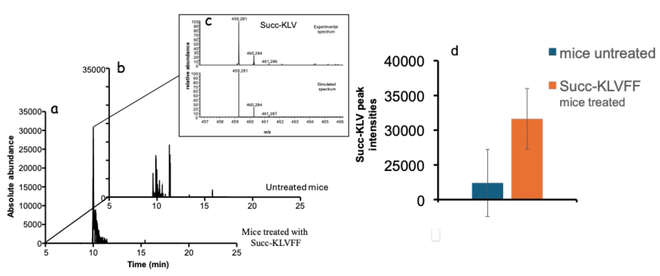


A) Representative immunoblot of pCREB from differentiated SH-SY5Y after 30 min exposure to 5μM Memantine (Mem), Memantine-Succ-KLVFF (Mem-Succ-KLVFF) and Succ-KLVFF. B) Densitometric values of pCREB, normalized on CREB signals, are represented in the respective graph bars. Bars represent means ±SEM of 4 independent experiments with n=3 each.

IN VITRO STUDIES: HR-ESI-MS



Chromatographic profiles reported as extracted-ion chromatograms (XIC) of Mem (m/z=180.17-180.18) of a) homogenate brain tissue obtained from mice treated with Mem and b) homogenate brain tissue obtained from mice not treated with Mem. The inset c) shows the comparison between the experimental and theoretical isotopic distribution of the m/z peak corresponding to the Mem.



Chromatographic profiles reported as extracted-ion chromatograms (XIC) (m/z=459.27–459.29) of a) homogenate brain tissue obtained from mice treated with Mem + Succ-KLVFF and b) homogenate brain tissue obtained from mice not treated with Mem + Succ-KLVFF. The inset c) shows the comparison between the experimental and theoretical isotopic distribution of the m/z peak corresponding to the Succ-KLV; d) comparative analysis of the chromatographic peak intensities averaged over 3-6 replicate measurements.

CONCLUSIONS

The results suggest that co-administration of Mem and KLVFF-based peptides behave as cognitive enhancers in normal cognition and therefore might have great potential to treat cognitive impairment resulting from neurodevelopmental and neurodegenerative disorders.

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