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Compounds Interfering with Embryonic Lethal Abnormal Vision (ELAV) Protein−RNA Complexes: An Avenue for Discovering New Drugs

Miniperspective

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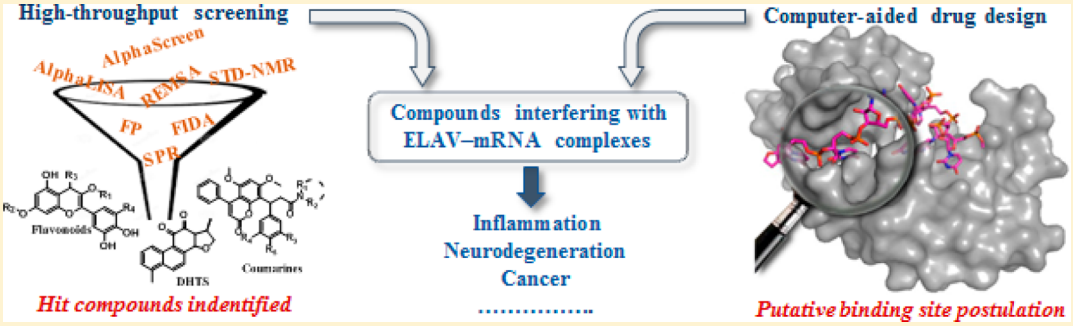
Supporting Information



ABSTRACT: RNA-binding proteins play a key role in post-transcriptional processes. Among these proteins, embryonic lethal abnormal vision (ELAV) proteins are among the best described. ELAV proteins predominantly act as positive regulators of gene expression, and their dysregulation is involved in several pathologies, such as cancer, inflammation, and neurodegenerative diseases. Only a few structurally unrelated compounds interfering with ELAV protein−mRNA complexes have been identified by applying high-throughput screening approaches. Considering the structural diversity of the compounds discovered so far and the different techniques employed for screening their ability to interfere with ELAV protein−mRNA complexes, drawing conclusions from structure−activity relationships remains a challenge. We performed docking studies to understand the interactions of compounds reported over the past decade to be inhibitors of ELAV proteins and to evaluate the potential of computer-aided drug design to target this family of proteins for further drug discovery.

1. INTRODUCTION

Post-transcriptional processes have a crucial role in controlling gene expression. Indeed, they shape the fate of each transcript, starting from the nucleus (i.e., alternative splicing, polyadenylation, and nuclear export) and continuing through its final journey to a ribosome (i.e., cytoplasmic localization, stability, and rate of translation).[1−4] Therefore, dysfunctions within these processes may have a causative role in several pathologies, such as neurodegenerative diseases and cancer.[5−9]

Over the past decade, progress in genome-wide experimental and computational techniques allowing the study of protein−RNA and RNA−microRNA (miRNA; a small noncoding RNA also involved in the control of gene expression) interactions with high specificity and resolution has significantly improved understanding of the role of post-transcriptional control of gene expression.[10,11] Within this context, the scientific community has recently focused on RNA-binding proteins (RBPs). To date, hundreds of RBPs (some examples are reported in Table 1) affecting the fate of target messenger RNAs (mRNAs), whose corresponding proteins are fundamental for several key cellular functions, have been identified.[12] Some RBPs are ubiquitous, while others are expressed in specific tissues, such as the nervous system, and/or they are preferentially localized in subcellular compartments (i.e., the cytoplasm or nucleus).[13−23] Deregulation of the content/function of RBPs may contribute to the pathogenesis of several diseases, including neurological disorders and cancer.[2,4,24−29]

Embryonic lethal abnormal vision (ELAV) proteins, or Hu proteins, are RBPs that play pivotal roles not only in neuronal

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Table 1. Role and Localization of Selected Well-Known RBPs

RBP	localization	role
Cytoplasmic polyadenylation element-binding protein (CPEB)	Neuronal dendrites	Regulation of mRNA translation[13]
Embryonic lethal abnormal vision (ELAV) proteins	Neurons (HuB, HuC, HuD), gonads (HuB), ubiquitous (HuR)	Regulation of mRNA stability, translation, splicing, and polyadenylation[14,15]
Fragile x mental retardation protein (FMRP)	Brain	Control of mRNA transport and translation at the synapse level[16]
Fused in sarcoma/translocated in liposarcoma (FUS/TLS)	Nucleus	Regulation of RNA biogenesis[17]
Neuro-oncological ventral antigen (NOVA-1, NOVA-2)	Differentiated neurons; spinal cord and hindbrain (NOVA-1), neocortex (NOVA-2)	Regulation of alternative splicing of RNA[18,19]
Polypyrimidine tract-binding protein 2 (PTBP-2)	Early postmitotic neurons, muscles and testes	Repression of alternative splicing[20]
Transactive response DNA-binding protein 43 (TDP-43)	Nucleus	Involvement in pre-mRNA splicing, mRNA trafficking and turnover[21]
Lin28a-b	Nucleus/cytoplasm	Suppressor of microRNA (miRNA) biogenesis[22]
Tristetraprolin (TTP)	Nucleus	Regulation of mRNA stability[23]

development and maintenance but also in cancer.[30] In mammals, four ELAV proteins are known: HuR (ELAVL1), HuB (ELAVL2), HuC (ELAVL3), and HuD (ELAVL4).[30] HuR is ubiquitously expressed, while HuB, HuC, and HuD are predominantly expressed in the nervous system and are classified as neuronal ELAV (nELAV) proteins. In addition to neuronal expression, HuB is also expressed in the gonads, and HuD is expressed in insulin-producing pancreatic β cells.[31] Mammalian ELAV proteins are characterized by a high degree of sequence homology (70−85%) and are approximately 40 kDa in size. They contain three RNA recognition motif-type (RRM) domains, each approximately 90 amino acids long.[32] The first two consecutive domains (RRM1 and RRM2) are near the N-terminus and are linked to the third domain (RRM3) by an unconserved hinge region. Notably, the hinge region is mainly responsible for the nuclear/cytoplasmic shuttling that occurs after protein activation.[33−35]

Studies in the literature have demonstrated that the RRM1 and RRM2 domains directly interact with the target transcript through the highly conserved ribonucleoprotein (RNP) 1 and 2 sequences, which have 8 and 6 amino acids, respectively.[36,37] Aside from being involved in binding to the poly(A) tail of the target mRNAs, the RRM3 domain has a crucial role in the homo- and heteromultimerization processes of ELAV proteins.[38−40]

ELAV mammalian genes are orthologues of the *Drosophila melanogaster elav* gene family, which includes three paralogous genes: *elav* (embryonic lethal, abnormal visual system), *rbp9* (RNA binding protein 9), and *fne* (found in neurons).[41] In 1985, the *elav* gene was the first member of this family to be identified in *Drosophila*. Campos et al. renamed the relative locus l(1)EC7 to "*elav*" in virtue of its essential function in the eye: indeed, elav is required for a physiological neuronal differentiation, and *elav* gene mutations lead to abnormal visual system phenotype and lethality of the fruit fly.[41,42] ELAV protein is expressed in the nervous system and localized in the nucleus of neuronal cells, where it regulates splicing and polyadenylation. It is expressed at all stages of neural development, and its function is essential also for the maintenance of the adult nervous system of the fruit fly.[42] With respect to their counterpart in *Drosophila*, besides nuclear localization and relative functions, mammalian ELAV proteins can also be found in the cytoplasm, where they control mRNA target stability, translation, and transport by preferentially binding to the adenine-uracil-rich element (ARE) sequences of specific transcripts. However, other consensus sequences may also be implicated.[43,44] The neuronal members of the ELAV family are mainly localized in the cytoplasm, while HuR remains mostly within the nucleus in basal conditions.[45] However, it is important to point out that there is no strict compartmentalization for both localization and function, since ELAV proteins can shuttle between the cytoplasm and the nucleus under stimuli, thus affecting any step of the post-transcriptional gene regulation (PTGR). It is likely that the processing of the target transcripts is mainly affected by alteration in the expression and/or subcellular localization of ELAV proteins and only marginally by a modified RNA-binding specificity.[46] All these aspects may have implications for drug interference and should be pondered when deciphering the molecular mechanism of ELAV-targeting drug actions.

It is well-known that the four mammalian ELAV proteins can interact with many mRNAs, giving rise to various ELAV protein−mRNA complexes with different physiological roles.[15,28,47,48] High-throughput screening (HTS) studies have highlighted that although ELAV proteins share some common target transcripts, they also possess a certain degree of specificity. In this regard, mRNA-encoding proteins involved in neuronal plasticity, outgrowth, and neuronal metabolism maintenance are mainly targeted by nELAV proteins, while HuR has been described to possess a greater binding affinity for transcripts implicated in oxidative stress defense, inflammation, cell growth, and cell cycle regulation.[43,44] Notably, the importance of HuR in these processes is so relevant that HuR has been proposed as a prognostic factor for different types of cancer.[49−51] Additionally, in the nervous system, nELAV protein impairments have been suggested to contribute to the development of neurodegenerative disorders, such as Alzheimer's disease[52] and paraneoplastic neurological syndrome.[26]

Overall, this evidence suggests that ELAV proteins have potential as pharmacological targets in several pathologies. Nevertheless, to date, only a few compounds interacting/interfering with ELAV−mRNA complexes have been discovered; hence, the use of small molecules can be challenging for medicinal chemists.

This review provides an overview of the efforts directed toward the identification of natural and synthetic compounds able to interfere with ELAV protein−mRNA complexes, from the pioneering work of Meisner in 2007[53] until today. The druggability of ELAV proteins, which may provide valuable information for rational drug design, is also discussed.

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2. IDENTIFICATION OF COMPOUNDS INTERFERING WITH ELAV PROTEIN−MRNA COMPLEXES VIA HTS

Research in the field of ELAV protein and RBP modulators is still considered pioneering and challenging for medicinal chemists. Only a few research groups have focused on the ELAV protein−mRNA system to identify potential therapeutic agents. The related research studies were generally conducted by screening a large number (or a library) of commercially available compounds against a specific "protein−RNA complex" in accordance with HTS approaches. As a result, only a few hit compounds have yet been discovered using various biological assays based on different detection technologies. In the following sections, we will discuss the most relevant findings.

2.1. Dehydromutactin (1), Okicenone (2), and Compound MS-444 (3). In 2007, Meisner and co-workers for the first time approached the ELAV protein−mRNA system from a medicinal chemistry perspective, focusing particularly on HuR.[53] Within a nature-aided drug-discovery program, 50 000 different extracts derived from microbial, mycological, and plant sources were screened against both the full-length HuR protein and a shortened variant of recombinant HuR (rHuR), HuR_{12}, amino acids 2−189 equivalent to RRM1/RRM2 domains. A fluorescence intensity distribution analysis was used for the screening. The extracts showing an inhibitory effect on the HuR protein−mRNA interaction were then purified, thus allowing the isolation of five compounds, which were subsequently fully characterized. Compounds **1**−**3** (Figure 1) displayed an in vitro

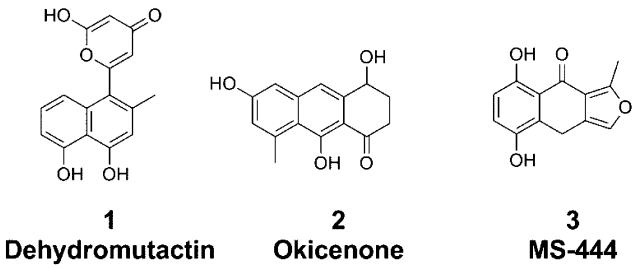

Figure 1. Compounds isolated from microbial extracts and identified as the first HuR protein inhibitors.[53]

inhibitory effect on the HuR−mRNA complex in a concentration-dependent manner. The experiments were conducted on both the full-length HuR and on the fragment HuR_{12}, employing ARE-bearing RNA sequences of comparable lengths within the transcripts of tumor necrosis factor α (TNFα), interleukin 2 (IL-2), and cyclooxygenase 2 (Cox-2), whose corresponding proteins play key roles in inflammation and the immune response. Compounds **1**−**3** clearly showed an inhibitory effect on the formation of both HuR−mRNA and HuR_{12}−RNA complexes, suggesting that **1**−**3** interact with the RRM1 and RRM2 domains. In light of these results and with the aim of clarifying the mechanism of interaction, the authors studied the HuR_{12}−RNA interaction by applying a mathematical approach. Combining experimental and in silico results, they postulated the following: (i) HuR exists as a homodimer; (ii) HuR binds mRNA in a stoichiometric ratio (2HuR:1mRNA and 2HuR:2mRNA); (iii) the RRM domains could mediate homodimerization. The in silico studies also confirmed that compounds **1** and **2** can interfere with HuR homodimerization, whereas compound **3** has a weak binding affinity.

Taken together, these observations partially clarify the mechanism of interaction of exogenous ligands and ELAV proteins, highlighting the importance of the RRM sequences. The binding of compounds **1** and **2** to the RRM sequences may prevent homodimerization of the protein, thus impeding interactions with mRNA ARE sequences. In light of these findings, these studies represent a milestone in this field, providing initial proof of the chemical druggability of the ELAV protein family.

2.2. Flavonoids: (−)-Epigallocatechin Gallate (4), (−)-Epicatechin Gallate (5), Ellagic Acid (6), Rhamnetin (7), Myricetin (8), and Quercetin (9). In 2009, Park and co-workers identified flavonoids that inhibit HuC−mRNA complexes.[54] They screened a library of 52 natural compounds with certain anti-inflammatory and antitumorigenic activities (source not reported) by employing a nonradioactive RNA electrophoretic mobility gel shift assay (REMSA), which is a technique useful for characterizing protein−RNA interactions.[55] REMSA is considered a versatile, useful, and informative tool, especially when combined with other biochemical, biophysical, or genetic approaches. Interestingly, only compounds with druglike properties [i.e., high membrane permeability, low toxicity, and suitable size, with a molecular weight (MW) of <500] were screened, and the effects on HuC−artificial AU-rich RNA sequence complexes were evaluated. The library was initially tested at a concentration of 100 μM, and the most active compounds were further assayed at lower doses, leading to the identification of 14 compounds effective at low concentrations (20 μM and 4 μM). For the most active compounds, the IC_{50} values were determined (Figure 2). Briefly, flavonoids **4**−**9** showed IC_{50} values ranging from 0.2 to 1.8 μM.

Encouraged by these results, the same research group studied the complex formed by HuR and ARE-bearing RNA sequences from TNFα transcripts (ARE-$RNA^{TNF\alpha}$) to identify new compounds potentially useful for the treatment of inflammatory diseases.[56] A first screening using the rHuR−cloned-ARE-$RNA^{TNF\alpha}$ complex was performed at a concentration of 100 μM by REMSA using 179 compounds of a chemical library at the Korea Research Institute of Technology. Thereafter, nine candidates were selected based on a cutoff of >25%, which was indicative of an inhibitory effect, and the IC_{50} values were evaluated by a filter-binding assay. In this way, compounds **9**−**11** (Figures 2 and 3) were further selected. Interestingly, the IC_{50} value of compound **9** (1.4 μM) was approximately comparable with the previously found value (1.8 μM).[50] The capability of compounds **9** and **10** to prevent the formation of the HuR−RNA complex was subsequently evaluated using other cloned ARE sequences from different mRNAs (i.e., those of IL-6, Cox-2, and the proto-oncogene c-*fos*). Remarkably, compound **9** only inhibited the complex HuR−$ARE^{TNF\alpha}$ at 0.5 μM, whereas compound **10** inhibited both complexes HuR−$ARE^{TNF\alpha}$ and HuR−$ARE^{c\text{-}fos}$ at a lower concentration (0.25 μM). In addition, the stability of the HuR−$ARE^{IL\text{-}6}$ and $ARE^{Cox\text{-}2}$ complexes studied in this work was not affected by compounds **9** and **10**. Considering these experimental data, compounds **9** and **10** seem to preferentially bind to specific complexes, exerting their activity depending on the complex studied.

2.3. Mitoxantrone (12) and 15,16-Dihydrotanshinone-I (13). Provenzani and co-workers recently focused on a research program aimed at identifying new compounds able to interfere with the formation of HuR−RNA complexes, and they proposed the amplified luminescent proximity homogeneous assay screen (AlphaScreen) as a new biochemical tool for HTS.[57] As a first step, they confirmed that HuR dimerization occurs before the binding to ARE−RNA at a stoichiometry of 2:1 (HuR:RNA).

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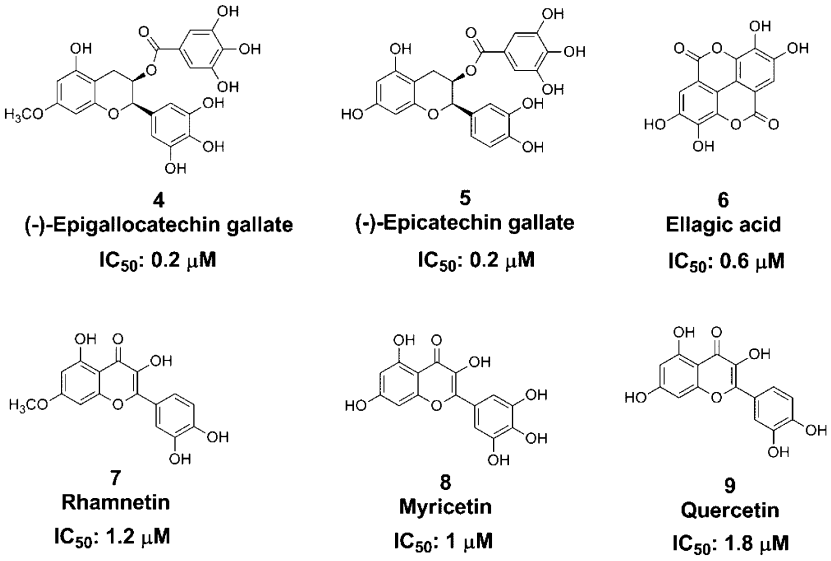

Figure 2. Compounds **4**–**9** as inhibitors of the complex HuC–artificial ARE sequences (IC_{50} values determined by REMSA).[54]

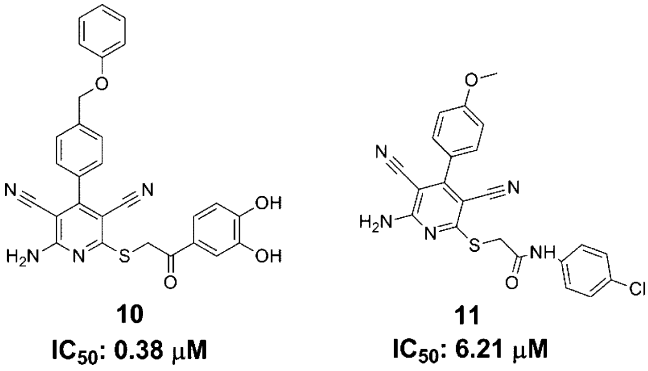

Figure 3. Compounds **10** and **11** as inhibitors of HuR–ARE$^{TNF\alpha}$ (IC_{50} values determined by a filter-binding assay).[56]

By applying the AlphaScreen technology, they successfully screened a library of 2000 molecules (source not described) against rHuR in the presence of a biotinylated single-stranded ARE-TNFα (Bi-TNF) probe, resulting in 16 compounds (hit rate of 10%) for use in an in-depth investigation. To exclude false-positive results due to technical artifacts, as well as to better discriminate between interfering compounds and real inhibitors, the selected compounds were evaluated by REMSA. Interestingly, mitoxantrone (compound **12** in Figure 4) can disrupt HuR–RNA complexes, which is in agreement with the results previously reported by Meisner, who already proposed that compounds characterized by anthracenedione scaffolds, such as **12**, are inhibitors of HuR function.[53] After validating the proposed approach, the authors screened a library of 107 anti-inflammatory agents against the complex formed by HuR and ARE-RNA$^{TNF\alpha}$, again using the AlphaScreen assay for selecting hits and REMSA for confirming the most interesting compounds.[58] Among the eight hits identified, compound **13** (Figure 4) emerged with the most potency for preventing complex formation. To evaluate its selectivity, the interaction of **13** with other RBPs, such as Lin28b, TTP, TDP-43, and HuD, was then investigated. As expected due to the high degree of structural similarity with HuR, only the HuD–RNA complex was altered by **13** at the reference dose (5 μM). Additionally, the authors replicated the experiments using two different rHuR mutants retaining two distinct RRM domain arrangements,

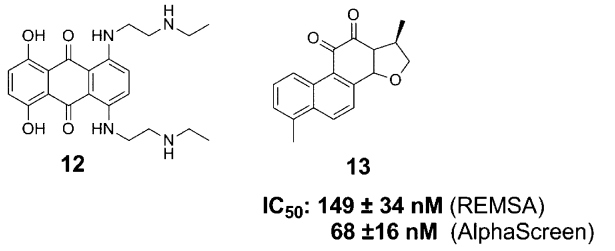

Figure 4. Compounds **12** and **13** (IC_{50} values of **13** determined by REMSA and AlphaScreen assays).[57,58]

M1_M2 and M2_M3 constructs, in the presence of **13** and the ARE-RNA$^{TNF\alpha}$ probe; the results showed that **13** inhibited the formation of M1_M2–RNA complexes with an affinity similar to that of the full-length HuR, while a less potent effect of **13** was observed on the binding to the M2_M3 construct. To better understand the interaction mechanism, the authors tested the binding of each HuR RRM domain (i.e., RRM1, RRM2, and RRM3) to the ARE-RNA$^{TNF\alpha}$ probe in the presence of compound **13**. They found that **13** affects the interaction between RRM1 and the ARE-RNA$^{TNF\alpha}$ probe only marginally at a concentration of 5 μM, without altering the binding between the probe and RRM2 or RRM3. These results suggest that **13** interferes with complex formation possibly by interacting with a site other than the RRMs.

2.4. Coumarins. In 2015, Xu and co-workers[59] screened 6000 compounds present in the KU Chemical Methodologies and Library Development Center and in a library of FDA-approved drugs by applying a fluorescence polarization (FP) binding assay to a full-length HuR and the ARE sequence of the Musashi1 transcript (ARE-RNA^{Msi1}), which encodes an RBP protein whose expression correlates with the grade of the malignancy and proliferative activity of gliomas and melanomas.[60] In this way, they selected 38 candidate compounds and analyzed them using ChemMine, a free database that groups compounds according to their structural similarities and physicochemical properties. To compare the different clusters and choose the best ones, the authors selected atom pairs as structural descriptors and Tanimoto coefficients as similarity coefficients. Applying this approach, they identified six coumarin derivatives (compounds **14**–**19**) able to disrupt the complex

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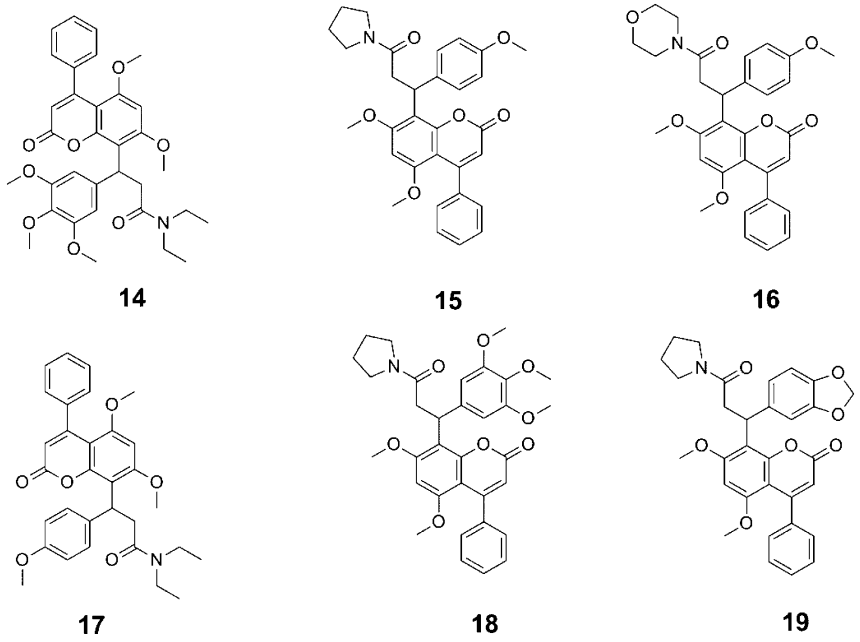

Figure 5. Compounds **14**–**19** as inhibitors of the HuR–ARE^{Msi1} interaction.[59]

formed by HuR and the ARE-RNA^{Msi1} probe (Figure 5). Compounds **14**–**19** were further investigated [by amplified luminescent proximity homogeneous assay linked immunosorbent assay (AlphaLISA)] to assess their ability to interfere with the binding between the ARE-RNA^{Msi1} probe and the full-length HuR (using FP) or the HuR RRM1 and RRM2 domains. As shown in Table 2, all the compounds generally have lower

Table 2. IC_{50} and K_i Values of Compounds 14–19 Determined by FP and AlphaLISA.[55]

	assay			
	FP full-length (10 nM) HuR–ARE-RNA^{Msi} (2 nM)		AlphaLISA HuR RRM1/RRM2 domains (100 nM)–ARE-RNA^{Msi} (25 nM)	
compd	IC_{50} (μM)	K_i (μM)	IC_{50} (μM)	K_i (μM)
14	4.0 ± 1.5	0.59 ± 0.13	12.2 ± 2.4	5.1 ± 0.9
15	2.4 ± 0.5	0.35 ± 0.03	8.2 ± 1.0	3.4 ± 0.4
16	5.1 ± 1.5	0.80 ± 0.12	15.7 ± 0.5	6.5 ± 0.2
17	3.7 ± 0.9	0.57 ± 0.07	11.0 ± 0.8	4.6 ± 0.3
18	6.7 ± 2.2	1.10 ± 0.21	17.6 ± 1.3	7.3 ± 0.5
19	6.6 ± 1.8	1.05 ± 0.18	16.5 ± 1.3	6.9 ± 0.5

IC_{50} and K_i values for the full-length protein than for the RRM1 and RRM2 domains (for comparison purposes, we assumed that apparent binding affinities are not influenced by the detection method). To gain further insight into the interaction of HuR with mRNAs, the authors employed surface plasmon resonance and obtained similar findings. The same results were observed when using ARE-bearing probes from B-cell lymphoma 2 (Bcl-2) and X-linked inhibitor of apoptosis protein (XIAP) transcripts, whose corresponding proteins are involved in anti-apoptotic mechanisms. Although these data do not provide information about the binding region involved, they collectively indicate the preferential binding of compounds **14**–**19** to the full-length HuR protein with respect to the relative RRM1 and RRM2 domains. In addition, these compounds may represent a starting point from which to design novel proapoptotic molecules useful as potential adjuvant therapies in oncology.[60,61]

2.5. Other Compounds. In 2015, Wang and co-workers[62] screened 1597 compounds belonging to the "NCI diversity set V library" by FP to evaluate their ability to inhibit HuR–RNA$^{c\text{-}fos}$ complex formation. The authors identified 12 compounds with IC_{50} values ranging from 2.7 to 97.4 μM, but they only reported the structures of the four compounds shown in Figure 6 (compounds **20**–**23**). They performed a nuclear magnetic resonance (NMR)-based secondary screening assay, which provided evidence for a direct interaction with the target protein, thus removing a false-positive effect due to the direct influence of the compound on the fluorescent tag. In particular, saturation transfer difference-NMR (STD-NMR) and chemical shift perturbation using two-dimensional $^{1}H-^{15}N$ heteronuclear single quantum correlation (HSQC) are powerful tools for monitoring small molecule–protein interactions. The study was first conducted on compounds **21** and **23** and was then extended to the other compounds using HuR at 10 μM. In the $^{1}H-^{15}N$ HSQC spectrum of the ^{15}N-labeled full-length protein,

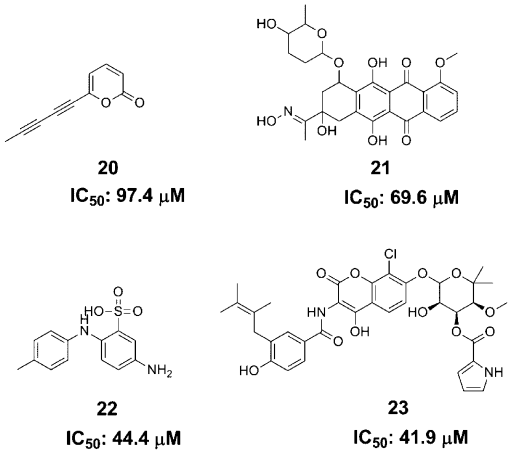

Figure 6. Compounds **20**–**23** (IC_{50} values determined by FP).[62]

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very few signals were visible, and they exhibited poor spectral dispersion. These signals were only due to the flexible portions of the protein, given that the folded domains could be involved in the oligomerization process.[63] The successive addition of **23** to this solution at a ratio of 2:1 (**23**:HuR) led to a change in the HSQC spectrum, most likely due to disrupted protein oligomerization. Moreover, the improvement in the protein spectrum confirmed direct interactions of compound **23** with HuR protein dimers. Regarding the other compounds, only **21** showed a slight effect on the formation of an HuR dimer, whereas **20** and **22** did not show a particular effect on the HSQC spectrum. Further studies showed the capability of **21** to interact with the RRM1 and RRM2 domains alone and in the presence of unlabeled, single-stranded 11-mer RNA. The interference of **21** on the HuR (RRM1/RRM2 domains)–$RNA^{c\text{-}fos}$ interactions is indicated by the reappearance of peaks usually broadened by the interaction of the protein with the RNA, thus suggesting the direct interaction of **21** with the complexes. The authors concluded that some hit compounds disrupt HuR oligomer formation, whereas others seem to block HuR–RNA binding.

3. IDENTIFICATION OF COMPOUNDS INTERFERING WITH ELAV PROTEIN–MRNA COMPLEXES VIA RATIONAL DRUG DESIGN

The fact that a binding pocket for target mRNAs in most RBPs remains not well-defined can explain the difficulties in identifying new chemical entities able to interfere with mRNA–protein complexes. Regarding ELAV proteins, some studies in past years have improved the potential druggability of these targets, as follows:

(I) The primary sequence of all ELAV proteins is highly conserved, especially regarding the first two RRM domains, each containing the RNP1 and RNP2 sequences, which have 8 and 6 amino acids, respectively (Figure 7).[37]

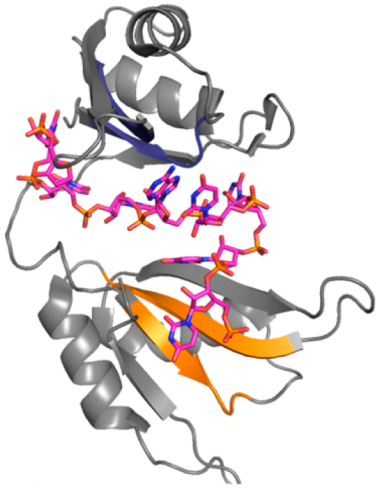

Figure 7. Cocrystal structure of RRM1-RRM2 of HuD–$ARE^{TNF\alpha}$ (PDB code 1G2E). Color code: protein backbone, gray; RNP1 and RNP2 sequences of the RRM1 domain, blue; RNP1 and RNP2 sequences of the RRM2 domain, orange. ARE-$mRNA^{TNF\alpha}$ skeleton: C, purple; N, blue; O, red; P, orange.

(II) The X-ray crystal structures of the RRM1 and RRM2 domains of HuD when complexed with a target transcript have been reported, and the results showed that the four RNPs (the RNP1 and RNP2 sequences of both the RRM1 and RRM2 domains) are directly involved in mRNA binding (Figure 7). The same sequences are involved in HuR–$RNA^{c\text{-}fos}$ [Protein Data Bank (PDB) code 4ED5].[37,64]

(III) The profile of the RNA recognition sequence was predicted using the RRM1 and RRM2 domains of HuD bound to 11-nucleotide fragments of $RNA^{c\text{-}fos}$ and $RNA^{TNF\alpha}$. Analysis of the steric restriction and the availability of hydrogen-bonding partners allowed the identification of a repeated nucleotide sequence of adenine (A) and uracil (U) (AUUUA).[37] Further studies on HuR showed that the sequence necessary for binding by HuR corresponds to two stretches of eight to nine nucleotides consisting of uracil and adenine.[65]

(IV) In 2008, we synthesized four peptides corresponding to the RNP sequences in the RRM domains of HuD (Table 3). The ability of these four peptides (P1–P4) to stabilize $mRNA^{NOVA1}$ and $mRNA^{VEGF}$ was then demonstrated when they were used in a mixture.[66] Successive in vitro studies combined with molecular modeling experiments showed that the peptide sequences P1 and P2 play greater roles in binding to RNA than do P3 and P4.[67] Further confirmation was provided by NMR studies [diffusion-ordered spectroscopy and STD-NMR]. Interestingly, the NMR experiments confirmed that each peptide alone is unable to bind the biological target but that the peptide mixture P1 + P2 is the most effective for stabilizing mRNA.[68] These results are in agreement with molecular modeling studies and biological tests.[66,67]

Table 3. Amino Acid Sequences of Peptides P1–P4 and Their Corresponding Sequence within the RRM

domain	peptide	amino acid sequence
RNP1_RRM1	P1	LGYGFVNY
RNP2_RRM1	P2	LIVNYL
RNP1_RRM2	P3	RGVGFIRF
RNP2_RRM2	P4	LYVSGL

Considering the above-mentioned studies, it seems possible to adopt a rational approach for designing ELAV protein–mRNA-interfering compounds starting from the current knowledge of the region that is mainly involved in stabilizing mRNA.

3.1. Definition of an ELAV Ligand Pharmacophore.

Considering the structural variability of the compounds discovered so far (compounds **1**–**23**, Figures 1–6) and the different techniques employed for screening their ability to interfere with ELAV protein–mRNA complexes, drawing conclusions from structure–activity relationship (SAR) studies remains challenging. The molecular weight of the ligands usually ranges between 250 and 500 Da, with the exceptions of compounds **21** and **23** (MW > 700). All compounds are characterized by the presence of one or more aliphatic or aromatic rings fused in the same scaffold, which endows the molecules with structural rigidity and a hydrophobic character, which are most likely relevant for protein interactions. Moreover, each ligand is characterized by the presence of other functional groups, such as carbonyl, carboxylic acid, amine, and hydroxyl groups, which confer an additional polar character. Extensive efforts are still needed to identify new compounds and elucidate their ligand–target interactions.

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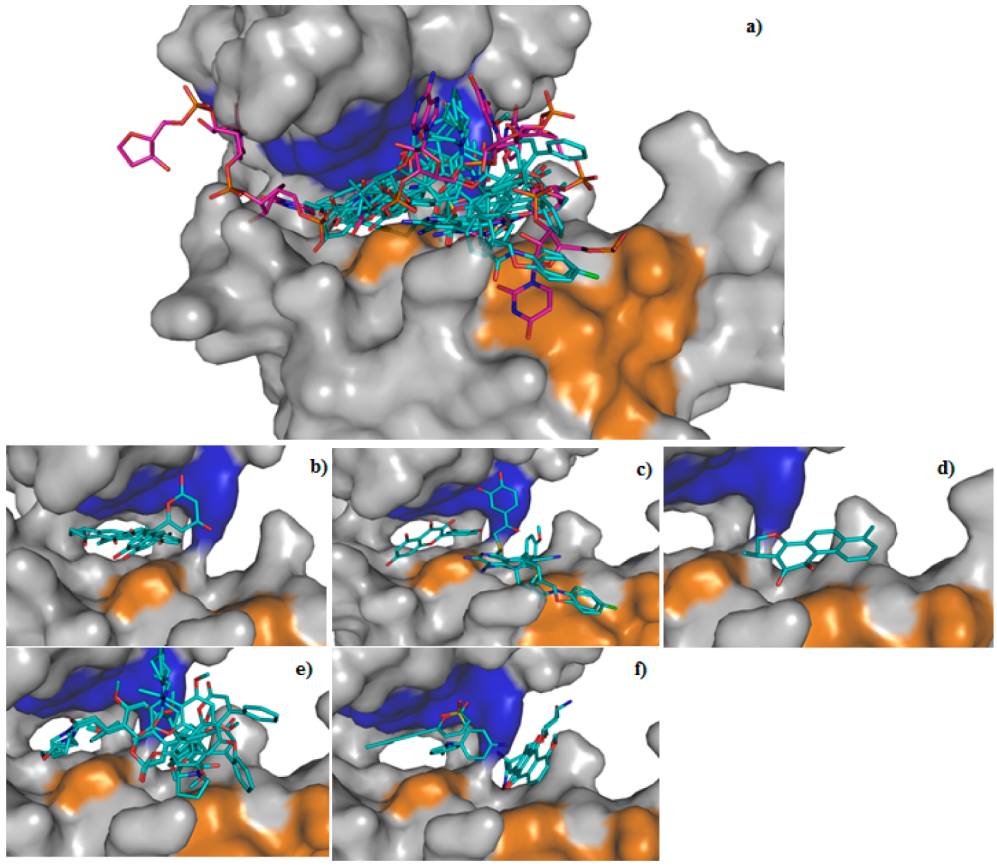

Figure 8. (a) Docking studies of compounds **1**−**3**, **9**−**11**, and **13**−**22** using the X-ray cocrystal structure of the RRM1 and RRM2 domains of the HuR−RNA complex (PDB code 4ED5) generated by the FlexX docking module in the LeadIT suite, followed by evaluations using the HYDE scoring function in SEESAR.[70] Top-ranked poses of compounds are the following: (b) **1**−**3**; (c) **9**−**11**; (d) **13**; (e) **14**−**19**; (f) **20**−**22**. Color code, RNA skeleton: C, purple; N, blue; O, red; P, orange. Compounds **1**−**3**, **9**−**11**, and **13**−**22** skeleton: C, cyan; N, blue; O, red; protein backbone, gray; RNP1 and RNP2 sequences of the RRM1 domain, blue; RNP1 and RNP2 sequences of the RRM2 domain, orange.

3.2. Docking Studies of Compounds Interfering with HuR−mRNA Complexes. We believe that the experimental evidence described above is a good starting point for understanding the ELAV protein region responsible for mRNA interactions. It could reasonably be identified between the RRM domains, especially in the site involved in hosting the RNA recognition sequence (AUUUA). Exactly at this point, the two asymmetric units of the protein are in proximity, providing a deeper and narrower pocket, which is partially basic owing to the presence of arginine and asparagine residues. Therefore, we consider a rational approach through computer-aided drug design (CADD) techniques a suitable tool for targeting this specific pocket. We used docking studies to examine the possible interactions of the compounds already identified as inhibitors of HuR−mRNA interactions and to evaluate the potential of CADD for use in future drug-discovery programs. Accordingly, we performed docking studies only for compounds **1**−**3**, **9**−**11**, and **13**−**23** using the FlexX docking module in the LeadIT[69] suite; these studies were followed by evaluations using the scoring function HYDE in SEESAR.[70] Our starting point was the X-ray cocrystal structure of RNA$^{c\text{-}fos}$-bound HuR (PDB code 4ED5). For the docking studies, the binding site in the protein was restricted to 6.5 Å around the cocrystallized RNA, and the 30 top-scored (FlexX) solutions were retained and subsequently postscored with SEESAR, and the best-scored poses were then selected (Supporting Information). The docking studies revealed that inhibitors **1**−**3**, **9**−**11**, and **13**−**22** bind to the same pocket of the HuR protein, benefiting from interactions with the RNP1 and RNP2 sequences of the RRM1 and RRM2 domains of the protein, as in the HuR−RNA$^{c\text{-}fos}$ complex (Figure 8a). For compound **23**, we performed the same docking protocol; however, we could not generate any poses, indicating that inhibitor **23** may not bind to this specific region. This finding is in agreement with the experimental data demonstrating that this compound does not interact with the RRM1 and RRM2 domains.[62] Although all the compounds occupy different sites of the binding pocket, they are mainly involved in interactions with the amino acid sequences of RNP1 and RNP2 of both domains (Table 4). Moreover, with a few exceptions, most of the compounds occupy the deep part of the binding pocket (Figure 8a). The first class of compounds **1**−**3** is hosted in the deep part of the binding site (Figure 8b). Compounds **14**−**19** are superimposed in the same way, featuring similar structural motifs (Figure 8e). In the cases of compounds **9**−**11**, we observed different binding poses for each compound. Compound **9** occupies the deep part of the pocket, most likely due to its smaller size compared with compounds **10** and **11**, which are bulkier and more flexible (Figure 8c). We observed the same trend for compounds **20**−**22**. While compounds **20** and **22** are involved in interactions with amino acids lining the deep part of the pocket, compound **21**, which has a more rigid and larger structure, may occupy outer parts of the binding site (Figure 8f). Unlike the other small scaffolds, compound **13** is hosted in the outer hydrophobic pocket (Figure 8d), where it

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Table 4. List of Amino Acids Involved in the Interactions with Compounds 1−3, 9−11, and 13−22 and the Corresponding Nucleotide(s) within the ARE Sequence Located in the Hypothesized Binding Pocket

compd	main interactions	corresponding nucleotide(s)
1	Asn 25, Tyr 26, Asn 134, Arg 153	U8
2	Ile 23, Asn 25, Lys 92, Ala 96, Arg 97, Ile 133, Arg 153	U8−U9
3	Ile 23, Asn 25, Lys 92, Arg 97, Ile 133	U8
9	Ile 23, Asn 25, Tyr 26, Lys 92, Ile 103, Asn 134, Arg 153	U8
10	Tyr 26, Asn 107, Tyr 109, Asn 134, Arg 136, Arg 153, Phe 151, Ala 184, Ala 185,	U3−U4
11	Asn 25, Tyr 26, Gly 62, Asn 107, Asn 134, Arg 153, Phe 151, Ala 184, Ala 185	U3−U4
13	Tyr 26, Asn 107, Arg 136, Leu 138, Phe 151	U4
14	Tyr 26, Lys 89, Asn 134, Arg 136, Phe 151	U4−U5
15	Ile 23, Asn 25, Tyr 26, Tyr 63, Lys 92, Asn 134, Arg 153,	U5 and U8
16	Ile 23, Asn 25, Tyr 26, Tyr 63, Lys 92, Ile 133, Asn 134, Arg 153	U5 and U8
17	Ile 23, Asn 25, Tyr 26, Tyr 63, Lys 92, Ile 133, Asn 134, Arg 153,	U5 and U8
18	Tyr 26, Gly 62, Asn 134, Arg 136, Leu 138, Phe 151	U4
19	Tyr 26, Leu 61, Gly 62, Tyr 63, Asn 134, Ser 135, Arg 136, Phe 151, Arg 153, Ala 184	U4
20	Asn 25, Tyr 26, Tyr 63	A7−U8
21	Tyr 26, Asn 134, Ser 135, Arg 136, Phe 151	U4−U5
22	Asn 25, Tyr 26, Lys 61, Lys 92, Ile 133, Asn 134	U8

might be involved in $\pi-\pi$-stacking interactions with some aromatic amino acids located in that region (Table 4) (Supporting Information). This may be due to the hydrophobic features present in its structure with respect to the other small molecules. Although all of the docked compounds occupy different positions in the identified pocket, each of them can be superimposed onto at least one nucleotide of the RNA$^{c\text{-}fos}$ sequence, as shown in Table 4.

Our docking results suggest that these compounds bind to the HuR protein in this region. Thus, the pocket identified based on our previous hypotheses may be taken into consideration for the design of specific ligands targeting this new site.

4. CONCLUSIONS

Post-transcriptional mechanisms can affect every aspect of the fate of a given transcript, playing fundamental roles in the modulation of gene expression. Hence, disruptions of these pathways may lead to disease development and contribute to pathologies. Therefore, drugs acting at the post-transcriptional level could represent an innovative pharmacological approach for the treatment of several diseases. In particular, the identification of compounds able to interfere with ELAV protein−RNA complex formation opens up a fascinating route for the discovery of new drugs. Nevertheless, this strategy still represents a challenge for medicinal chemists, and the few compounds that have been identified so far have been yielded from HTS techniques. Several structurally unrelated compounds able to interfere with ELAV protein−RNA complexes have been reported over the past several years.[53,54,56−59,62] Given that different techniques and experimental conditions were used (Table 5) for screening compounds, comparing the results of various studies is not straightforward. Specifically, in almost all the experiments, either the full-length HuR protein or its truncated form containing the RRM1 and RRM2 domains of HuR was studied when complexed with different RNA sequences. These sequences were selected based on the corresponding protein involved in the pathology under investigation (i.e., TNFα, IL-2, IL-6, Cox-2, c-fos, and Msi1); RNA$^{TNF\alpha}$ was the most studied RNA.

The results obtained thus far suggest the following:

(i) HuR exists in monomeric and dimeric forms, as confirmed by theoretical and experimental data.[53,57]

(ii) The RRM domains of HuR are mainly involved in the interaction with ARE−RNA. In particular, compounds **1−3**, **13**, **14−19**, and **21** act on the RRM1/RRM2 domains.

(iii) The ability to interfere with ELAV protein−RNA complexes depends on both the structure of the compounds and the transcripts, as demonstrated by reports by Park et al.[56]

(iv) NMR experiments are suitable for studying the direct interactions between HuR−RNA complexes and potentially interfering compounds,[62] thus providing insight into the structural features required for interactions with the complexes.

(v) Studies performed over the past few years have laid the foundation for the druggability assessment of ELAV proteins and therefore for designing new compounds.

Given that ELAV proteins have pleiotropic functions, targeting them (especially HuR) may have detrimental or positive impacts.[48] Moreover, as mentioned, ELAV proteins are nucleocytoplasmic shuttling proteins virtually affecting all the steps of PTGR; thus molecules targeting ELAV should be carefully evaluated for their effects on processes taking place in the nucleus and/or the cytoplasm. These aspects need to be carefully evaluated in the specific pathological setting in which

Table 5. HTS Techniques, RNA Sequences, and ELAV Proteins Used for Discovering Compounds 1−23

	complex		
compd	ELAV protein	RNA sequence	assay
1−3	HuR (RRM1/RRM2 domains)	TNFα, IL-2, Cox-2	FIDA[53]
4−9	HuC (full-length)	Artificial ARE	REMSA[54]
9−11	HuR (full-length)	TNFα, IL-6, Cox-2, c-*fos*	REMSA
			Filter-binding assay[56]
12, 13	HuD	TNFα	REMSA/AlphaScreen[57,58]
	HuR (full-length)		
	HuR (RRM1/RRM2 domains)		
14−19	HuR (full-length)	Msi1, Bcl-2, XIAP	FP
	HuR (RRM1/RRM2 domains)		Alpha LISA[59]
20−23	HuR (full-length)	c-*fos*	FP
	HuR (RRM1/RRM2 domains)		STD-NMR[62]

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pharmacological modulation is desired. In this context, the pioneering studies of Park et al.[56] are particularly relevant, showing that the compounds may specifically affect the fate of different ELAV protein−mRNA complexes at various levels, in both the nucleus and the cytoplasm, and also depending on the transcript in question, as well as on other modulating factors (i.e., other RBPs and miRNAs). Nevertheless, it is important to realize that the deficit in terms of content and/or function of one specific ELAV protein could be compensated by others, or even by RBPs belonging to other families. On the other hand, compounds interfering with all ELAV proteins may have dramatic side effects; for this reason, the tailored design and tissue-targeted delivery of selective compounds will be essential.

In summary, new potent and selective compounds able to act on ELAV protein−mRNA complexes may represent useful tools for validating the therapeutic potential of such proteins, and they may open up an unexplored avenue for the discovery of novel and effective drugs with unprecedented modes of action.

■ ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/acs.jmedchem.6b01871.

Top-ranked poses of compounds **1**−**3**, **9**−**11**, and **13**−**22** (PDF)

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Marialaura Amadio obtained her Specialization in Applied Pharmacology in 2004 and Ph.D. in 2007. She has a permanent position as Aggregate Professor at the Department of Drug Sciences, Pharmacology Section, University of Pavia. Her research mainly focuses on the biology of aging and retinal diseases. Her interest is in cellular and molecular pharmacology, in particular on the role of ELAV proteins in physiopathological contexts. Her studies also point to the possibility of modulating the ELAV cascade upstream, through the activation/inhibition of PKC and directly by the use of ELAV-like compounds or siRNA technology. Another field of interest is the autophagy process and its role in neurodegeneration.

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■ ABBREVIATIONS USED

ELAV, embryonic lethal abnormal vision protein; mRNA, messenger ribonucleic acid; miRNA, microribonucleic acid; RBP, RNA binding protein; ARE, adenine-uracil rich element; RRM, RNA-recognition motif; RNP, ribonucleoprotein; CADD, computer-aided drug design; PDB, Protein Data Bank; HTS, high-throughput screening; CPEB, cytoplasmic polyadenylation element binding protein; FMRP, fragile-X mental retardation protein; FUS/TLS, fused in sarcoma/translocated in liposarcoma; NOVA, neuro-oncological ventral antigen; PTBP-2, polypyrimidine tract binding protein 2; TDP-43, transactive response DNA-binding protein 43; TTP, triestetrapolin; FIDA, fluorescence intensity distribution analysis; TNFα, tumor necrosis factor α; IL-2, interleukin-2; IL-6, interleukin-6; COX-2, cyclooxygenase-2; PTGR, post-transcriptional gene regulation; REMSA, RNA electrophoretic mobility shift assay; MW, molecular weight; FP, fluorescent polarization;

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AlphaScreen, amplified luminescent proximity homogeneous assay screen; rHuR, recombinant HuR; Alpha LISA, amplified luminescent proximity homogeneous assay linked immunosorbent assay; Msil, Musashi l; Bcl 2, B-cell lymphoma 2; XIAP, X-linked inhibitor of apoptosis protein; NMR, nuclear magnetic resonance; VEGF, vascular endothelial grown factor; SPR, surface plasmon resonance; STD-NMR, saturation-transfer difference nuclear magnetic resonance; HSQC, heteronuclear single quantum correlation; DOSY, diffusion-ordered spectroscopy; NCE, new chemical entity

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