Novel compounds targeting the RNA-binding protein HuR. Structure-based design, synthesis and interaction studies.

Serena Della Volpe,[†,#] Rita Nasti,[†,#] Michele Queirolo,[†] M. Yagiz Unver,[‡,§] Varsha K. Jumde,[‡,§], Alexander Dömling,[∥] Francesca Vasile,[¶,*] Donatella Potenza,[¶] Francesca A. Ambrosio,[⍰] Giosué Costa,[⍰] Stefano Alcaro,[⍰] Chiara Zucal,[⊥] Alessandro Provenzani,[⊥] Marcello Di Giacomo,[†] Daniela Rossi,[†] Anna K. H. Hirsch,[‡,§,*] Simona Collina.[†,*]

[†]Department of Drug Sciences, Medicinal Chemistry and Technology Section, University of Pavia, Via Taramelli 12, 27100, Pavia, Italy

[‡]Helmholtz Institute for Pharmaceutical Research Saarland (HIPS) - Helmholtz Centre for Infection Research (HZI), Department of Drug Design and Optimization and Department of Pharmacy, Saarland University, Campus building E8.1, 66123, Saarbrücken, Germany

[§]Stratingh Institute for Chemistry, University of Groningen, Nijenborgh 7, NL-9747 AG Groningen

[∥]Department of Drug Design, University of Groningen, A. Deusinglaan 1, Groningen, 9713 AV, Netherlands

[¶]Department of Chemistry, University of Milan, Via Golgi 19, 20133, Milano, Italy

[⍰]Department of Health Sciences, University "Magna Græcia" of Catanzaro, Viale Europa, 88100, Catanzaro, Italy

[⊥]Department of CIBIO, University of Trento, Via Sommarive 9, 38123, Povo, TN, Italy

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ABSTRACT: The key role of RNA-binding proteins (RBPs) in regulating post-transcriptional processes and their involvement in several pathologies (*i.e.*, cancer and neurodegeneration) have highlighted their potential as therapeutic targets. In this scenario, Embryonic Lethal Abnormal Vision (ELAV) or Hu proteins and their complexes with target mRNAs have been gaining growing attention. Compounds able to modulate the complex stability could constitute an innovative pharmacological strategy for the treatment of numerous diseases. Nevertheless, medicinal-chemistry efforts aimed at developing such compounds are still at an early stage. As part of our ongoing research in this field, we hereby present the rational design and synthesis of structurally novel HuR ligands, potentially acting as HuR–RNA interferers. The following assessment of the structural features of their interaction with HuR, combining saturation-transfer difference NMR and *in silico* studies, provides a guide for further research on the development of new effective interfering compounds of the HuR–RNA complex.

RNA is an important regulatory element of many cellular processes and, thus, so-called RNA-binding proteins (RBPs), play a prominent role in affecting the fate of target messenger RNAs (mRNAs) coding for proteins pivotal in key cellular functions.[1-5] Therefore, a dysregulation of RBPs may be related to the pathogenesis of several diseases.[2,4,6] In 2018, the first candidate drug targeting RBPs, called H3B-8800, reached the clinical phase for the treatment of acute myelogenous leukemia and chronic myelomonocytic leukemia.[7] Among RBPs, the family of ELAV (Embryonic Lethal Abnormal Vision) proteins is involved in controlling the functional activities of diverse RNA populations. In particular, HuR regulates splicing, stability and translation of thousands of coding and non-coding RNAs and is therefore considered a valid drug target for anticancer therapy.[8] HuR is a nuclear protein but, upon cell stress such as DNA damage, it shuttles into the cytoplasm where it regulates the fate of cargo mRNAs and determines the abundance of the encoded proteins. Over-expression of HuR is associated with tumor progression and poor prognosis in various cancer types.[8] For this reason, compounds able to bind HuR and to inhibit the formation of the HuR–RNA complex may have anticancer properties. So far, various high-throughput screening campaigns afforded several natural products. [9-12]

After studying the concept of druggability of ELAV proteins and related complexes with mRNA,[13-15] we analyzed the literature concerning the main findings on the ELAV–RNA complexes from a medicinal-chemistry standpoint and defined the interaction features of HuR and a small series of natural products.[16,17] In this letter, we report on the structure-based design and the synthesis of compounds with different "core structures" and the investigation of their interactions with HuR using a combination of saturation-transfer difference (STD)-NMR and *in silico* studies. While on the RNA side the interaction of HuR and RNA is mediated by AU-rich elements (AREs), on the protein side two consecutive RNA recognition motif-type (RRM1 and RRM2) domains are involved; these interact directly with target RNAs through highly conserved ribonucleoprotein (RNP1 and RNP2) sequences.[18,19] For the de-

sign of new compounds, we focused on a pocket-like region hosting small HuR ligands, formed by the two asymmetric units of the protein, which belong to RNP1 and RNP2 of RRM1. Inspection of the cocrystal structure of the HuR RRM1 and RRM2 domains in complex with the ARE sequence of RNA$^{c\text{-}fos}$ (PDB code 4ED5), confirms that this region corresponds to binding site of RNA uridine residues 8 and 9 (U8-U9)(Figure 1a).

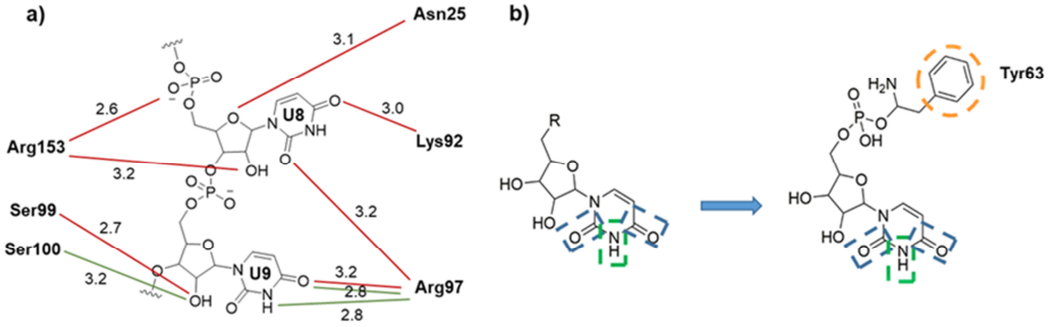


Figure 1. a) HuR–RNA contacts (PDB code 4ED5) for U8-U9. Red lines show side-chain contacts, green lines show main-chain contacts. Distances are expressed in Å;[20] b) Modification applied to the anchor to increase the predicted affinity. Dashed blue rectangles indicate hydrogen-bond acceptors and dashed green rectangles hydrogen-bond donors on both the original and modified anchor, while the dashed orange circle shows the anchor modification potentially allowing for additional hydrophobic interactions with the selected Tyr63 residue.

We based our search for new scaffolds for HuR ligands on the key interactions shown in Figure 1a and employed the free web-based virtual screening platform AnchorQuery^{TM}.[21-24] This program is specifically designed for targeting protein–protein interactions (PPIs) with small molecules by combining the anchor concept with one-pot multicomponent reaction (MCR) chemistry. Briefly, PPI inhibitors are characterized by specific moieties able to mimic amino acid side chains of the donor protein, called "anchor motifs". Since the contact surfaces involved in PPIs are typically large and flat, similar to protein–RNA interactions, we used NucleoQuery, a nucleoside derivative of the web application AnchorQuery^{TM} for the rational structure-based design of HuR–RNA targeting compounds. We selected U8 and U9 as anchors, and investigated whether one of the two nucleotides may be a pharmacophore in terms of occupied position and interactions. To increase the probability of discovering new ligands, the anchors were modified so as to bear an additional phenyl group to establish a π–π-stacking interaction with Tyr63 (Figure 1b). As a result of four runs in NucleoQuery, a large library (800 molecules) featuring a wide range of structurally diverse derivatives was obtained. To select the candidates for synthesis, we relied on visual inspection and molecular-recognition studies. This way, we selected 17 compounds featuring piperidinones, aromatic heterocycles, *N*,*N*-disubstituted amides and sulfonamides as scaffolds (see SI, S1.2). For all compounds, the anchor shows good overlap with the corresponding uracil ring, preserving the same hydrogen bonds with Arg97 and Lys92, while the additional aromatic rings should afford the desired interaction with Tyr63. Moreover, selected compounds are engaged in additional interactions with the protein, including hydrophobic interactions with residues Ile23, Asn25, Phe65, Ile103, Ile133 (docking poses of exemplary compounds for each scaffold can be found in SI, S1.3). Taking into account the synthetic feasibility and commercial availability of the corresponding starting materials, we selected the most promising scaffolds and replaced the uracil moiety withan easy-to-handle starting material. We evaluated several hydrophobic/aromatic portions as anchors, potentially able to interact with HuR in the position occupied by U8 and preserving its main contacts. Thus, we designed and synthesized compounds **1**–**4** (Figure 2), characterized by different scaffolds.

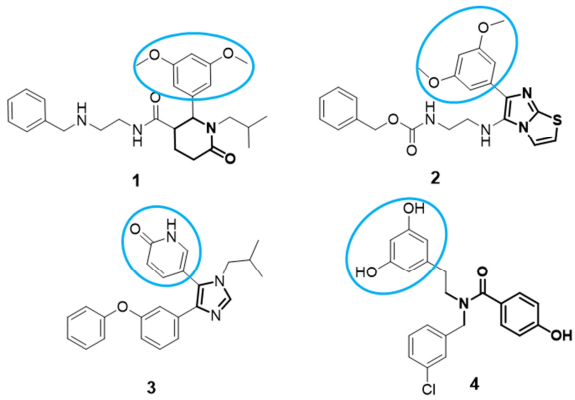
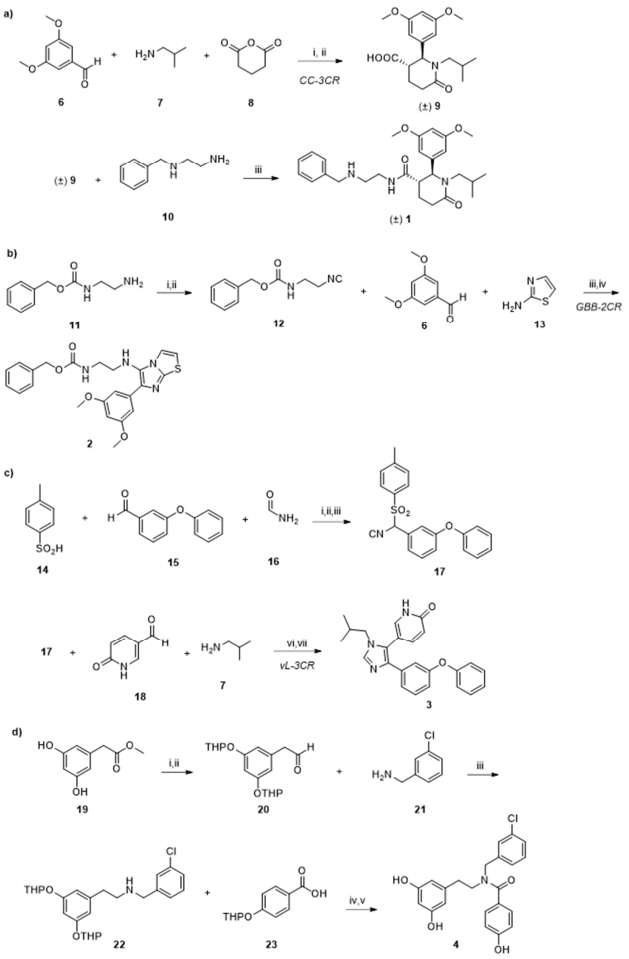


Figure 2. Structures of the four compounds selected for the experimental study. The modified anchors are highlighted in light blue. The different scaffolds (piperidinones, heterocycles, N,N-disubstituted amides) are highlighted in bold.

The computationally predicted structures can be easily prepared by a short synthesis using MCR or equally efficient processes. Thus, compound **1** was synthesized by a Castagnoli-Cushman reaction (CC-3CR)[25] followed by amidation; **2** by a Groebke-Blackburn-Bienaymé (GBB-2CR);[26] **3** by a Van Leusen reaction (vL-3CR),[27] and **4** by a reductive amination[28] followed by acylation. Herein, we will briefly describe the synthetic strategies adopted to obtain compounds **1**–**4**. Further details on protocols employed and compound characterization are reported in the supporting information (SI, S2).

Amidation of the acid intermediate *trans* (±)-**9** with amine **10** afforded compound (±)-**1** as racemate.[29] The key intermediate *trans* 6-oxopiperidine-3-carboxylic acid (**9**) was obtained in good yield and diastereoselectivity, applying a microwave-aided one-pot CC-3CR on benzaldehyde (**6**), isobutylamine (**7**), and glutaric anhydride (**8**) (Scheme 1a).[25] A microwave-assisted GBB-2CR,[26,30] involving isocyanide **12**, aldehyde **6** and amino-thiazole **13** produced compound **2**. Isocyanide **12** was obtained starting from amine **11**, which was converted into the corresponding formamide and subsequently dehydrated (Scheme 1b).



Scheme 1. Synthetic pathways of compounds 1–4. Reagents and conditions: a) Compound (±)-**1**: i) **6**, **7**, HCOOH, ACN, mw 120°C, 50 W, 30 min; ii) **8**, p-xylene, reflux, 10 h, yield 62%; iii) TBTU, DIPEA, THF, rt, 12 h, yield 71%. b) Compound **2**: i) Ethyl formiate, 10h; ii) TEA, $POCl_3$, DCM, rt, 4 h, yield 62%; iii) **6**, **13**, HCOOH, ACN, mw 120°C, 30 min; iv) **12**, $ZrCl_4$, 80 °C, 10 h, yield 21%. c) Compound **3**: i) H_2O, 30 min; ii) HCl, MTBE, 30 min; iii) **15**, **16** Me_3SiCl, ACN, Toluene, N_2, 50 °C, 5 h; iv) **14**, 16 h, yield 62%; v) $POCl_3$, triethylamine, THF, 10°C, 45 min, yield 85%; vi) **18**, **7** dry DMF, rt, 4 h; vii) **17**, K_2CO_3, rt, 72 h, yield 15%.

A vL-3CR[27] on commercially available aldehyde **18**, isobutylamine (**7**) and substituted TosMIC derivative **17** led to compound **3**. This MCR allows to access functionally rich imidazoles in a single pot via cycloaddition of TosMIC reagents on imines, generated *in situ* from an aldehyde and an amine under mildly basic conditions. We synthesized **17** starting from *p*-toluenesulfinic acid (**14**), which afforded the intermediate formamide derivative upon reaction with commercially available 3-phenoxybenzaldehyde (**15**), formamide (**16**) and chlorotrimethylsilane; subsequent dehydration furnished isocyanide **17** (Scheme 1c).[31] Compound **4** was obtained by reductive amination of aldehyde **20** followed by an amide coupling with acid **23** followed by removal of the THP protecting groups (Scheme 1d).[28] Prior to the interaction study, compounds **1**–**3** were converted into the corresponding hydrochloride salts, and their solubility in both the buffer and time-ranges required for STD-NMR experiments was evaluated. Under these conditions, compound **2** proved to be poorly soluble and had to be excluded from the interaction study.

The interaction of compounds **1, 3** and **4** with HuR was studied by STD-NMR spectroscopy. This technique, based on the nuclear Overhauser effect, is a well-established epitope-mapping methodology for studying the target–ligand interactions.[32] Briefly, the method relies on the selective irradiation of the protein, which allows magnetization to be transferred to the bound ligand; the observation of the ligand signals in the NMR spectrum provides an indication of the interaction. Those ligand protons that are nearest to the protein are more likely to become highly saturated, and therefore show the strongest signal in the mono-dimensional STD spectrum. Therefore, the intensity of the STD signal (expressed as absolute STD percentage) reflects the proximity of the ligand to the protein surface.[33-38] The group epitope mapping illustrates which chemical moieties of the ligand are key for molecular recognition in the binding site. The analysis of STD data shows that compounds **1** (Figure S3.2.1 and Table S3.2.1), **3** (Figure S3.2.2 and Table S3.2.2), and **4** (Figure 3 and Table S3.2.3) interact with the protein.

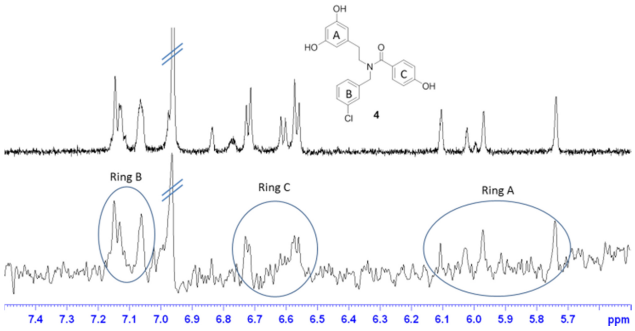


Figure 3. STD-NMR spectrum (bottom) and 1H-NMR (top) of compound **4**. The strong interaction observed for aromatic rings A, B and C is evidenced.

For ligand **1**, the isopropyl group shows the strongest interaction (0.40 STD %), while the STD signal related to ring B is the least intense (0.03 STD %); ring A contributes to the interaction (0.20 STD %), while the protons of the amino chain (H14, H15 and H16) do not give STD signals. The binding of compound **3** to HuR is mediated by the pyridone anchor (1.20 STD %) and by the isopropyl moiety (0.30 STD %). For compound **4**, we observed STD signals of similar intensity for the three aromatic rings (0.40 % for ring A, 0.36 % for ring B and 0.25% for ring C, respectively), suggesting that they are positioned within a protein pocket.

In parallel, we performed docking studies on the crystal structure of HuR RRM1-2 domains according to the approach we published.[17] We used STD-NMR in combination with *in silico* studies (applied to the "closed" HuR conformations, see SI) to enable a more detailed description of the ligand–protein behavior in solution. Molecular docking simulations were performed to elucidate the binding mode and the interactions between compounds **1**, **3**, and **4** with HuR protein, using Maestro tools[39]. We observed that the anchor moiety of each compound is predicted to be superimposed with U8, establishing pivotal interactions with the HuR protein. The interactions of compounds 1 and 3 with HuR is described in the SI (Section S4.3, Figures S4.3.1 and S4.3.2). As for compound **4**, we report strong hydrophobic interactions for all aromatic rings as evidenced by STD data. The amide carbonyl group establishes double hydrogen bonds with Arg97 and hydrophobic interactions with the same residue and Ile103 (Figure 4). Regarding ring B, it establishes a π–π-stacking interaction with

Phe65 and hydrophobic interactions with Arg97, Pro98 and Phe65. Ring C is involved in a double hydrogen bond with Arg153 and Ile103 and different hydrophobic interactions. The zoomed-in view in Figure 4 highlights the full overlap of U8 and the anchor motif (ring A) of compound **4**.

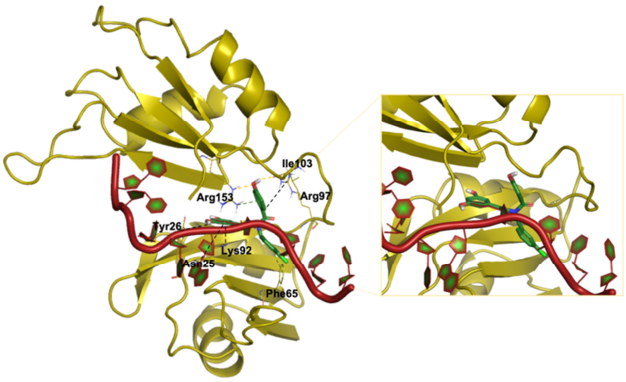


Figure 4: 3D representation of HuR–4 interactions. Hydrogen bonds and hydrophobic interactions are displayed as yellow and dark dashed lines, respectively; a π–π-stacking interaction is shown as a blue dashed line. RNA and HuR are represented as a brown and yellow cartoon, respectively. Compound **4** is shown in green sticks.

In this letter, we report for the first time on the structure-based design and synthesis of potential HuR ligands. The structure-based design strategy used in the present study enabled the identification of three novel compounds that interact with HuR, establishing contacts with the RNP regions of RRM1 and RRM2 domains of the protein. Targeting this region is pivotal for the inhibition of the protein activity and so the identification of compounds able to bind to this site plays a critical role in the development of new potential HuR inhibitors. Biophysical experiments (STD-NMR) are in agreement with computational results, confirming that the designed compounds **1**, **3**, and **4** are indeed binders of HuR. Furthermore, theoretical experiments carried out by molecular dynamics and docking studies confirm that compound **4** is the best binder of HuR protein, in terms of theoretical binding affinity (SI, S4.2). The identified hit compounds will be further optimized in forthcoming studies, building an *in silico* focused library. Our findings represent a step forward in discovering novel compounds potentially useful to counteract several pathologies such as cancer, inflammation, and neurodegeneration, in which ELAV–RNA complexes play a pivotal role.

Experimental Procedures

Design of new scaffolds. We report all protocols in the SI, S1.
Compound synthesis. Procedures are reported in the SI, S2.
Protein expression and purification. HuR aliquots utilized in the STD-NMR study were prepared as already described.[10,40]
Interaction study with HuR. STD-NMR experiments, Molecular dynamics simulations and docking studies were carried out as reported in the SI (S3 and S4, respectively).

ASSOCIATED CONTENT

Supporting Information

Protocols (compound design, synthesis, characterization, and interaction studies) are reported in the supporting information.
The Supporting Information is available free of charge on the ACS Publications website.

AUTHOR INFORMATION

Corresponding Author

*S.C. e-mail: simona.collina@unipv.it
*F.V. e-mail: francesca.vasile@unimi.it
*A.K.H.H. email: Anna.Hirsch@helmholtz-hzi.de

Author Contributions

The manuscript was written through contributions of all authors. All authors have given approval to the final version of the manuscript. #S.D.V and R.N. contributed equally to the work.

Present Addresses

R. N.: Department of Environmental Science and Policy (ESP), University of Milan, via C. Golgi 19, 20133 Milano-Italy M.Q.: Agro business Park 10, 6708 PW Wageningen, The Netherlands

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ABBREVIATIONS

ELAV, Embryonic lethal abnormal vision; RBP, RNA-binding protein; STD-NMR, saturation transfer difference-NMR; RRM, RNA recognition motif-type; RNP, ribonucleoprotein; MCR, multi-component reaction; CC-3CR, Castagnoli-Cushman reaction; GBB-2CR, Groebke-Blackburn-Bienaymé reaction, vL-3CR, Van Leusen reaction.

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