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Discovery of Small Peptides Derived from Embryonic Lethal Abnormal Vision Proteins Structure Showing RNA-Stabilizing Properties

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Abstract: Four peptides corresponding to highly conserved motives within the first two RNA recognition motif-type domains of ELAV proteins were prepared, and their effect on the stability of NOVA-1 and VEGF ELAV-target mRNAs was evaluated. Biological results show that in the presence of phorbol 12-myristate 13-acetate (a PKC activator triggering the ELAV pathway), an equimolar mixture of peptides induces a statistically significant stabilization of the selected transcripts, suggesting a synergic effect of the two stimuli.

Evidence in the literature indicates an increasing interest in post-transcriptional regulatory mechanisms because of their fine, localized effects on gene expression. Within this context, modulation of mRNA decay appears to be an efficient post-transcriptional way of controlling gene expression because small changes in mRNA half-life can drastically alter the amount of the corresponding protein. Among the different players, the RNA-binding proteins (RBPs[a]) can affect the processing of selected transcripts, their transport and subcellular localization in the cytoplasm, and their stability and translatability. Among the best characterized RBPs are the ELAV (embryonic lethal abnormal vision) proteins that preferentially interact with adenine and uridine-rich elements (AREs) present in the 3′-untranslated region of a subset of mRNAs. ARE-containing mRNAs are prone to a rapid degradation, but following the binding of ELAV, an increase in their cytoplasmic stability and rate of translation occurs.[1,2] In vertebrates, HuB, HuC, and HuD are neuron-specific (nELAV) while HuR is ubiquitously expressed.[3]

Biologically, ELAV are pleiotropic proteins that are important in various contexts, since they can affect the fate of some mRNAs whose corresponding proteins are fundamental for key cellular functions, such as maintenance of cellular homeostasis, growth and development, survival/apoptosis, and stress response.[4−6] In particular, within brain physiology nELAV positively control (spatiotemporally) the fate of target mRNAs whose products are necessary for neuronal differentiation and plasticity, thus making nELAV major determinants of brain development and memory processes.[7−9] Consequently, a deficit in the nELAV cascade may play a key role in pathologies associated with memory disturbances as indicated by recent findings on Alzheimer's dementia,[10] strongly suggesting the importance of post-transcriptional regulatory mechanisms on gene expression in the etiology of the disease.

On the basis of these observations, we addressed our efforts to the discovery of ELAV-mimicking molecules that can be useful for a basic research aimed at better understanding of mRNA−protein interactions and can constitute the first step toward a highly innovative pharmacological approach to increase or recover the expression of ELAV-target mRNAs that are defective in some pathological conditions. To date, the ELAV/mRNA complex remains completely unexplored from a medicinal chemistry point of view. Currently in the literature there is no evidence of molecules that may affect the fate of mRNAs and thus the amount of the corresponding proteins. Therefore, to find ELAV-mimicking molecules, we followed a rational design approach based on ELAV proteins structure analysis and on the resolved 3D-structure of HuD in complex with a target transcript.

The four vertebrate ELAV proteins are characterized by a high degree of sequence homology (70−85%). They are about 40 kDa in size and contain three ∼90-amino-acid-long RNA recognition motif-type (RRM) domains. The first two RRMs, positioned at the N-terminal, are separated from the third one, at the C-terminus, by a hinge segment.[3,11] In vitro binding studies indicate that the first and second RRMs are primarily implicated in the interaction of ELAV RBPs with the ARE sequences present in the target mRNA.[12,13] Structural investigation of the first two RRMs bound to ARE shows that they form a cleft with their β-sheet-containing surfaces in which the mRNA element is inserted. Nothing is known about the binding mode of the third RRM to mRNA. This RRM seems to have affinity for the poly(A) tail of the messenger, thus contributing to the stability of the ELAV/mRNA complex.[4] Moreover, sequence alignment studies of the different ELAV proteins clearly indicate that the first two RRM domains each contain two highly conserved sequences of eight and six amino acids, named RNP1 and RNP2, respectively. Additionally, X-ray crystallography reveals that the four RNPs (RNP1 and RNP2 sequences of both RRM1 and RRM2 domains) are directly involved in the mRNA binding.[14]

On the basis of these observations and considering that peptides are adequate molecules for mimicking protein binding sites,[15] we focused our attention on the preparation and

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[a] Abbreviations: ARE, adenine and uridine-rich elements; DRB, 5,6-dichlorobenzamidazole riboside; ELAV, embryonic lethal abnormal vision; Fmoc, 9-fluorenylmethyloxycarbonyl; HBTU, 2-(1*H*-benzotriazol-1-yl)-1,1,3,3-tetramethyluronium hexafluorophosphate; HOBt, *N*-hydroxybenzotriazole; HPLC, high performance liquid chromatography; LC−MS, liquid chromatography−mass spectrometry; nELAV, neuron-specific embryonic lethal abnormal vision proteins; NOVA-1, neuro-oncological ventral antigen-1; PKC, protein kinase C; P_{1-4}, equimolar mixture of peptides **1**−**4** (1 μM each peptide); PMA, phorbol 12-myristate 13-acetate; RBPs, RNA-binding proteins; RNP, ribonucleoproteic; RRM, RNA recognition motif type; RT-PCR, real time polymerase chain reaction; SAR, structure−activity relationship; TBTU, *O*-(benzotriazol-1-yl)-*N*,*N*,*N*′,*N*′-tetramethyluronium tetrafluoroborate; VEGF, vascular endothelial growth factor.

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Table 1. Peptide Sequences and Their Correspondence in ELAV Structure

peptide	sequence	ELAV ref[a]
1	LGYGFVNY	RRM1_RNP1
2	LIVNYL	RRM1_RNP2
3	RGVGFIRF	RRM2_RNP1
4	LYVSGL	RRM2_RNP2

[a] Correspondence in ELAV structure.

Table 2. Peptide Crude Purities

	crude purity,[a] %	
peptide	method 1	method 2
1	75.1	90.1
2	66.4	89.3
3	43.6	85.3
4	72.4	90.3

[a] Determined by analytical reverse-phase HPLC using a Phenomenex Jupiter C18 column with gradient elution (water:acetonitrile:trifluoroacetic acid, see Supporting Information); UV detection at 230 nm.

biological evaluation of four peptides corresponding to ELAV RNP1 and RNP2 sequences of RRM1 and RRM2 domains (peptides **1**−**4**, Table 1).

Peptides were synthesized according to the solid phase approach using the Fmoc-based methodology. A parallel solid-phase procedure was first experimented in an automated parallel organic synthesizer (Vantage-aapptec) at room temperature, using HBTU and HOBt as the coupling reagents (method 1; see Supporting Information). After cleavage and side chain deprotection (trifluoroacetic acid/dichloromethane, 95:5 v/v), peptides **1**−**4** were obtained as main reaction products (reverse-phase LC−MS analysis; see Supporting Information) with satisfactory crude purities (>65%, Table2), with the only exception of **3** (43.6%, Table 2). A more convenient synthetic procedure, combining solid-phase peptide synthesis with microwave irradiation, was then developed using the Liberty microwave-assisted automatic peptide synthesizer (CEM, Matthews, NC) and successfully applied to the preparation of **1**−**4** (method 2; see Supporting Information). Coupling reactions were performed under microwave irradiation (80 °C, 25 W, 300 s), using TBTU. Cleavage and side chain deprotection were then carried out (trifluoroacetic acid/water/triisopropylsilane, 95:2.5:2.5 v/v/v, 40 °C, 15 W, 25 min), providing the desired peptides with purities higher than 85.0% (Table 2). Interestingly, a noticeable improvement of the peptide crude purities was observed when our optimized microwave-assisted protocol was applied instead of the conventional parallel one. As clearly reported in Table 2, this effect was particularly pronounced for peptide **3**.

Crudes **1**−**4** were finally purified by preparative reverse-phase LC−MS, using a Waters Simmetry C18 column (Waters autopurification system; see Supporting Information). Fractions of adequate HPLC homogeneity and with the expected mass were combined and lyophilized, obtaining **1**−**4** with a purity higher than 95.0%, as evidenced by the reverse-phase HPLC analysis (see Supporting Information).

Moving from chemistry to biological investigation, our strategy consisted of evaluating the capability of peptides **1**−**4**, corresponding to the highly conserved sequences within the first two ELAV RRM domains, to affect the mRNA stability without the remaining portion of the protein. According to our approach, we performed the biological

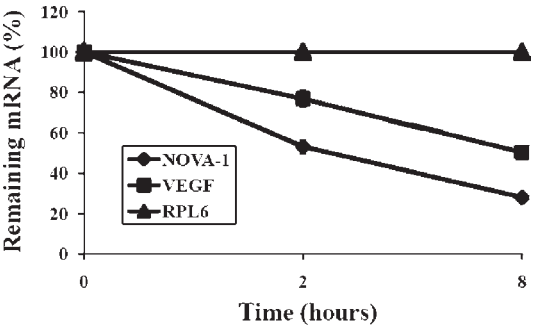

Figure 1. Evaluation of NOVA-1 and VEGF mRNAs decay by real-time quantitative PCR in SH-SY5Y human neuroblastoma cells in an 8 h time interval. RPL6 was used as reference mRNA for NOVA-1 and VEGF normalization, remaining substantially stable in the 8 h time frame.

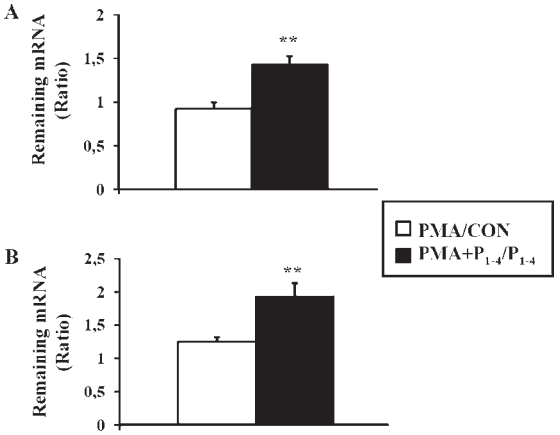

Figure 2. Effect of P_{1-4} on VEGF and NOVA-1 mRNA stability: ratios between percentages (PMA/CON and (PMA + P_{1-4})/P_{1-4}) of remaining NOVA-1 (A) and VEGF (B) mRNAs in SH-SY5Y cells after 2 h. The treatments were performed in the presence of transcriptional blocking: (**) $p < 0.005$, two-tailed t test, $n = 5$, CON = control.

investigation by assaying **1**−**4** in mixture and not individually, even if this is not the most commonly used procedure. In detail, we evaluated the effect of the equimolar (1 μM) mixture of **1**−**4** (P_{1-4}) on the stability of two ARE-bearing ELAV target mRNAs, NOVA-1 (neuro-oncological ventral antigen-1)[9] and VEGF (vascular endothelial growth factor).[16]

NOVA-1 is a neuron-specific splicing factor that regulates the alternative processing of a set of transcripts essential for proper neural development and for synaptic plasticity in the nervous system.[9,17] Indeed, the lack of expression of *Nova-1* is responsible for severe defects in the development of the motor system in the null mice.[18] VEGF is a protein with well-documented effects on blood vessel formation; moreover, it has also been related to repairing processes after brain injury.[19] Indeed, the deletion of a single *Vegf* allele causes embryonic lethality.[20]

Preliminary kinetic studies aimed at evaluating NOVA-1 and VEGF mRNAs decay (within 8 h in conditions of transcriptional arrest) led us to set 2 h as the optimal experimental time for performing mRNA stability experiments, since the mRNA levels of both transcripts are easily detectable at this time (Figure 1).

The effect of P_{1-4} on NOVA-1 and VEGF mRNAs stability was then evaluated in vitro on SH-SY5Y human neuroblastoma cells by quantitative real time PCR (RT-PCR),

after transcriptional block, with or without PMA (phorbol 12-myristate 13-acetate) stimulation (100 nM for 15 min) (see Supporting Information). PMA is an activator of protein kinase C able to trigger the downstream phosphorylation/activation of ELAV proteins.[21] Regarding NOVA-1 mRNA, PMA alone does not produce any change in its stability with respect to the control (Figure 2A), in accordance with previously published data.[9] Instead, the increase in VEGF mRNA stability after PMA alone (Figure 2B) is in line with the higher levels of VEGF protein found in the same experimental conditions in another cell line.[16] Results reported in Figure 2 clearly indicate that the concomitant cell treatment with P_{1-4} and PMA gives rise to a statistically significant stabilization of both ELAV-target transcripts with respect to PMA alone, while P_{1-4} without PMA does not show any detectable mRNA stabilizing effect. This result is of particular relevance in the case of NOVA-1: only the copresence of the P_{1-4} and PMA leads to significant stabilization of its mRNA, with the PMA alone proving ineffective. In short, results strongly suggest a synergic effect of P_{1-4} and PMA stimuli on mRNA stability, according to our model in which P_{1-4} acts in concert with the PMA-activated ELAV proteins physiologically present in the cells, through the binding to ARE sequences and the following stabilization of the target mRNAs. However, further investigation is needed to better clarify the mechanism of action of these peptides.

To conclude, we have reported the synthesis and the biological investigation of four peptides as prototypes of the first class of ELAV-mimicking molecules. The surprising discovery made in this study is that the mixture of peptides **1**−**4** induces a statistically significant stabilization of the two considered ELAV-target transcripts with respect to PMA alone. Actually, this is the first time that ELAV mimicking properties have been clearly indicated for nonphysiological molecules. Notably, given the pleiotropic action of ELAV,[7] the availability of specific molecules able to modulate the fate of a subset of mRNAs may have relevance in many physiologic and pathologic fields.

Our current efforts are directed toward a deeper understanding of the mRNA-stabilizing activity of **1**−**4** by testing them individually or in different compositions. Successively, we intend to address our attention to peptide structure−activity relationship (SAR) studies and to peptide bioactive conformation identification followed by design and synthesis of **1**−**4** analogues, with the final aim of obtaining new molecules with improved ELAV mimicking properties.

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Supporting Information Available: Details of syntheses, purification, characterization, and biological evaluation of peptides **1**−**4**; HPLC chromatograms. This material is available free of charge via the Internet at http://pubs.acs.org.

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