HuR-targeted agents: an insight into medicinal chemistry, biophysical, computational studies and pharmacological effects on cancer models.

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Declarations of interest: none

ABSTRACT

The Human antigen R (HuR) protein is an RNA-binding protein, ubiquitously expressed in human tissues, that orchestrates target RNA maturation and processing both in the nucleus and in the cytoplasm. A survey of known modulators of the RNA-HuR interactions is followed by a description of its structure and molecular mechanism of action – RRM domains, interactions with RNA, dimerization, binding modes with naturally occurring and synthetic HuR inhibitors. Then, the review focuses on HuR as a validated molecular target in oncology and briefly describes its role in inflammation. Namely, we show ample evidence for the involvement of HuR in each of the eight recognized hallmarks of cancer, reporting findings from *in vitro* and *in vivo* studies; and we provide abundant experimental proofs of a beneficial role for the inhibition of HuR-mRNA interactions through silencing (CRISPR, siRNA) or pharmacological inhibition (small molecule HuR inhibitors).

Keywords
HuR modulators, Hallmarks of cancer, HuR role in immunity, Chemical features of HuR modulators, Structural interactions of HuR modulators

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1. Introduction

1.1. ELAVL family conservation through evolution

Human antigen R (ELAVL1, HuR or HuA) belongs to the ELAVL (Embryonic Lethal Abnormal Vision-like) protein family [1] that includes also HuB (or Hel-N1), HuC and HuD [2]. A characteristic feature of these proteins is the tripartite architecture of three functionally distinct RNA Recognition Motif (RRM) domains, which individually contribute to mRNA binding [3,4]. Elav-like orthologous genes are present in all metazoans with a high identity score (>45%), but the number of paralogous genes in the different species varies from one to four, without a clear relationship between the complexity level of the organism and its size, development, and brain structure [5]. These observations suggest that the diversification of ELAVL members could have occurred before or at the first stages of metazoan evolution [5-7]. In mammals, the four ELAV-like proteins show a 70–85% identity. The most conserved sequences are contained in the RRM domains and include a conserved octapeptide termed ribonucleoprotein motif (RNP-1) and a conserved hexapeptide termed RNP-2, both responsible for the binding with nucleotides of their RNA targets. The four human ELAVL paralogous genes have different roles and different cellular localizations. HuB, HuC and HuD are mainly neural (HuC and HuD are strictly neural-specific, HuB targets are neural-specific RNA species although several transcripts are also expressed in ovary and testis), and are localized mainly in the cytoplasm, although they can translocate into the nucleus. HuR, conversely, is ubiquitously expressed in all human tissues [8-10], is localized mainly in the nuclear compartment, but shuttles to the cytoplasm under certain stimuli. The roles of neural ELAVL (nELAVL) proteins are overlapping, undergo strict tissue- and cell-specific modulation, and their expression is time regulated throughout development [5,8,9,11]. HuR knock-out (KO) leads to embryonic lethality in mice, due to extra-embryonic defects of placenta, showing abnormalities in skeleton and spleen development in the surviving embryos. These findings suggest that HuR is involved in regulating the fate of mRNAs encoding proteins involved in key processes, such as organ development and tissue homeostasis, and highlights its relevance for the entire organism [12,13].

1.2 HuR expression, localization and molecular functions

In the nucleus, HuR binds target pre-mRNA introns, promoting splicing and alternative polyadenylation events [14-17]. Upon intrinsic (such as DNA damage) [18] or extrinsic stimuli (such as UV irradiation), HuR acts as a shuttle, exporting associated-mature target mRNAs to the cytoplasm, where it mainly stabilizes and promotes the translation of such mRNAs. In so doing, HuR regulates the fate of thousands of coding and noncoding RNAs containing AU/UU-rich elements (AREs) sequences primarily located in their 3' untranslated regions (UTR) (**Figure 1**).

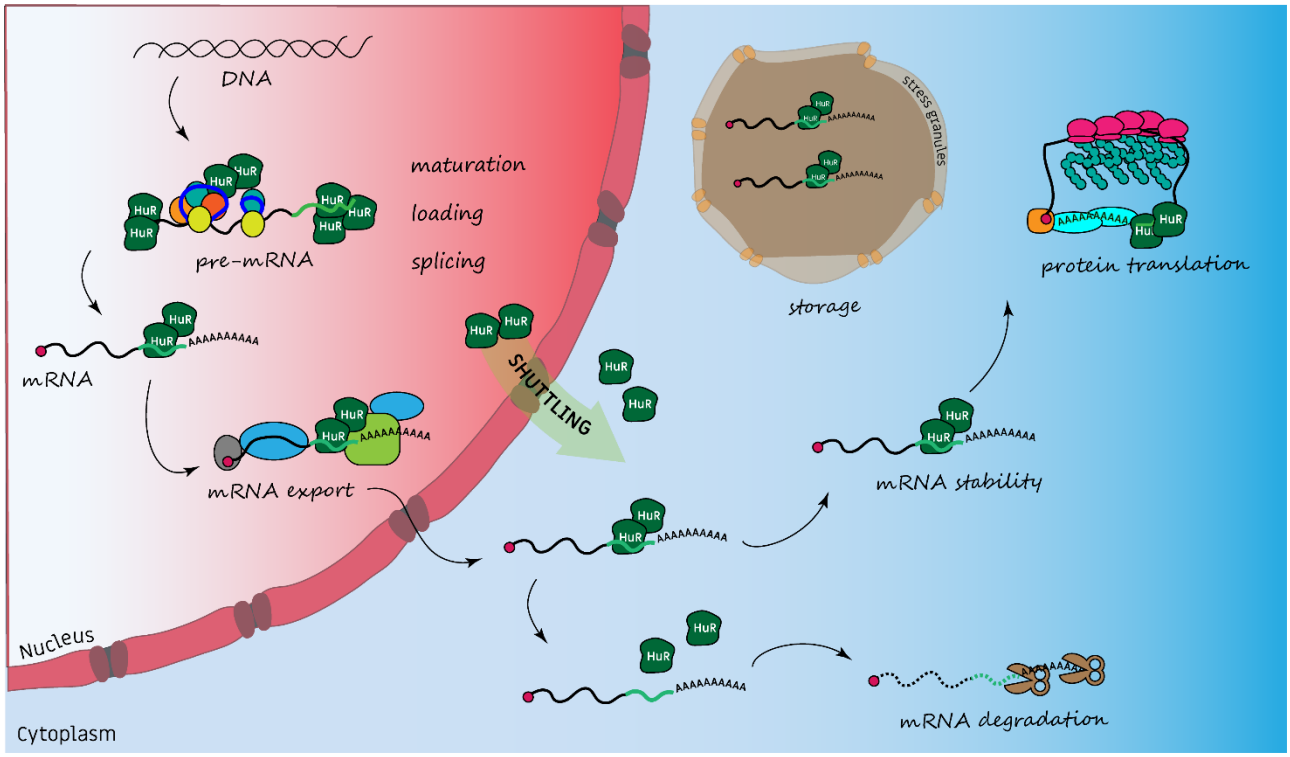


Figure 1: Schematic representation of HuR functions within the cell. Inside the nucleus HuR (green) binds pre-mRNA introns (light blue thin lines) and untranslated regions (light green lines), promoting nuclear processing and mRNA maturation events. HuR cooperates with splicing factors (round colored dots), guiding splicing and alternative splicing events, and favors mRNA export to the cytoplasm by interacting with transportation factors (colored shapes). In the cytoplasm HuR promotes mRNAs stability, helps its storage (as in stress granules), and modulates target translation.

The expression of HuR is finely regulated at multiple levels. HuR expression depends on the transcription factor Nuclear Factor kappa-light-chain-enhancer of activated B cells (NF-kB) [19] and on Smad family proteins [20], but the mechanism of transcriptional regulation of HuR still remains unclear. It was shown that HuR binds and stabilizes its own mRNA [21], and that *HuR* mRNA shows alternative polyadenylation variants to protect itself from degradation, decay and nucleus-cytoplasmic export promotion [22,23]. HuR translation can be negatively controlled by several microRNAs such as miR-519 and miR-125a, as observed in human MCF7, WI-38 fibroblast and Hela cells [24,25]. Furthermore, HuR activity is regulated by multiple post-translational modifications (PTMs), such as phosphorylation and methylation. In particular, HuR can be phosphorylated by the serine/threonine-kinase ChK2, the mitogen-activated protein kinase (MAPK), p38, the protein kinase C alpha (PKC-α) [26], the protein kinase C delta (PKC-δ), and the IkB kinase α (IKKα) [18,27-30], can be methylated by coactivator associated methyltransferase (CARM1) [31] and neddylated by murine double minute 2 (MDM2) [32]. Moreover, HuR activity can be regulated by PARylation through the poly(ADP-ribose) polymerase 1 (PARP-1) [33,34]and by sulfhydration through the cystathionine δ-lyase (CSE) [35]. PTMs on HuR can occur at its RRMs, at the hinge region, and at the HuR nucleocytoplasmic shuttling domain (HNS), affecting either its binding ability and its localization [36]. Furthermore, HuR can be degraded *via* the proteasome after ubiquitination [37] or cleaved by caspases during apoptosis [38].

As HuR is involved in the regulation of post-transcriptional gene expression for thousands of targets (approximately 7% of the human protein-coding gene transcripts), it is not surprising that an altered expression or localization of HuR leads to the emergence of multiple pathologic phenotypes.

1.3 HuR in healthy tissues and human pathologies

HuR is crucial in promoting a proper differentiation of different cellular lineages, including spermatocytes, myocytes, and adipocytes [39-42]. In testes, HuR is essential for spermatogenesis by specifically binding the heat shock protein A2 (HspA2), and while its ablation causes sterility, HuR overexpression causes the failure of spermatid differentiation [43]. In adipose tissue, HuR positively regulates the expression of adipose triglyceride lipase (ATGL), thus promoting lipolysis, while HuR ablation increases the risk of obesity [44]. Furthermore, in muscle, HuR plays a critical role in myogenesis by positively regulating myogenic factors such as *MyoD*, *myogenin*, and *p21* [39], although during muscle wasting, HuR is involved in the repression of myogenic differentiation [45].

In vivo studies targeting HuR functions clearly demonstrated its key functions in development, differentiation and control of tissue homeostasis, and its importance in the pathogenesis of various disorders. HuR is essential for mouse embryogenesis, as genetic ablation of HuR in the germ line using Deleter-Cre lines [12] or Hrpt-Cre lines [46] both lead to embryonic lethality. Global ablation of HuR using tamoxifen-inducible Cre recombinase–mediated gene excision in adult mice also resulted in lethality within 10 days, showing its non-redundant function in postnatal life. In these mice, a severe depletion in progenitor cell populations in hematopoietic and intestinal systems was observed [46].

Subsequent studies over the years, targeting HuR and using cell-type-specific Cre lines in various cells ranging from immune to neuronal cells, from hepatocytes to adipose cells have further shown the exquisite role of HuR in regulating many aspects of cellular differentiation and functions in various cell types (**Table 1**).

Table 1: Cellular studies showing cell-specific HuR functions, major observations and genes regulated by HuR.

Targeted cell type	***Cre line used***	**Major observations**	***Regulated genes***	**Refs**
Ubiquitous	*Morpholinos (Zebrafish)*	Knockdown of Elavl1a using specific morpholinos results in a striking loss of primitive embryonic erythropoiesis.	*Gata1*	[47]
All tissues (global)	*Hrpt-Cre; Rosa26Cre/ERT2*	Postnatal global deletion of HuR1 induces atrophy of hematopoietic organs, extensive loss of intestinal villi, obstructive enterocolitis, and lethality within 10 days.	*Mdm2*	[46]
Germ line, epiblast cells; endothelial cells	*Deleter-Cre; Sox-Cre; Tie 1-Cre*	Targeted HuR ablation leads to defects in placental labyrinth morphogenesis, skeletal specification patterns, and splenic ontogeny.	*Fgf10, Tbx4, Ets2, Hoxd13, Hoxb9*	[12]
Ubiquitous	*HuR-transgenic*	Transgenic overexpression of HuR, prevents the production of fully functional gametes.	-	[40]
Reproductive tissue	*Sycp1-Cre; Vav-Cre; Nestin-Cre; Vasa-Cre; HuR-transgenic*	Targeted deletion of HuR specifically in germ cells leads to male but not female sterility. Mutant males are azoospermic because of the extensive death of spermatocytes at meiotic divisions and failure of spermatid elongation. The latter defect is also observed upon HuR overexpression.	*Hspa2*	[48]
Epiblast cells; epithelial compartment of the lung endoderm	*Sox-Cre; Sftpc-Cre*	The loss of HuR blocks lung branching morphogenesis in the mouse by controlling mesenchymal but not epithelial responses during branching.	*Fgf10, Tbx4,*	[49]
Hepatocytes	*Albumin-cre*	HuR knockout in hepatocytes reduces liver lipid transport and ATP synthesis, and aggravates high fat diet (HFD)-induced NAFLD.	*Apob, Uqcrb, Ndufb6*	[50]
Adipose tissue; brown adipose tissue	*Adipoq-Cre; UCP1-Cre*	Fat-specific knockout of HuR significantly enhances adipogenic gene program in adipose tissues, accompanied by a systemic glucose intolerance and insulin resistance. HuR knockout also results in depot-specific phenotypes: it can repress myogenesis program in brown fat, enhance inflammation program in epididymal white fat and induce browning program in inguinal white fat.	*Insig1*	[51]

Adipose tissue	*Adipoq-Cre*	Mice lacking HuR in adipose tissue show obesity when induced with a high-fat diet, along with insulin resistance, glucose intolerance, hypercholesterolemia and increased inflammation in adipose tissue.	*adipose triglyceride lipase (ATGL)*	[44]
Vascular smooth muscle cells	*alpha-smooth muscle actin (α-SMA)-Cre*	Mice lacking expression of HuR in vascular smooth muscle cell show hypertension and cardiac hypertrophy.	*RGS (regulator of G-protein signaling) protein(s) RGS2, RGS4, and RGS5*	[52]
Endothelial cells	*VE-cadherin-Cre*	Endothelial-specific HuR knockout mice exhibit reduced revascularization after hind limb ischemia and tumor angiogenesis in oncogene-induced mammary cancer, resulting in attenuated blood flow and tumor growth, respectively.	*Eif4enif1*	[53]
Cardiomyocytes	*α-MHC-Cre*	Deletion of HuR in cardiomyocytes aggravates the effect of isopentol-induced myocardial hypertrophy and cardiac fibrosis.	*PLB; β1-AR*	[54]
Cardiomyocytes	*αMHC-mER-Cre-mER*	HuR deletion reduces left ventricular hypertrophy, dilation and fibrosis while preserving cardiac function in a transverse aortic constriction (TAC) model of pressure overload-induced hypertrophy	*Tgfb*	[55]
Muscle cells	*MyoD-Cre*	Muscle-specific HuR knockout mice have high exercise endurance that is associated with enhanced oxygen consumption and carbon dioxide production. These mice exhibit a significant increase in the proportion of oxidative type I fibers in several skeletal muscles.	*KSRP*	[56]
Muscle cells	*MyoD-Cre*	Genetic ablation of HuR in muscle cells protects mice from tumor-induced muscle loss (cachexia)	*STAT3*	[57]
Skeletal muscle	*Myl1-Cre*	Male, but not female, mice lacking HuR in skeletal muscle exhibit metabolic inflexibility, with mild obesity, impaired glucose tolerance, impaired fat oxidation and decreased in vitro palmitate oxidation.	-	[58]
Intestinal - epithelial cells	*Villin-Cre*	Mice lacking intestinal expression of HuR display reduced cell proliferation in the small intestine and increased sensitivity to DOXO-induced acute intestinal injury. Intestinal deletion of HuR also decreases tumor burden in genetic and pharmacological models.	-	[59]
Intestinal epithelial cells	*Villin-Cre*	Intestinal epithelium-specific HuR knockout inhibits repair of damaged mucosa induced by mesenteric ischemia/reperfusion in the small intestine and by dextran sulfate sodium in the colon.	*cdc42*	[60]
Intestinal epithelial cells	*Villin-Cre*	Intestinal tissues from intestinal epithelium-specific HuR knockout mice have reduced numbers of Paneth cells, and Paneth cells have fewer lysozyme granules per cell.	*Cnpy3*	[61]
Intestinal epithelial cells	*Villin-Cre*	Targeted deletion of HuR in intestinal epithelial cells disrupts mucosal regeneration and delays repair after injury.	*Nucleophosmin (NPM)*	[62]
TH17 cells	*OX40-Cre*	Knockout of HuR reduces the number of pathogenic IL-17+IFN-γ+CD4+ T cells in the spleen during experimental autoimmune encephalomyelitis, impairs splenic Th17 cell migration to the CNS and abolishes the disease.	*Irf4, Runx1, iL12rb1*	[63]
TH17 cells	*OX40-Cre*	Targeted deletion of HuR in Th17 cells delays initiation and reduces disease severity in the onset of experimental autoimmune encephalomyelitis.	*IL-17*	[64]
CD4+ T cell	*distal lck-Cre*	Conditional HuR knockout in CD4+ T cells results in loss of IL-2 homeostasis and defects in JAK–STAT	*Il2ra*	[65]

		signaling, Th2 differentiation, and cytokine production.		
B lineage cells	*Mb1-Cre*	Mice lacking HuR have reduced numbers of immature bone marrow and mature splenic B cells.	-	[66]
B lineage cells	*Mb1-Cre*	In the absence of HuR, defective mitochondrial metabolism resulted in large amounts of reactive oxygen species and B cell death, showing that HuR controls the balance of energy metabolism required for the proliferation and differentiation of B cells.	*Dlst*	[67]
Myeloid cells	*LysM-Cre (Mice): Morpholinbos (zebrafish)*	Tumor growth, angiogenesis, vascular sprouting, branching, and permeability are significantly attenuated in HuR-knockout mice, suggesting that HuR-regulated myeloid-derived factors modulate tumor angiogenesis. Zebrafish embryos injected with an elavl1 morpholino show angiogenesis defects in the subintestinal vein plexus.	*Vegf-a*	[68]
Myeloid cells	*LysM-Cre (Mice): HuR-transgenic*	Mice lacking HuR in myeloid-lineage cells, which include many cell types of the innate immune system, display enhanced sensitivity to endotoxemia, rapid progression of chemical-induced colitis, and severe susceptibility to colitis-associated cancer. Conversely, myeloid overexpression of HuR reduces inflammatory profiles, and protects mice from colitis and cancer.	*Tnf, Il10, Ccl2, Ccl7*	[69]
Myeloid cells	*HuR-transgenic*	HuR overexpression in murine innate compartments suppresses inflammatory responses in vivo.	*Tnf, Cox2*	[70]
Myeloid cells	*LysM-Cre*	Macrophage-specific HuR knockout mice display a markedly diminished microvascular angiogenic response to an inflammatory stimulus, and blood flow recovery and ischemic muscle neovascularization after femoral artery ligation.	*Vegf, MMP-9*	[40]
Thymocytes	*Lck-Cre*	Mice with genetic deletion of HuR in thymocytes possess enlarged thymi but display a substantial loss of peripheral T cells.	*Tnf, Dr3*	[71]
Microglia/macrophages	*Cx3cr1 -Cre*	Targeted deletion of HuR in microglia/macrophages reduces tumor growth and proliferation associated with prolonged survival in a murine model of glioblastoma.	-	[72]
Excitatory neurons; pyramidal neurons of the cerebral cortex and hippocampus	*AAV-CaMKIIamCherry-Cre; Nex-Cre*	HuR is a critical modulator of stress-induced synaptic plasticity. In adult mice, AAV-Cre-mediated knockout of HuR prevents anxiety-like and depression-like behaviors induced by chronic stress, and is required for stress-induced dendritic spine loss and synaptic transmission deficits. Genetic inactivation of HuR during embryonic development leads to enhanced synaptic functions.	*RhoA*	[73]
Radial glia neural progenitors; neuroepithelial cells	*Emx1–Cre ; Foxg1-Cre*	Deletion of HuR before embryonic day 10 disrupts both neocortical lamination and formation of the main neocortical commissure, the corpus callosum.		[74]
Neurons	*Thy1Cre-ERT2-EYFP*	Inducible, neuron-specific HuR-deficient mice develop a phenotype consisting of poor balance, decreased movement, and decreased strength.	*Immediate Early Response 2 (IER2)*	[75]

These are just some examples of the physiological role of HuR in regulating tissues homeostasis. Indeed, physiological functions of HuR were also reported in other organs. Namely, de-regulation of HuR was shown in cardiovascular [76,77] and retinal diseases [78,79], nephropathies [80] and neurological disorders [81].

In vivo models have been pivotal to better understand the role of HuR in diseases. In some cases, HuR has a protective function during disease initiation and progression, since its ablation leads to disease aggravation, as for intestinal epithelial cells [59,60,62]. In other circumstances, HuR knockdown leads to beneficial effects,

most notably in experimental models of multiple sclerosis [63,64]. Such a complex functional profile for HuR is further demonstrated by surprisingly opposite effects in the same cell type, depending on the insult. In cardiomyocytes, for example, HuR ablation aggravates the effect of isopentol-induced myocardial hypertrophy and cardiac fibrosis [54]; conversely, in a transverse aortic constriction (TAC) model of pressure overload-induced hypertrophy HuR deletion reduces left ventricular hypertrophy, dilation, and fibrosis while preserving the cardiac function [55]. Importantly, being one of the major regulators of gene expression, a de-regulation of HuR has been also associated in the development of a variety of cancers. Indeed, the majority of its mRNA targets encodes for extremely relevant proteins in oncogenesis and tumor progression, such as p21 [82], c-FOS [83], the vascular endothelial growth factor (VEGF) [84], SIRT1 [85], tumor necrosis factor alpha (TNF-α) [86], B-cell lymphoma 2 (Bcl-2) [87], cyclooxygenase 2 (COX-2) [88] and p53 [89]. An aberrant overexpression of HuR and a disturbance of its nuclear/cytoplasmic localization have been associated with malignant transformations [90] in a significant number of human cancers, including breast [91], colon [92], ovarian [93,94], prostate [95], pancreatic [96] and oral cancer [97] among others.

1.4. HuR nucleus to cytoplasm transport and association with diseases

The functions of HuR in the nucleus are still to be fully elucidated, only recently being targeted in multiple studies. Conversely, the role of HuR in the cytoplasm and the mechanisms driving its shuttling have been clarified. Indeed, several PTMs regulate HuR subcellular localization, influencing its interactions with several transporters, and thus impacting on its regulatory feedback [36,98-103]. A correct and balanced subcellular localization for HuR, either in the nucleus or in the cytoplasm, is pivotal for normal development and organism homeostasis. As an example, a proper cytoplasm to nucleus redistribution influences the 3' UTR driven alternative splicing of different mRNAs, such as focal adhesion kinase (FAK) guiding adult neurodevelopment in mice neural stem cells [104]. As to bone marrow derived macrophages (BMDMs), HuR retention in the nucleus caused by its interaction with macrophage associated atherosclerosis lncRNA sequences (*MAARS*) increases macrophage apoptosis, leading to a decrease in their efferocytosis and, consequently, exacerbating the pathophysiology of atherosclerosis [105]. When liberated from the *MAARS* sponging effect and after translocation to the cytoplasm, HuR can then appropriately regulate mRNAs (e.g *p53, BCL-2*) that drive beneficial apoptotic processes. Furthermore, nuclear HuR regulates the alternative splicing of 3' UTRs in different oncogenic mRNAs (e.g. CENPN), so that treating MCF7 cells with doxorubicin (DOXO) prevents HuR nuclear localization, impairing cell cycle progression and thus cancer development [16]. Therefore, abnormal HuR subcellular localization and accumulation correlates with multiple diseases. Many studies on histological and clinical samples demonstrated a positive correlation between cytoplasmic HuR accumulation and *VEGFA, VEGFC, COX2* and *IL-8* levels in human tumor samples [88,106-109]. HuR is also correlated with a high-density of blood microvessels, and its presence in the cytoplasm is associated with large different tumors [53]. HuR cytoplasmic accumulation is mostly associated with cancer onset and progression, along with worse prognosis and poor outcomes in renal, urothelial and esophageal carcinomas, and small-lung cancer [90,110-114]. It also correlates with an overexpression of oncogenes and pro-tumorigenic factors [115,116]. Conversely, HuR nuclear accumulation is reported as a causative factor in the onset of gallbladder carcinoma [117].

1.5 HuR targeting as a therapeutic strategy

Considering the earlier described pathogenic functions of HuR, its inhibition *via* either small-interfering RNAs or small molecules has emerged as a putative therapeutic approach to ameliorate the outcome of multiple diseases. Many reports focused on the identification and characterization of HuR targeting agents, each exhibiting their advantages and disadvantages. The modulation of either the expression, the translocation and the PTMs profile of HuR, and its silencing were found to be effective in a context-dependent manner, and rely on the activation of specific factors [111,118]. Conversely, inhibitors (mostly small molecules)

impairing the HuR-mRNA interaction are less context-dependent, although their potency may depend on the PTM profile of HuR [33,119]. Their use could lead to unpredictable side effects due to the ubiquitous expression of HuR, its pleiotropic and controversial functions, and the lethal phenotype connected with its complete ablation. Indeed, the *in vivo* efficacy and tolerability of these HuR-mRNA modulators must still be completely determined [12,13]. Moreover, HuR conditional KO animal models (Table 1) suggest that its presence is essential for tissue homeostasis (i.e. hepatocytes, hematopoietic stem cells and epithelia), therefore a strong inhibition of HuR function by small molecules may cause potentially severe side effects. On the other side, pharmacological modulation of HuR only partially recapitulates a complete HuR KO condition and may be less impactful. In addition, considering the structural similarity of HuR with other ELAVL-like proteins, specificity remains a largely unknown issue. The aim of this review is to critically review the HuR modulators discovered so far, focusing mainly on naturally occurring and synthetic small molecules, describing their physico-chemical and structural properties, and commenting on their bioavailability. Their *in vitro* and *in vivo* anti-tumorigenic activities is recapitulated in association with eight Hallmarks of Cancer [120,121], providing evidence for a modulating role of HuR targeting agents on all of them. In addition, a summary guide on the molecular and cellular tools used so far for the identification and characterization of authentic HuR-mRNA modulators is presented (**Figure 2**).

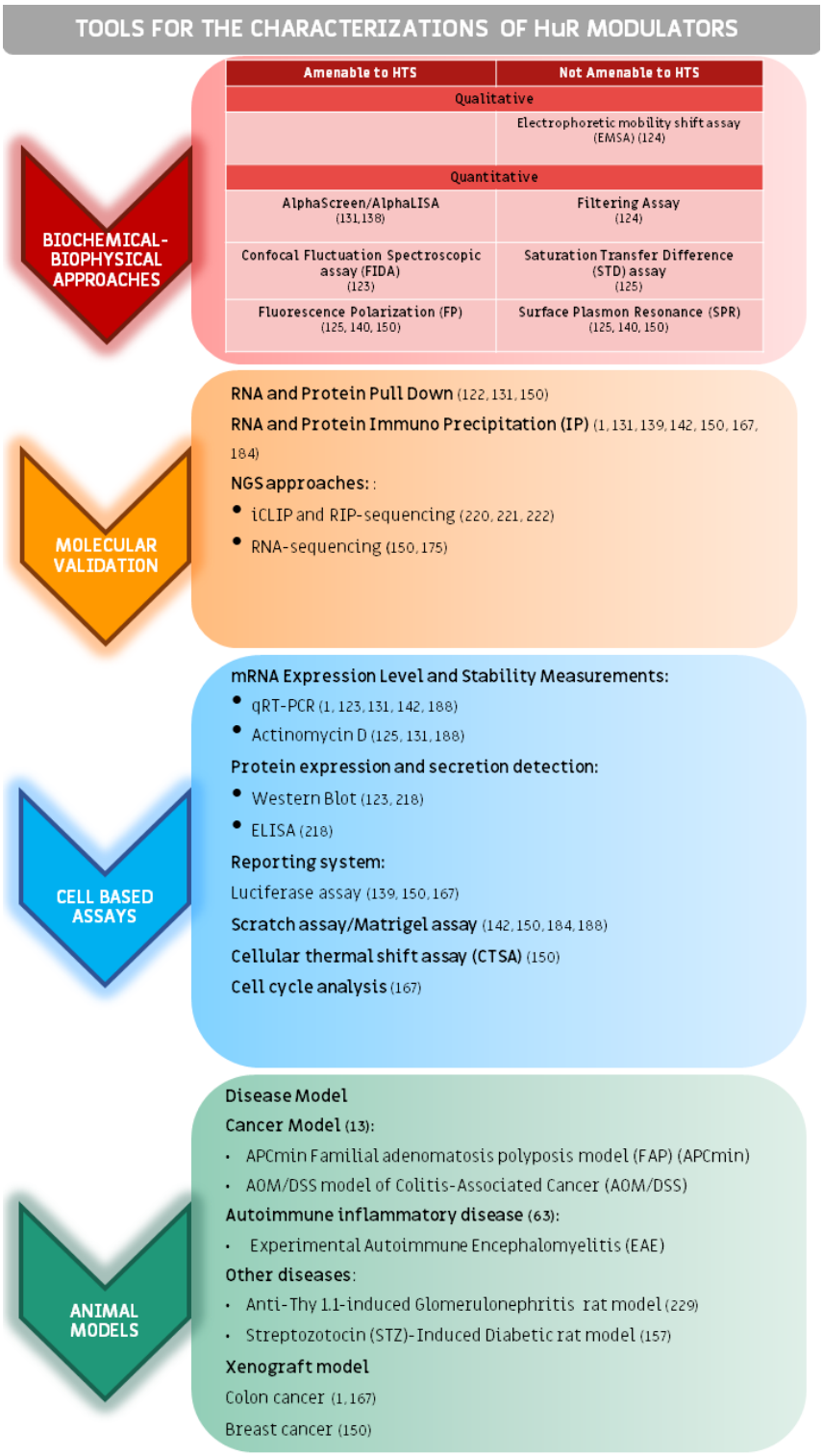


Figure 2. Tools for the discovery, development and characterization of HuR modulators. This pipeline lists techniques, strategies, cellular and *in vivo* models mostly applied for the identification and characterization of HuR inhibitors. A number of biochemical and biophysical approaches (red boxes) were used for the identification (e.g. high-throughput screening-HTS methods) and *in vitro* validation of HuR modulators (e.g., EMSA assays). Molecular validation assays (orange boxes) were then used to elucidate the molecular mechanisms leading to HuR inhibition (e.g., RNA immunoprecipitation). Finally, cell-based assays (e.g., scratch assays) (blue boxes) and *in vivo* cancer models (green boxes) used to preclinically evaluate HuR hits and leads as anti-cancer and inflammatory candidates are listed.

2 HuR modulators from various sources: chemical classes, structural and mechanistic features

This Section focuses on small molecule HuR inhibitors, divided in three classes depending on their source. *Naturally occurring compounds* (**Paragraph 2.1**, **Table 2** entries **1n**-**13n**) were the first to be reported in literature; they include a number of heavily oxygenated chemotypes with varying potency on HuR and limited bioavailability. *Synthetic compounds* (**Paragraph 2.3**, **Table 3** entries **1s**-**17s**) resulted either from HTS campaigns on large compound collections, or from rational drug design efforts using computational tools. Finally, *nature-inspired synthetic compounds* (**Paragraph 2.2**, **Table 4** entries **1ns**-**7ns**) were discovered either by testing semi-synthetic derivatives of biologically active natural compounds, or by rationally designing and synthesizing analogues inspired by known, naturally occurring HuR inhibitors.

We decided to include in our review both direct/HuR-binding inhibitors, and indirect modulators, acting to reduce HuR functional activity. Conversely, we only included a brief **Paragraph 2.4** to HuR inhibitors other than small molecules (e.g., siRNA or miRNA sequences, antisense nucleotides, nano-objects containing or decorated with HuR-interacting moieties). Some recent reviews [122,123] can be accessed to cover in details these areas.

2.1 Natural Compounds

A first study, aimed at demonstrating the druggability of HuR with small organic molecules, was initially reported [124]. A HTS campaign, taking advantage of a confocal fluctuation spectroscopy homogeneous assay format, was run on ≈50,000 microbial, mycological and plant extracts tested on a shortened variant of recombinant HuR (HuR_{12}). RP-HPLC-fractionation of 13 most active extracts led to the identification of chrysantone-like MS-444, dehydromutactin and okicenone as HuR-RNA binding inhibitors (**Table 2,** respectively entries **1n**-**3n**). Namely, cell-free inhibition constants in the low-medium micromolar range were determined for the three hits; HuR-dependent cellular effects were confirmed for MS-444 and dehydromutactin. Through RNA electrophoretic mobility assay (EMSA) screening of 179 chemicals representing a subset from a Korean compound library, quercetin (**Table 2,** entry **4n**), a flavonoid extracted from more than 20 plant varieties, was identified together with the synthetic compounds b-40 and b-41 (**Table 4,** entries **1s** and **2s** respectively) as inhibitors of the binding of HuR to the ARE domain of TNF-α mRNA [125]. A 1.4 μM IC_{50} was subsequently determined by measuring their effect on HuR-mRNA formation in a filter binding assay; a good specificity vs. other RNA-binding proteins was also observed.

Additional, naturally occurring flavonoids and coumarins were reported as HuR-mRNA interaction modulators. Clorobiocin/C11 [126] (**Table 2,** entry **5n**) – a natural aminocoumarin extracted from *Streptomyces roseochromogenes*, known to inhibit DNA gyrase [127] – was identified together with semi-synthetic daunomycin 3-oxime/C10 (**Table 3,** entry **2ns**) through a high throughput fluorescence polarization (HT-FP) assay run on the National Cancer Institute (NCI) diversity set V (≈1,600 compounds). Twelve selected hits were validated using saturation transfer difference (STD)-NMR [128] and analytical ultracentrifugation [129], which confirmed a direct HuR-hit interaction for clorobiocin and daunomycin 3-oxime and a medium micromolar inhibition constant for both (41.9 μM and 21.7 μM respectively).

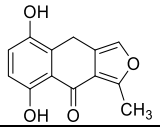
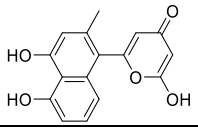
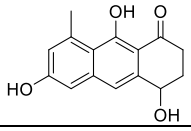
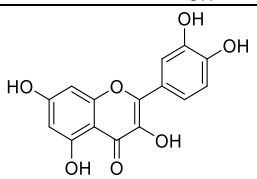
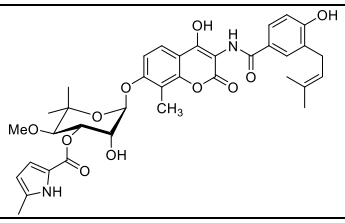
A computational- and NMR-driven effort on a small subset of 28 validated, naturally occurring HuR inhibitors run a first stability-solubility test for STD-NMR studies, selecting 13 drug-like hits; STD-NMR confirmed the interaction between HuR and twelve hits, and rutin and novobiocin (**Table 2,** entries **6n** and **7n** respectively) were selected as the most prospective validated hits. Inhibition constants were not reported [130].

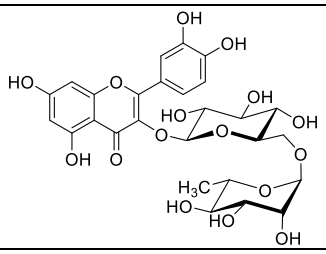
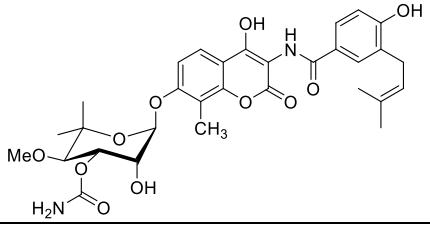
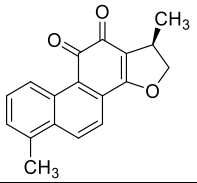
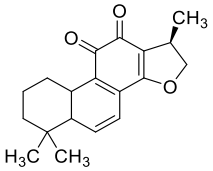
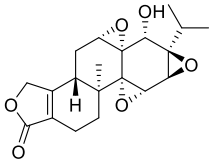
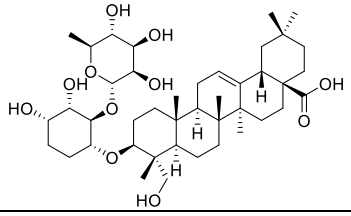
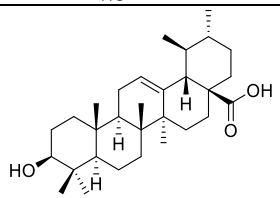
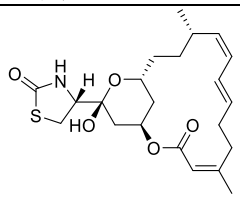
A validated amplified luminescent proximity homogeneous assay (AlphaScreen) format, measuring the inhibition of an interaction between human HuR and the ARE domain of TNF-α mRNA, was used to screen a set of 107 commercially available anti-inflammatory compounds [131]. Out of eight hits, after further validation with an RNA EMSA assay, dihydrotanshinone I (DHTS I) (**Table 2,** entry **8n**) was identified as a nM

inhibitor of recombinant HuR (rHuR)-mRNA complex formation (0.149 μM in REMSA, 0.068 μM in AlphaScreen assay), endowed with cytotoxic cellular activity [1,131]. A few other, naturally occurring tanshinones (i.e., cryptotanshinone – **Table 2,** entry **9n**) resulted to be slightly less potent [131,132].

In addition to validated HuR interactors, several compounds of natural origin affected HuR functions; although no direct HuR-compound interaction was proven for any of them, they are described here. Several naturally occurring terpenoids, well known in traditional Chinese medicine as anti-inflammatory and anti-cancer compounds [133], were submitted to mechanistic studies in order to identify their molecular target; they were found to interfere with the cytoplasm-nucleus translocation of HuR, reducing stability and expression of various HuR-interacting, tumor-related mRNAs. Triptolide (**Table 2,** entry **10n**) was characterized as a TNF- α-dependent COX-2 expression inhibitor [134], due to a reduction of cytoplasmic HuR in A549 cells. Similarly, kalopanax saponin A (KPS-A) (**Table 2,** entry **11n**), used in traditional medicine against rheumatoid arthritis and diabetes, inhibited phorbol 12-myristate 13-acetate (PMA)-induced cytoplasmic translocation of HuR, and reduced HuR-dependent matrix metalloprotease 9 (MMP-9) mRNA stability and expression [135]. Ursolic acid (UA) (**Table 2,** entry **12n**), isolated from the leaves of many plants, fruits and flowers, was also characterized as being able to reduce adriamycin resistance by promoting HuR cytoplasm-nucleus translocation, and by decreasing the mRNA stability of the HuR interactor multidrug resistance gene 1 (MDR1), thus reducing MDR1 expression [136]. A few modulators of actomyosin cytoskeleton assembly such as latrunculin A (**Table 2,** entry **13n**), an actin polymerization inhibitor toxin extracted from the Red Sea sponge *Negombata magnifica*, and blebbistatin (**Table 4,** entry **16s**), a synthetic highly selective inhibitor of non-muscle myosin II ATPase activity, were able to reduce the translocation of HuR from nucleus to cytoplasm in HepG2 and Huh7 hepatocarcinoma (HCC) cells [137].

Table 2: Naturally occurring HuR inhibitors: chemical structures, identification process. P = proven HuR binders, O = others.

	Entry	Molecule	Structure	Origin	P/O	Ref
Natural compounds	**1n**	**MS-444**		HTS of ≈50,000 microbial, mycological and plant extracts using a confocal fluctuation spectroscopic assay	P	[124]
	2n	**Dehydromutactin**		As **1n**	P	[124]
	3n	**Okicenone**		As **1n**	P	[124]
	4n	**Quercetin**		EMSA screening of 179 molecules from a chemical library at the Korea Research Institute of Chemical Technology (KRICT).	P	[125]
	5n	**C11/Clorobiocin**		Fluorescence polarization (FP)-assay followed by STD-NMR validation, using the NCI diversity set V.	P	[126]

	6n	**Rutin**		STD-NMR and molecular modelling.	P	[130]
	7n	**Novobiocin**		As **6n**	P	[130]
	8n	**DHTS I**		AlphaScreen assay of 107 commercially available anti-inflammatory compounds	P	[1]
	9n	**Cryptotanshinone**		Known DHTS analogue	P	[132]
	10n	**Triptolide**		Known anti-inflammatory and anticancer activities	O	[134]
	11n	**Kalopanax saponin A (KPS-A)**		*In vitro* biological profiling of KPS-A	O	[135]
	12n	**Ursolic Acid**		*In vitro* biological profiling of ursolic acid	O	[136]
	13n	**Latrunculin A**		Known antimitotic effects of latrunculin A	O	[137]

2.2 Nature-inspired synthetic compounds

In addition to previously mentioned daunomycin 3-oxime/C10 (**Table 3**, entry **2ns**) [126], several nature-inspired synthetic compounds have been identified as HuR inhibitors.

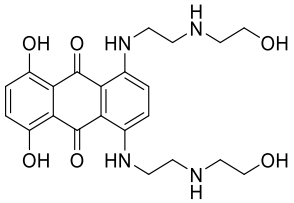
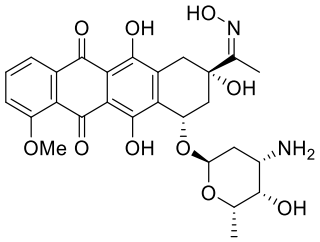
Using the experimental setup described earlier (AlphaScreen homogeneous assay, followed by an RNA EMSA assay) a library of 2,000 small molecules, including clinically tested candidates and natural products, was screened [138]. The anthraquinone mitoxantrone (**Table 3,** entry **1ns**) resulted to be able to interfere with rHuR – TNFα mRNA complex formation.

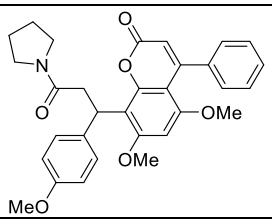
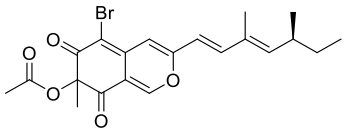
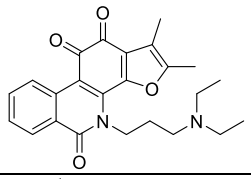
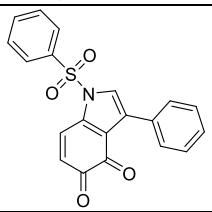
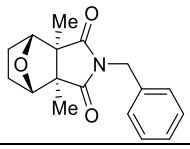
Two years later, the coumarin-like derivative CMLD-2 (**Table 3,** entry **3ns**) was identified through a FP-based HTS on a 6,000-membered library containing FDA-approved drugs and in house made compounds [139]. CMLD-2 was then validated through a rich profiling cascade including a biochemical AlphaLISA assay, surface plasmon resonance (SPR), ribonucleotide immunoprecipitation (RNP IP), and luciferase reporter functional studies, displaying a dose-dependent effect on HuR. Another library of ≈2,000 molecules, including compounds from the NCI Diversity Set II, a small set of natural products, FDA-approved oncology drugs and a few in-house compounds was screened using the same FP-based biochemical competition assay [140]. Azaphilone 9 (AZA-9) (**Table 3,** entry **4ns**), a semi-synthetic derivative built on the fungal natural asperbenzaldehyde scaffold, was characterized as the most potent hit through SPR and NMR (1.2 µM for full length HuR, 7.4 µM for HuR RRM1/2); computational studies were carried out to better pinpoint the molecular interaction between HuR and azaphilone 9.

Using previously mentioned **8n** (**Table 2**) as structural guidance, two research groups successfully obtained synthetic analogues endowed with higher potency and better bioavailability. A small set of lactam tetracyclic compounds inspired by **8n** was synthesized [141], out of which compound 22h (**Table 3,** entry **5ns**) was selected as a bioavailable early lead. Rational design and medicinal chemistry led us to a small array of bicyclic indolesulfonamide tanshinone mimics, out of which azatanshinone 6a (**Table 3,** entry **6ns**) was selected as an early lead [142]. Namely, a profiling cascade entailing biochemical Alpha-Screen and RNA EMSA assays, followed by cytotoxicity assays in breast MCF7 and MDA-MB-231 cancer cell lines, and on pancreatic ductal adenocarcinoma PANC-1 cell lines, established the potent cellular antitumoral activity of compound 6a.

As to inhibitors of HuR functions without a proven direct interaction, *N*-benzyl-cantharidinamide (**Table 3,** entry **7ns**), a synthetic analogue of the topic, naturally occurring cantharidine drug, was reported to reduce MMP-9 expression and the invasive potentials of hepatoma Hep3B cells by inhibiting cytosolic translocation of HuR, thus reducing HuR-mediated MMP-9 mRNA stability [143].

Table 3: HuR inhibitors inspired by natural products: chemical structures, identification process. P = proven HuR binders, O = others.

	Entry	Molecule	Structure	Origin	P/O	Ref
Nature-inspi	**1ns**	**Mitoxantrone**		HTS of ≈2000 molecules and secondary verification with RNA-EMSA.	P	[138]
	2ns	**C10/ Daunomycin 3-oxime**		As **5n** – **Table 2**	P	[126]

r e d s y n t h e t i c c o m p o u n d s	**3ns**	**CMLD2**		FP-based HTS of ≈6000 compounds, validation by an AlphaLISA assay, SPR, RNP IP, and luciferase reporter functional studies	P	[139]
	4ns	**AZA-9**		FP-based HTS of ≈2000 compounds from the NCI library plus in-house compounds, validation by SPR, NMR, and computational modeling	P	[140]
	5ns	**22h**		DHTS I-inspired modifications	P	[141]
	6ns	**AzaTanshinone 6a**		DHTS I-inspired modifications	P	[142]
	7ns	***N*-Benzyl-cantharidinamide**		Known antitumoral effects of cantharidine	O	[143]

2.3 Synthetic compounds

In addition to previously mentioned b-40 [125], b-41 [125] and blebbistatin [137] (**Table 4**, entries **1s**, **2s** and **16s** respectively), a few fully synthetic compounds have been characterized as effective HuR inhibitors.

At first, a representative diversity set of 89,000 compounds from a large, 2.2M bead-based library was screened by confocal nanoscanning/bead picking (CONA) [144]; after decoding by MS the structures of 46 most recurring hits, they were re-synthesized and validated as single compounds with full-length HuR and HuR_{12}. Out of six confirmed hits, polyamidoamine H1N (**Table 4**, entry **3s**) was identified as the first RRM3-targeted, low molecular weight HuR inhibitor, while the vast majority of reported HuR inhibitors bind to HuR RRM1 and RRM2.

In a glioma-targeted project, an AlphaScreen assay measuring hinge phosphorylation and subsequent oligomerization of HuR was used for a HTS campaign [145]; no details were given about size and nature of the screened collection. Benzimidazoleamide compound 5 (**Table 4**, entry **4s**) was identified as a putative disruptor of HuR multimerization. Suramin (**Table 4**, entry **5s**), a polysulfonated naphthylurea known for its antitrypanosomal, anticancer, and antiviral properties [146], was identified through a differential scanning fluorimetry (DSF)-based screening of a 1570-membered library of FDA- approved compounds; 55 initial hits were further profiled in more demanding DSF conditions, and suramin resulted as a single, fully validated hit. A low affinity HuR-suramin interaction (K_d = 0.24 μM) was confirmed by SPR [147].

The first structure-based rational design of HuR ligands, based on a virtual screening (vHTS) campaign using a platform specifically set to identify novel scaffolds/molecules as inhibitors of macromolecular interactions, was recently reported [148]. Ranking among 200 virtual chemotypes led to the selection of 17 specific hits, which were docked into the HuR binding site and more extensively studied. As a result four compounds were synthesized, three (**Table 4,** entries **6s, 7s** and **8s**) were confirmed as HuR interactors using a combination of

STD-NMR and *in silico* studies, identifying contacts with the RNP regions of RRM1 and RRM2 HuR domains, and polyphenol benzamide 4 (**Table 4,** entry **8s**) was qualitatively suggested being the most potent based on the intensity of the STD signal.

Aiming to improve the activity of polyphenol benzamide 4 (**8s**), compounds 2 and 3 (**Table 4,** entries **9s** and **10s** respectively) were designed on the basis of a SPR-fragment screening [149]. Their interaction with HuR was then also evaluated by STD-NMR, and their interference with the HuR–RNA complex was quantitated with an FP assay (IC_{50} =105 μM and 92 μM for compound 2 and 3 respectively).

Following the earlier described discovery of CMLD-2 [139] and Aza-9 [140], using the same FP-based HTS – AlphaLISA and SPR profiling cascade on a ≈2000-membered compound collection, benzothiophene hydroxamate KH-3 (**Table 4,** entry **11s**) was identified as a HuR–ARE^{Msi1} (Musashi RNA-binding protein 1) disruptor with low micromolar potency (3.5 μM in an FP assay, 2.3 μM in an AlphaLISA assay) and functional HuR effects [150].

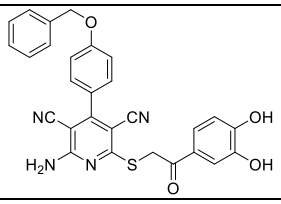
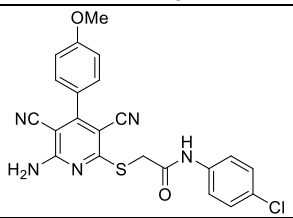
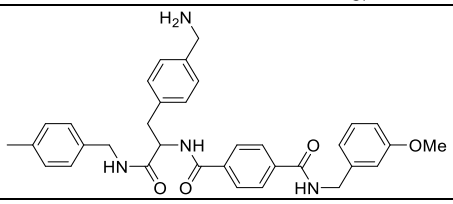
In a study directed towards the identification of novel HuR-mRNA binding inhibitors, 28 derivatives based on indole and caffeic acid phenethyl ester (CAPE) scaffolds were designed and synthesized [151]. Among them, indole-based compounds VP12/14 and VP12/110 (**Table 4,** entries **12s** and **13s** respectively) were confirmed as HuR interactors in an *in vitro* assay.

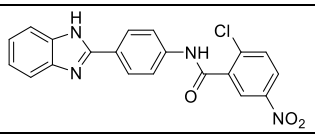
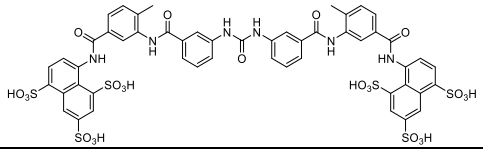
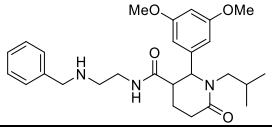
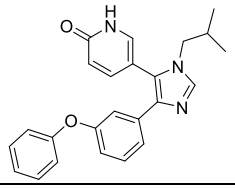
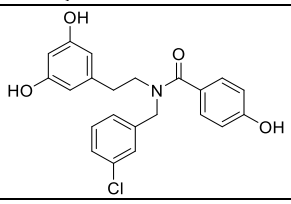
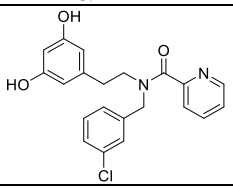
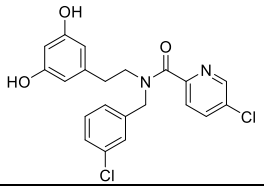
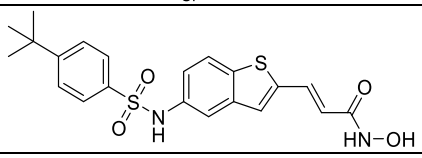
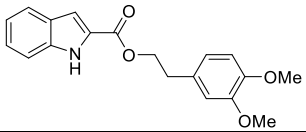
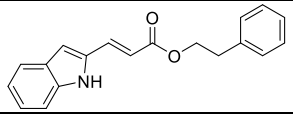
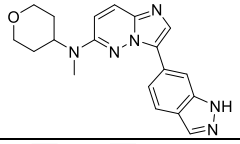
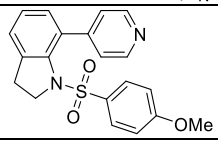
Recently, bisheteroaryl compound SRI-4217 (**Table 4**, entry **14s**) was found to bind and inhibit HuR dimerisation in primary patient-derived glioblastoma xenolines (PDGx) with a 1.2 μM IC_{50}. An interaction with the RRM1 and RRM2 domains was proposed through computational studies [152].

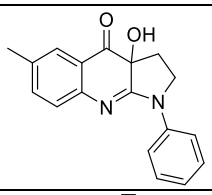
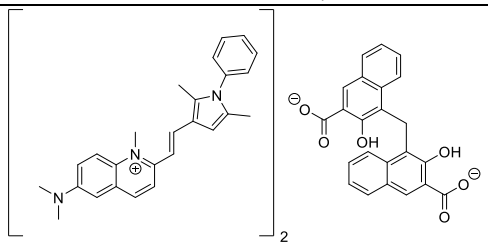
Few other synthetic HuR inhibitors were identified, such as Indoline sulfonamide MPT0B098 (**Table 4,** entry **15s**) [153]. In addition to its antimitotic activity through microtubule binding, MPT0B098 significantly decreased HuR translocation from the nucleus to the cytoplasm in A549, HONE-1 and PC3 tumor cells, subsequently reducing hypoxia-inducible factor 1-α (HIF-1-α) protein expression.

Finally, pyrvinium pamoate (**Table 4,** entry **17s**), an FDA-approved anthelminthic drug, was found to dose-dependently inhibit cytoplasmic accumulation of HuR by activating the AMP-activated kinase/importin α1 cascade and inhibiting the checkpoint kinase1/cyclin-dependent kinase 1 pathway [111].

Table 4: Fully synthetic HuR inhibitors: chemical structures, identification process. P = proven HuR binders, O = others.

	Entry	Molecule	Structure	Origin	P/O	Ref
Synthetic compoun	**1s**	**b-40**		As **4n** – **Table 2**	P	[125]
	2s	**b-41**		As **4n** - **Table 2**	P	[125]
	3s	**H1N**		HTS of ≈89,000 compounds by CONA (confocal nanoscanning/bead picking)	P	[144]

d s	4s	5		AlphaScreen assay	P	[145]
	5s	Suramin		DSF screening of an FDA approved library	P	[147]
	6s	1		Rational design by computational methods	P	[148]
	7s	3		Rational design by computational methods	P	[148]
	8s	4		Rational design by computational methods	P	[148]
	9s	2		Biophysical fragment-screening	P	[149]
	10s	3		Biophysical fragment-screening	P	[149]
	11s	KH-3		FP-based HTS of ≈2000 compounds from the NCI library plus in-house compounds, validation with an AlphaLISA assay and SPR	P	[150]
	12s	VP12/14		Rational design by computational methods	P	[151]
	13s	VP12/110		Rational design by computational methods	P	[151]
	14s	SRI-42127		Structural optimization of a HTS hit	P	[152]
	15s	MPT0B098		Known anticancer properties of MP70B098	O	[153]

	16s	**Blebbistatin**		Known antimitotic effects of blebbistatin	O	[137]
	17s	**Pyrvinium pamoate**		HTS of FDA-approved drugs for inhibition of HuR expression after UVC irradiation	O	[111]

2.4 Nanoparticle-encapsulated HuR siRNA as therapeutic agents

HuR was successfully targeted also through nanoparticle (NP) -based HuR-specific small interfering RNA (HuR siRNA) delivery. The efficacy of folate receptor-α (FRA)-targeted DOTAP:Cholesterol lipid NPs carrying HuR siRNA (HuR-FNPs) was tested against human lung cancer cells [154]. A folic acid (FA)-based FA-PEG-DSPE construct, 1,2-dioleoyl-3-trimethylammonium-propane chloride salt (DOTAP), and cholesterol were used to build liposomes with a particle size of 303 nm and a surface charge of +4.3 mV. Folate-conjugated liposomes showed efficient internalization in lung cancer cells *via* folate receptor endocytosis, and serum stability and gel retardation assays revealed that such liposomes protected HuR siRNA from rapid degradation [154]. A method to deliver siRNA against HuR *in vitro* and *in vivo* was set up and optimized by conjugating FA to 3DNA nanocarriers in ovarian cancer models [155]. A transferrin receptor-targeted, liposomal NP-encapsulated HuR siRNA (siRNA-Tf-NP) was investigated as a therapeutic agent against HuR by employing sulfhydryl reactive crosslinking chemistry to synthesize Tf-PEG-DSPE [156]. HuR siRNA was administered intraocularly as nano-based lipidic systems in streptozotocin (STZ)-induced diabetic rat models. Such lipoplexes caused an efficient decrease of pathologically elevated HuR and VEGF retinal levels. Finally, nanocarrier-transported siRNA showed an amelioration of the retinal damage caused by STZ, increasing retina thickness and the number of retinal ganglion cells (RGCs) up to homeostatic levels, compared to their reduction observed in diabetic rats alone and when receiving naked siRNAs [157].

In conclusion, the large number of reported small molecules demonstrates the druggability of HuR. HTS campaigns were at first employed to identify small molecules able to either directly or indirectly interfere with HuR activity, due to the lack of structural information about the HuR-mRNA interaction at that time. Although these structurally heterogeneous compounds, being either natural, synthetic or natural compound-inspired, often consist of complex molecular structures and/or show sub-optimal pharmaceutical properties, they were useful starting points to develop more specific HuR modulators. More recently, a detailed knowledge of HuR structure and binding modes to mRNA and small molecule modulators has enabled the rational design, synthesis and characterization of new ligands specifically designed through computational methods, as extensively described in the next Chapter. We do believe that both HTS campaigns / access to unpredictable structures of HuR inhibitors, and rational drug discovery / further exploitation of structural information on HuR will be exploited in future to enrich the panel of existing HuR modulators and their properties. Their potential as putative clinical candidates against oncological diseases will be commented upon in details in Chapter 4.

3 HuR structural features and interactions with low molecular weight inhibitors

Full-length HuR is a multi-domain protein constituted by three RRMs of about 90 amino acids long. Namely, two conserved RRMs near the N-terminus are named RRM1 (20-98) and RRM2 (106-186), linked by a 12 amino acid linker and preceded by an intrinsically disordered region of 20 amino acids; and a third recognition motif, named RRM3 (244-322), is located at the C-terminal region. RRM3 is separated from RRM2 by a longer basic linker that involves the 60 amino acid-long HNS, which is mainly responsible for the nuclear-cytoplasmic shuttling of HuR to stabilize and/or enhance the target mRNA translation efficiency (see **Figure 3A**) [3,4,130,158].

In the last decade ten 3D structures were deposited in the PDB databank (nine resolved by X-ray diffraction, one by solution NMR). Among them, three include only the RRM1 domain (PDB codes: 4FXV, 3HI9, and 5SZW for the NMR structure), two span the tandem RRM1-RMM2 domains (PDB codes: 4ED5 and 4EGL), and five relate to the RRM3 motif (PDB codes: 6GD2, 6GD3, 6G2K, 6GD1 and 6GC5); none of them include any complexed inhibitor in the crystal structure. Although structural guidance for HuR modulation is now available, most HuR inhibitors discovered so far (see Chapter 2) have been identified through experimental HTS.

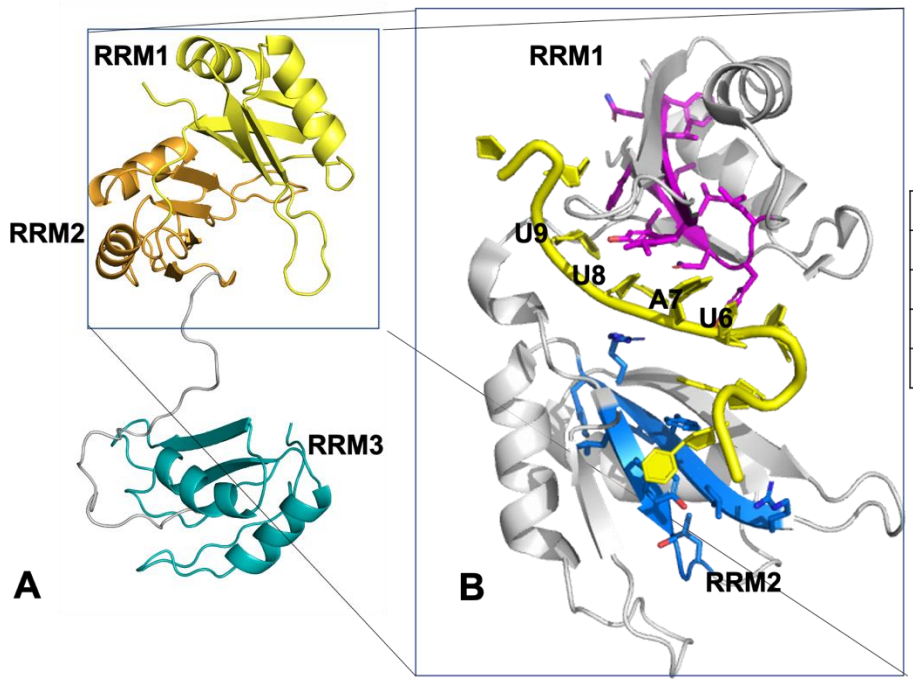
3.1 Structural insights on HuR

X-ray structures collected on the isolated domains and on the RRM1-RMM2 tandem domain of HuR reveal the typical architecture of the RNA recognition motifs in eukaryotic RNA binding proteins. They present four-stranded antiparallel β-sheets packed against alpha helices, adopting the canonical αβ structure with a β1-α1-β2-β3-α2-β4 topology [159,160]. Also, high structural similarity has been detected between the first two RRMs of HuR, with the exception of a different conformation involving a β-hairpin located at the α2-β4 loop, which in RRM1 adopts a β-turn-β conformation that is not present in RRM2 [158,159].

Characterization of the HuR-mRNA-binding activity allowed the understanding of the role for each RRM domain. The interactions between mRNAs and HuR are generally affected by any modification of the residues within the RRMs. Conversely, modifications on the residues within the HNS sequence alter the sub-cellular localization of HuR [10].

Information on the mRNA-bound structures of HuR have been obtained from the complexes of the protein with short AREs [3,4,158,160,161]. HuR RRM1 and RRM2 control the recognition of any target mRNAs. In particular, the RRM1-RMM2 tandem construct has been reported to bind a 11-base AU-rich strand with high nanomolar affinity, resulting from adding the micromolar affinity of RRM1 to the weak affinity of RRM2 and to further contacts established by the short interconnecting linker between them. The same tandem construct recognizes and binds U-rich sequences (U-rich RNA and U-rich DNA), with even higher affinity and with a preference for U-rich RNA sequences [162].

A detailed analysis of the experimental structure of HuR complexed with a 11-mer RNA oligonucleotide derived from c-fos mRNA (PDB code 4ED5) shows that the binding site is mostly hydrophobic. The residues involved in the interaction are placed at the two canonical ribonucleoprotein sequences named RNP1 and RNP2 (see **Figure 3B**) [163], located at the center of the β-platform (β1 and β3) in the RRM domain.



Domain	Sequence
RNP1_RRM1	$_{61}$LGYGFVNY$_{68}$
RNP2_RRM1	$_{22}$LIVNYL$_{27}$
RNP1_RRM2	$_{147}$RGVAFIRF$_{154}$
RNP2_RRM2	$_{108}$LYISGL$_{113}$

Figure 3. A) Representative 3D model of full length HuR (Chimera model obtained with Prime-Schrodinger software); the protein is shown in ribbons with RRM1, RRM2 and RRM3 displayed in yellow, orange, and cyan, respectively, and the long basic linker between RRM2 and RRM3 in grey. B) Co-crystal structure of the tandem RRM1 and RRM2 HuR-mRNA$^{c\text{-}fos}$ complex (pdb code 4ED5); the protein backbone is shown in grey, the RNP1 and RNP2 sequences of the RRM1 domain in magenta, the RNP1 and RNP2 sequences of the RRM2 domain in blue, mRNA$^{c\text{-}fos}$ in yellow.

Additional residues located around the β-strands and in the RRM1-RRM2 linker contribute to the binding with mRNA$^{c\text{-}fos}$ by establishing weaker interactions. Residues N24 and F65 located respectively at the β1 and β3 strands [158,159] are reported to play a binding role, while residues located at the α2 helix, β2-β3 and α2-β4 loops and belonging to the inter-domain linker experience conformational change upon RNA binding.

As to RNA, structural data show that the RRM1 domain recognizes up to 5 consecutive uracils, while the inter-domain linker and RRM2 bind only to one or two nucleotides [1,3]. A mechanism for the HuR-mRNA binding has been recently proposed [3]. It entails a first interaction with the RRM1 domain, followed by conformational changes involving the inter-domain linker and RRM2, inducing them both to bind the RNA strand, and thus stabilizing the HuR-mRNA complex.

As to the third recognition motif, despite previous studies suggested its negligible contribution to the interaction between HuR and mRNA strands, more recently an isolated RRM3 domain was also shown to recognize ARE sequences, contributing to the interaction with the target mRNAs by binding their poly-A tail, as well as being necessary for the cooperative assembly of HuR oligomers. In fact, disruptions at the dimerization interface result in a decreased binding affinity between HuR and its mRNA targets [10,160,164,165].

The RRM3 domain can bind both to AUUUA motifs and poly(U) sequences, with a preference for the U-rich strands [160]. Similarly, to other RRMs, the binding interface in RRM3 is located in RNP1 (β3), involving residues K285-M292, and in RNP2 (β1) involving residues I246-L251. Residues F287 and M292 from RNP1 and F247-L251 from RNP2 are crucial for the interaction. Later studies [4,161] confirmed both the binding site and preference for U-rich sequences and determined that HuR-mRNA complexes are stabilized by a combination of base stacking and polar and hydrogen bond interactions. Although there are conflicting reports on the number of binding pockets on the RRM3 domain, different studies agree that RRM3 can recognize the UUU/A motif. RNP2 is responsible for the direct interaction with uracil and RNP1 for other

nucleotides such as adenines. (A/U)UU(A/U) has been proposed as the RNA binding recognition sequence [161], while mutations at the binding interface of isolated RRM3 domains as well as within full-length HuR [4] confirmed the key role of F247 and Y249 in the HuR-mRNA interaction, and the minor contribution provided by F287 and F288 residues. Notably, ATP was identified as a natural RRM3 ligand responsible of a surprising, RNA-modifying, terminal adenosyl transferase activity of HuR, suggesting an additional role for HuR in the maturation and metabolism of mRNA targets [144].

As to the dimerization process of HuR, studies based on EMSA assays have also shown the propensity of isolated RRM1 domains to dimerize [166]. Interestingly, an equilibrium between multimeric forms of RRM1 has been described, with their predominance on monomeric and dimeric forms in the absence of mRNA sequences. The RRM1 domain region responsible for the dimerization process comprises the β-hairpin and α-helices. Besides, mutagenesis experiments reveal the involvement of a disulfide bridge at Cys13, which may have functional significance in redox modulation of HuR activity in response to oxidative stress [159]. Of note, the dimerization region is placed on the opposite site with respect to the mRNA binding surface. However, the interaction with mRNA sequences involves a slightly overlapped protein region, thus promoting the dissociation of the RRM1 dimeric form and shifting the equilibrium to the monomeric state [158,159].

Another important recognition motif for the dimerization of HuR is in the RRM3 domain. Recent studies on the C-terminal region of the protein proved the existence of a dynamic equilibrium between dimeric and monomeric forms of RRM3 even in the absence of RNA sequences. The extent of dimerization appears to be dependent on the concentration of RRM3 domains, and the dimeric form is stabilized by interaction with mRNA sequences. It is interesting to note that both sequence and length of the mRNA strand play a role in the stabilization of a HuR dimer. In particular, in presence of short mRNA constructs, the dimeric state of HuR might be disfavoured, while both RRM3 domains forming a dimer can bind to the same, long mRNA strand (more than 14 nucleotides) [4,160,161,164]. X-ray and NMR studies on the RRM3 domain, together with Molecular Dynamic (MD) calculations allowed the identification of a dimerization surface constituted by the α1 helix and the loop between α1-β2. Moreover, the W261 residue plays a crucial role, since any mutation involving this amino acid leads to an increase of HuR monomers [4,160,161,164].

The internal dynamics of HuR have been characterized in detail by NMR. Measurements of the longitudinal (R1) and transverse (R2) relaxation rates of the backbone amide nitrogens recorded on the RRM1-RRM2 tandem domains prove that this construct does not behave as a rigid body, but rather displays inter-domain flexibility with the two domains that can reorient with respect to one another [1].

Interestingly, the same relaxation data suggest that RRM1–RRM2 dimers are not present in solution as stable complexes. Another important finding from NMR studies concerns the RRM3 domain, that does not seem to interact with the other two recognition motifs in the absence of mRNA [160].

A comprehensive analysis of the experimental structures obtained by X-ray crystallography and NMR data indicates that in the absence of mRNA strands the three domains in full-length HuR, as well as RRM1 and RRM2 in the tandem domain construct, move independently and maintain an open/flexible conformation (**Figure 4**).

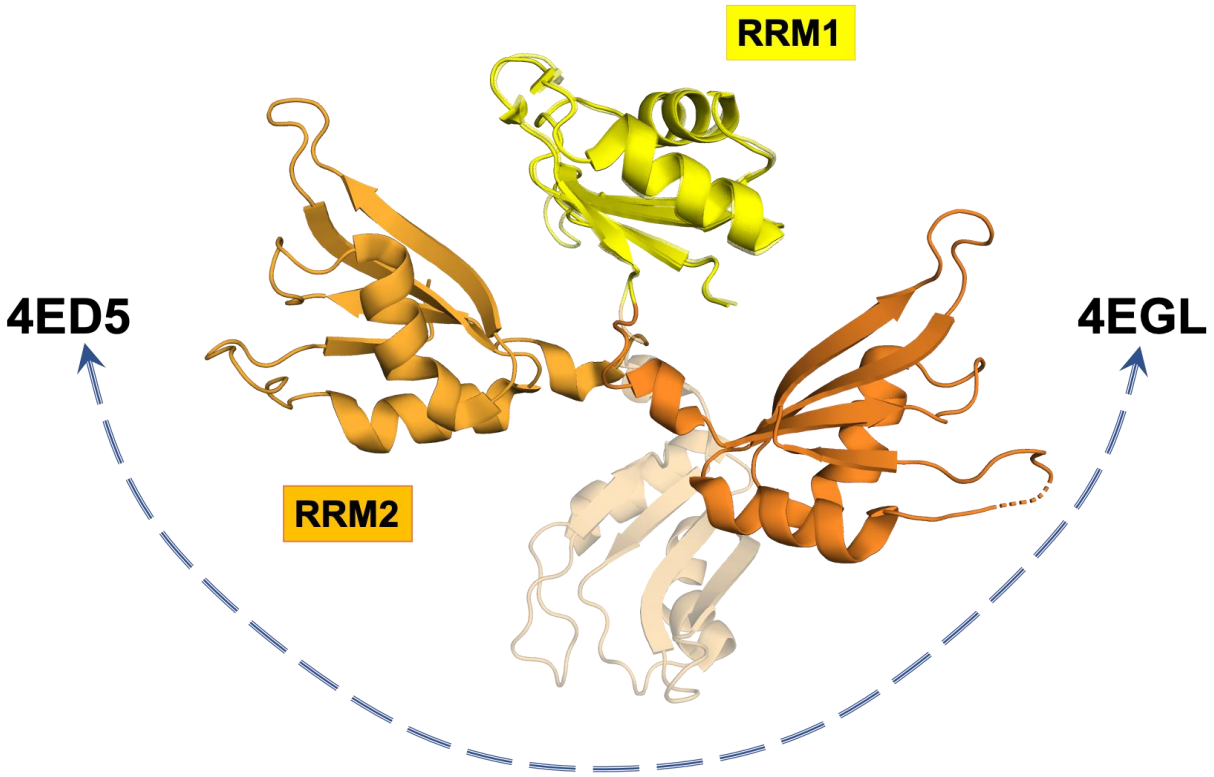


Figure 4. A superposition of NMR- (PDB code: 4EGL) and X-ray-derived (PDB code: 4ED5) HuR structures shows that RRM1 (yellow cartoon) and RRM2 (different shades of orange cartoon) domains experience conformational freedom with respect to each other [3].

After binding to a target mRNA, the protein adopts a closed globular conformation, forming a positively charged cleft where additional contacts between RRM1 and RRM2 can be detected. Namely, RNP1 and RNP2 sequences come in close proximity, especially around the U6, A7, U8 and U9 nucleotides, providing in this region a narrower binding pocket, possibly amenable to the rational design of organic Hur/RNA disruptors (see **Figure 3B**).

3.2 Computational and NMR studies to elucidate the interaction between HuR and small molecule inhibitors

Many HuR inhibitors were discovered and tested, especially *in vitro*, in the last decade. However, for most of them the molecular mechanism of HuR inhibition has been poorly investigated and characterized at the atomic level, and for a few it is completely unknown. It has been proposed, solely relying on docking studies, that small organic ligands should bind HuR at the cleft between the RRM1 and RRM2 domains [149,150,152,163,167]. This theory has been recently validated through combined NMR and computational studies [1,130,140,142].

For instance, using both NMR titration and docking calculations, the interaction of **4ns** (see **Table 3**) with HuR was elucidated. Specifically, NMR-titration experiments allowed the identification of a pool of residues interacting with the molecule (I103, L138, Y26, R97, I103, Y109 and R153) which delimit the mRNA binding cavity. In line with NMR results, docking of **4ns** into HuR suggests Y26, K55, R97, and R153 as residues surrounding **4ns,** establishing electrostatic, hydrogen bond, hydrophobic, and pi-stacking interactions with the small molecule (**Figure 5A**). Thus, **4ns** appears to disrupt the HuR-mRNA interaction by competitively binding to the RMM1-RRM2 interdomain cleft of HuR [140].

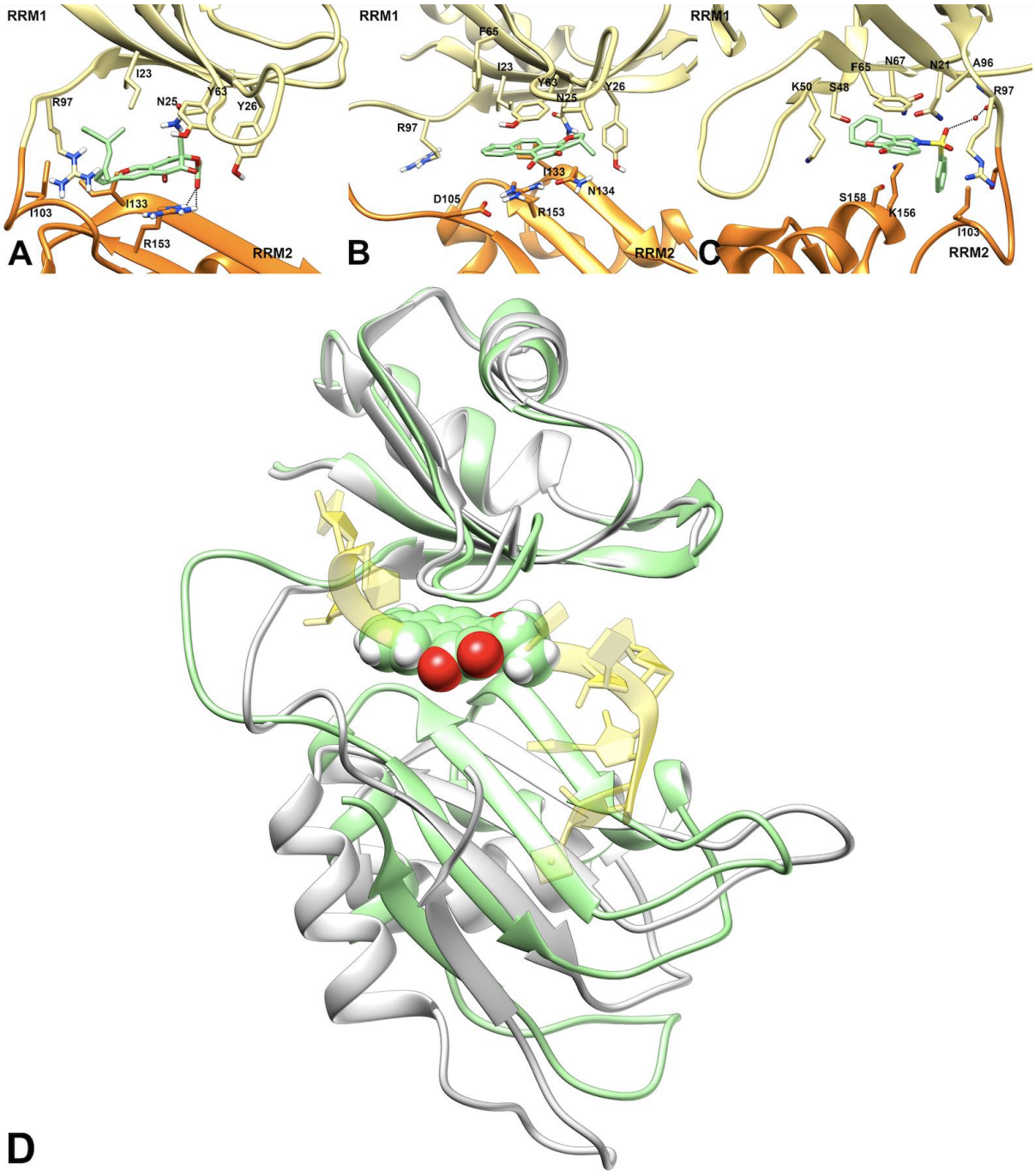


Figure 5. A),B),C) Binding modes of **4ns** (A), **8n** (B) and **6ns** (C) (green sticks) into HuR (yellow and orange cartoons), as determined by computational studies. HuR residues involved in binding interactions are displayed as sticks, and H-bonds are depicted as dashed black lines. D) 3D superposition of the HuR conformation (green cartoons) induced by **8n** (green spheres) upon binding, and the mRNA-bound HuR conformation (PDB code 4ED5, grey and transparent yellow ribbons for HuR and mRNA, respectively).

Similar results were subsequently obtained with twelve naturally occurring HuR inhibitors including flavones, flavonols and coumarins, which were studied through STD-NMR experiments and docking to explore the ligand-protein interaction mode [130]. These studies revealed that all compounds interacted with HuR in STD-NMR with different strengths (see for example **6n** and **7n** in **Table 3**); that all bind into the deep pocket between the RRM1 and the RRM2 domains, stabilizing a "closed" conformation of the protein; and that some

interactions are conserved among flavones, flavanols and coumarins, including those with Y63, R97, N25 and R153 side chains that have been described as important also for **4ns** and **6s** [148].

We reported structural details on the interaction mode of **8n** and **6ns** (see respectively **Table 2** and **Figure 5B**, and **Table 3** and **Figure 5C**) with HuR [1,142]. A combined approach using NMR, MD simulations and mutagenesis coherently indicated that these small molecules bind to the region between the interconnecting linker and the β-platform of both RRM1 and RRM2, altering the conformational freedom and the reciprocal reorientation of RRM1 and RRM2. This stabilizes an unproductive, "closed" conformation that hampers target mRNA binding (**Figure 5D**).

Noteworthy, a different mechanism of action has been described for compound **3s** [144]. Confocal nanoscanning-bead picking experiments showed that this molecule binds to the RRM3 domain. *In vitro* studies indicate that **3s** interferes with ATP as well as RNA binding within the RRM3 domain. Docking calculations of **3s** into the homology modeling structure of RRM3 (no RRM3 domain was crystallized at that time) showed that the relatively large ligand occupies both a conserved DxD motif (D_{254}, D_{256}), commonly used for recognition of ATP, and an adjacent shallow and positively charged cleft, very likely binding the target mRNA, thus hampering the accommodation of both RNA and ATP in line with experimental data.

3.3 Computer-aided hit discovery

In 2019, a first example of virtual screening has been reported [148] through which three structurally diverse ligands were found to inhibit HuR using the NucleoQuery application within the free Web-server platform AnchorQuery. Specifically, the authors selected the mRNA U8 and U9 nucleotides as anchor points and the NucleoQuery application detected all the possible interactions with the protein interface, allowing the final selection of several pharmacophore points. Such pharmacophoric query identified a library of 800 structurally diverse, synthetically accessible molecules as putative HuR inhibitors, all possessing a specific aromatic moiety (superimposable with the U8 of mRNA) as an anchor point. Four representative compounds were synthesized, three of which (**Table 4**, **6s**-**8s**) were characterized as HuR binders in an STD-NMR assay. Just recently, **8s** has been successfully optimized through SPR and STD-NMR fragment screening [149]. Docking experiments confirm that also optimized compounds **9s** and **10s** bind in the same region occupied by the U8 mRNA base. Most importantly, both studies demonstrate that computer based HuR ligand discovery is achievable, and that the molecular interaction between U8 in mRNA and Y63 in HuR represents an important anchor point to design small molecules suitable for HuR recognition and binding [148,149].

Another successful, recent example of computer-aided hit discovery on HuR consisted of a shape similarity screening using **8n** (**Table 2**) as a template and a proprietary database of 182 drug-like molecules [151]. As a result, 28 putative HuR ligands mostly built on indole or caffeic acid phenethyl ester scaffolds were found and clustered on the basis of their structural interaction fingerprints (SIFts). Two putative ligands (**12s** and **13s**, **Table 4**) were found in the same cluster of potent **1n** and **8n**, and thus were synthesized. Although a direct binding assay with HuR was not reported, cellular assays demonstrated that **12s** and **13s** modulate HuR expression and decrease VEGF and TNF-α release, similarly to **8n**. Noteworthy, MD simulations of **12s, 13s** and **8n** into the binding site of HuR demonstrated that **13s** and **8n** should compete with mRNA to bind to HuR, possibly inhibiting the functional effects of HuR on mRNAs.

NMR and modeling data reported so far for HuR inhibitors surely indicate, with the single exception of **3s**, their binding in the mRNA U6-U9 binding site of the RRM1-RRM2 construct, stabilizing a closed, unproductive conformation of HuR that in turn hampers mRNA accommodation in its binding site. Although HuR inhibitors are chemically diverse structures, and no pharmacophore hypothesis has been proposed up to now, some general, common features could be detected. Namely, one or more aromatic rings (rarely saturated rings) in the same scaffold, which confer structural rigidity and a hydrophobic character to HuR inhibitors, then fitting well in the mRNA U6-U9 binding region of the HuR protein; and functional groups such as carbonyls, hydroxyls, amines and carboxylic acid to increase polarity and interact with the hydrophilic residues in the

binding cleft. For instance, at least one polar contact with the side chains of R97 or R153 residues should be established, as compounds **6n**, **6ns** and **6s** possess a carbonyl group (a sulfonyl for **6ns**) that establishes a hydrogen bond with R97, while compounds **7n**, **4ns** and **6s** are endowed with a hydroxyl or a carbonyl moiety establishing one or two hydrogen bonds with R153. Other residues frequently interacting with active molecules are S99 and S100 on the interdomain linker, and Y63 and N25 in RRM1.

Taken together, the reported studies have laid the foundation for the rational finding / design of novel HuR inhibitors through a range of diverse methodologies. We expect that, now that diverse HuR inhibitors are known, a few pharmacophore models will be developed and used in virtual screening (vHTS) campaigns. Besides, taking advantage of recently released RRM3 X-ray structures, novel HuR ligands targeting the RRM3 domain should be soon discovered with the aid of receptor-based finding techniques.

4 HuR inhibitors and the hallmarks of cancer

HuR is often overexpressed in many human cancers, with increased levels associated with tumor aggressiveness and worse prognosis. HuR plays a critical role in controlling almost all key cancer-associated traits, including proliferation, survival and dissemination amongst others, by regulating the expression of a plethora of genes [106,122,168-170]. Due to this fundamental role in cancer progression and metastasis, HuR has received considerable attention as a therapeutic target. Remarkably, over the years hundreds of *in vitro* and *in vivo* studies (**Table 5**) have consistently shown that targeting HuR is a promising strategy for a variety of cancers. In addition, genetic silencing in several cancer models has shown benefits in terms of tumor regression, but, while these studies are promising, advancing siRNA-based therapy to the clinic remains a challenge. Thus, the importance of developing a potent, bioavailable and safe small molecule inhibitor directed against HuR to be tested and proposed for clinical studies cannot be understated.

Table 5: *In vivo* studies showing the effects of HuR targeting in different cancers.

Cancer type	In vivo model	HuR modulation	Major observations	Refs
Malignant peripheral nerve sheath tumors (MPNST)	– Tumor Xenograft – Lung metastasis model	*Genetic (Constitutive and inducible shRNAs)* *Pharmacological (**1n**)*	– HuR knockdown prevents formation of xenograft tumors and induces regression of already formed tumors. – Genetic ablation of HuR prevents formation and growth of metastatic lung nodules. – Pharmacological inhibition of HuR blunts growth of xenograft tumors and metastatic nodules.	[171]
Gastric cancer	– Tumor Xenograft	*Genetic (Overexpressing)*	Overexpression of HuR promoted gastric cancer cell growth *in vivo*.	[172]
Fibrosarcoma	– Tumor Xenograft	*Genetic (CRISPR/Cas9)*	HuR deletion markedly diminished tumor growth on its own whereas AGI-5198 (a mutant IDH1 inhibitor) treatment combined with HuR deletion had the greatest impact on tumor growth.	[173]
Pancreatic ductal adenocarcinoma (PDAC)	– Tumor Xenograft	*Genetic (DOXO-inducible shRNAs)*	HuR inhibition enhances PARP inhibitor olaparib-mediated reduction of PDAC tumor growth *in vivo*.	[174]
Colon carcinoma	– Tumor Xenograft	*Pharmacological (**8n**)*	Compound **8n** induces significant anti-tumor effects, with approximately a 4-fold reduction in tumor size.	[1]
Pancreatic ductal adenocarcinoma (PDA), colorectal cancer	– Tumor Xenograft	*Genetic (CRISPR/Cas9)*	HuR-deficient PDA cells were unable to engraft tumors *in vivo* compared with control cells, whereas HuR-deficient colon cancer cells showed significantly reduced *in vivo* tumor growth.	[175]
Lung cancer	– Tumor Xenograft – Lung metastasis model	*Transferrin receptor-targeted liposomal nanoparticle-based HuR siRNA (HuR-TfNP)*	HuR-TfNP treatment suppresses lung tumor growth *n vivo* and suppresses experimental lung metastasis	[154]
Bladder cancer	– Tumor Xenograft	*Pharmacological (**17s**)*	Combining compound **17s** with chemotherapeutic agents (e.g. cisplatin, DOXO, vincristine and oxaliplatin) synergistically suppressed the growth of patient-derived bladder tumor xenografts in mice	[111]
Colorectal cancer (CRC)	– Tumor Xenograft	*Pharmacological (**1n**)*	Compound **1n** led to an approximately 1.7-fold reduction in tumor size	[167]
Melano	– Tumor Xenograft	*Pharmacological (**9n**)*	Compound **9n** effectively inhibited tumor growth and angiogenesis,	[132]

			decreasing the cytoplasm translocation of HuR.	
Small intestinal and colon cancer	– Apcmin/– mice, a TG model of intestinal tumorigenesis. – inflammatory colon carcinogenesis protocol - AOM-DSS administration	*Conditional intestine-specific HuR deletor mice: Hurf/f villin-Cre-ERT2 mice*	Intestinal deletion of the HuR gene caused a three-fold decrease in tumor burden in Apcmin/– mice characterized by reduced proliferation and increased apoptosis, and a two-fold decrease in tumor burden in mice subjected to an inflammatory colon carcinogenesis protocol	[59]
Lung adenocarcinoma	– Tumor Xenograft	*Pharmacological (**15s**)*	Mice receiving compound **15s** significantly delayed tumor growth in a concentration-dependent manner	[153]
Glioblastoma (GBM)	– Intracranial injections	*Genetic (shRNA)*	HuR knockdown induces a significant attenuation of tumor growth and invasiveness	[176]
Conventional renal cell carcinoma (CRCC)	– Tumor Xenograft	*Genetic (siRNAs)*	HuR knockdown inhibits human CRCC tumor growth in nude mice *in vivo*.	[177]
Colon carcinoma	– Tumor Xenograft	*Genetic (siRNA & Overexpressing)*	HuR-overexpressing cancer cells produced significantly larger tumors; conversely, cells expressing reduced HuR developed significantly more slowly.	[92]

We decided to build our detailed description of biological and pharmacological studies on HuR and on its modulators by taking inspiration from the Hallmarks and Enabling Characteristics of Cancer [120,121]. A surprisingly long list of molecular targets impacted by HuR modulation through biologicals and/or small molecules, and referred to cancer hallmarks, is graphically depicted in **Figure 6**.

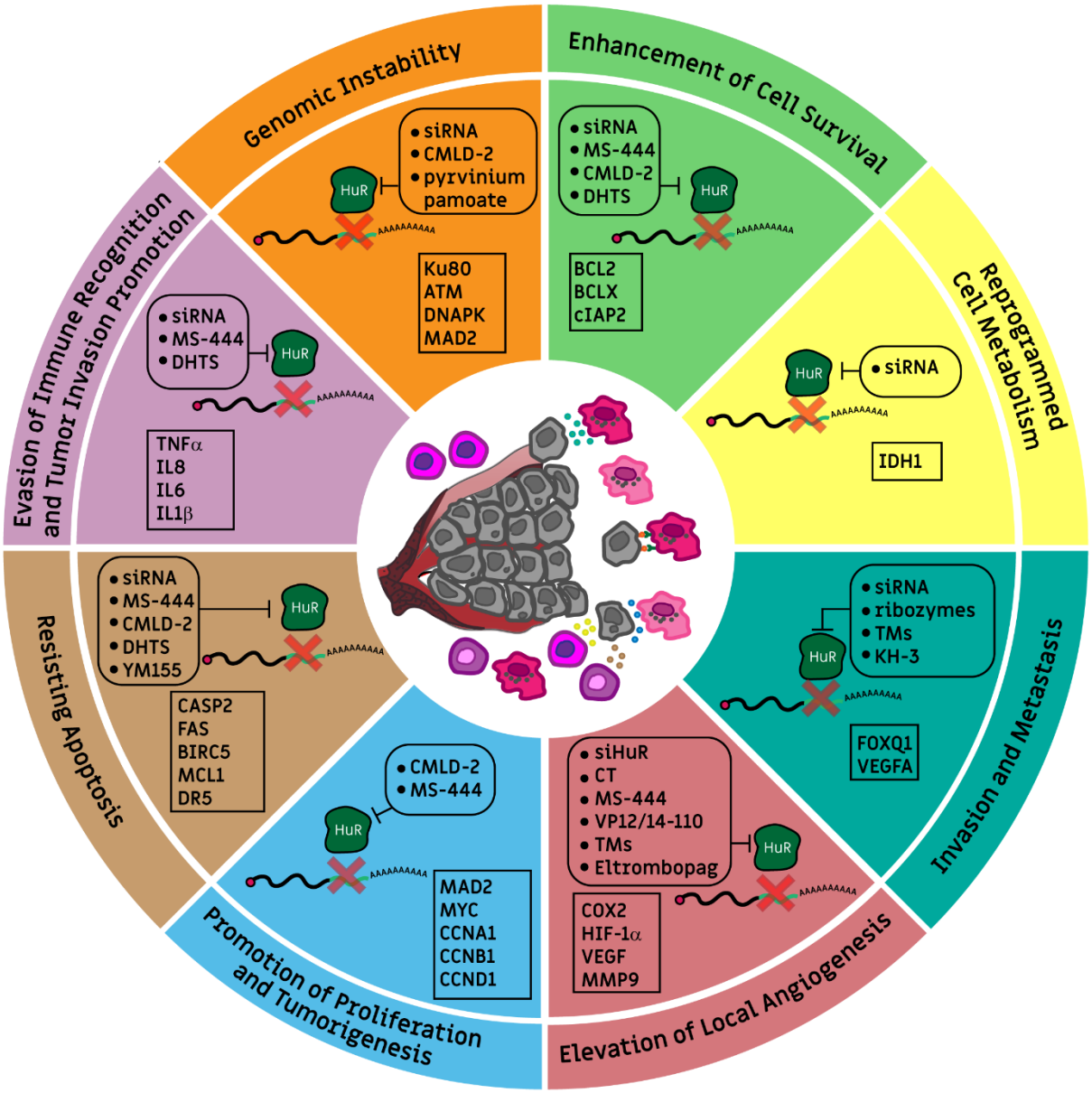


Figure 6. **HuR modulators antagonize the cancer traits.** Each feature is represented by a colored slice in a circle. Each slice contains a list of HuR modulators (e.g. siRNA, small molecules, confined in rounded boxes), and a list of molecular targets affected by HuR inhibition (cellular or *in vivo* models, confined in square boxes). In the middle of the figure, a schematic representation of the tumor progression, characterized by vascuolar tissues (in red) cancer cells (grey) and immune cells (pink and violet) is shown.

4.1 Genomic Instability

Genome instability embraces all the mutations which affect the genome of a cellular lineage, from changes of a single base in the nucleic acid sequence to chromosomal rearrangements or aneuploidy. Malfunctions of the DNA repair machinery are among the most common causes of genomic instability. Due to its consequences, genome instability plays a central role in the development of cancer.

Regulators of centrosome stability and integrity during mitosis, such as growth factors (i.e., EGF and fibroblast bFGF), induce tyrosine phosphorylation on HuR and association with centrosomes. A proper centrosomal

duplication represents a key point during mitosis. HuR dysregulations could therefore lead to strong chromosomal rearrangements [178]. The effect of HuR silencing on genomic instability has been studied through various approaches. HuR siRNA were used on triple negative breast cancer cells to study HuR knockdown in radiosensitization, and the consequent involvement of double strand break (DSB) repair [179]. HuR silencing correlates with a reduced expression of proteins involved in DNA repair, such as Ku80, ataxia telangiectasia mutated (ATM) and DNA-PK. However, the consequent positive or negative effects of HuR on cancer therapies triggering DNA damage is not straightforward. Indeed, HuR silencing combined with radiotherapy appears to improve the radio sensitization of tumor cells by decreasing DNA repair and enhancing radiation-induced ROS production. During DNA damage response (DDR) HuR translocates from the nucleus to the cytoplasm to regulate DNA repair genes upon chemotherapeutic stress, improving cancer cells' resistance to chemotherapy [103,111,180]. However, in pancreatic cancer cells, HuR overexpression favors the expression of deoxycytidine kinase (dCK) and the consequent metabolic activation of gemcitabine; therefore, HuR becomes a key determinant for gemicitabine activity [96]. Similarly, in breast cancer cells, HuR mediates doxorubicin efficacy by post-transcriptionally regulating topoisomerase IIα [181] and its downregulation can lead to doxorubicin resistance in *in vitro* cell models [182]. In ovarian cancer patients, HuR nuclear localization during chemotherapy correlates with a good outcome, while its cytoplasmic localization increases paclitaxel resistance [183].

The action of HuR inhibitors on genomic instability has also been tested. The coumarin-like HuR inhibitor **3ns** (**Table 3**) showed reduction of cell viability in thyroid cancer cells, impairing HuR binding with the microtubule regulator MAD2 [184]. Quinolinium salt **17s** (**Table 4**) has been observed to improve chemotherapy efficacy in urothelial bladder carcinoma (UCB) by affecting HuR's ability to translocate into the cytoplasm [111]. To sum up, any possible beneficial effect of HuR silencing on genomic instability for cancer therapy needs to be carefully evaluated within the cancer context, and the possible combinations with other treatments, due to the multifaceted profile of HuR.

4.2 Enhancement of cell survival

Tumor cells are characterized by enhanced cell survival thanks to resisting recognition and attack by immune cells and apoptosis [185]. HuR is a potential coordinator of this pathologic behavior, by controlling antiapoptotic genes such as *Bcl2, Bcl-xL*, and *cIAP2* [3]. Even if HuR effects on cell survival were most likely both tissue- and cancer type-dependent, silencing HuR expression utilizing HuR siRNA or shRNA significantly reduced tumor growth and inhibited tumor cells' survival *in vitro* and *in vivo* [137,184,186].

The therapeutic potential of targeting HuR for the treatment of ovarian [156] and pancreatic cancer [186] was evaluated in models stably expressing shHuR through a lentiviral gene transducing system. Ovarian OVCAR3 and MiaPaCa2 pancreatic ductal adenocarcinoma cancer cell lines stably expressed DOXO-inducible HuR-targeted shRNAs. The suppression of HuR expression reduced proliferation, anchorage-independent growth, and invasion of ovarian and pancreatic cancer cells *in vitro* [156,186]. MiaPaCa2 cells were also stably transfected with a tetracycline-responsive plasmid to overexpress HuR in response to DOXO treatment, without affecting cell proliferation [186].

Several small molecules showed inhibitory activity on HuR and cell survival, as observed with genetic silencing. Chrysantone-like **1n** (**Table 2**) interferes with the multimerization of HuR and formation of HuR-mRNA complexes, leading to a reduction in cancer cell proliferation and survival in glioblastoma, PDA and colorectal cancer [145,167,187,188]. Treatment with **1n** in inflammatory bowel disease (IBD) and inflammatory colon cancer mice models seems to increase tumor size and invasiveness by counteracting eosinophil recruitment into tumors, probably leading to enhanced cell survival [13]; notably, **1n** did not cause any relevant toxicity in mice models. Compound **3ns** (**Table 3**) disrupts the interaction between HuR and ARE oligomers in pancreatic, colon, lung and thyroid cancer cells [184,189]. At genome wide level, compound **8n** (**Table 2**) dysregulates HuR by switching it to preferential binding to longer, ARE-enriched mRNAs at the 3' UTRs, including mRNAs that encode apoptotic and cell-cycle regulatory proteins. Interestingly, it inhibits

colon cancer cell growth *in vivo* [1], and also reduces or disrupts HuR dimers in the cytoplasmic fraction of glioblastoma cells [145]; therefore, the exact, and possibly multifactorial mechanism of action of **8n** against cancer is still to be fully clarified. Microfilament inhibitors, such as macrocycle **13n** (**Table 2**) and fused pyridone **16s** (**Table 4**), mimic HuR silencing by interfering with HuR-dependent mRNA-stabilization and attenuating cytoplasmic HuR levels in hepatocellular carcinoma cells [137]. Thus, in conclusion, tumor cells may be dependent from the survival signals provided by HuR-regulated oncoproteins, thanks to HuR activity in the cytoplasm and its multimerization. This could be an adaptive survival mechanism of cancer cells under stress conditions, that can be interfered with by the ability of small molecules to inhibit the translocation of HuR in the cytoplasm, or to inhibit its dimerization.

4.3 Reprogrammed cell metabolism

So far, there is no clear evidence about any effect of small molecule HuR inhibitors on the regulation of cell metabolism. However, pancreatic tumor HuR-deficient cells were shown to be less resistant to glucose deprivation when compared to isogenic controls [190]. Changes in the cellular microenvironment, and in particular glucose deprivation, are a specific tumor signature of acute metabolic stress. HuR may activate a pro-survival signaling pathway in response to acute metabolic stress after translocation from the nucleus to the cytoplasm, where it regulates the expression of specific metabolic genes [190]. Three mRNAs that were selected for further validation (*GPI, IDH1*, and *PRPS2*) were observed as downregulated in HuR depleted pancreatic tumor cells.

In a similar manner, HuR was identified as the main regulator of the *IDH1* gene by deleting HuR in PDAC cells deprived of nutrients and performing RNA deep sequencing [191]. PDAC cells reprogrammed their metabolism in response to nutrient deprivation, and caused the development of resistance to chemotherapy, that induced an increase in oxidative stress. Namely, HuR regulation of *IDH1*, the NADPH-generating enzyme, activated a rapid antioxidant response that enhanced PDAC cell survival, thus resulting in a potential therapeutic target for this cancer type.

HuR shows an important role in the regulation of metabolic pathways, and most importantly its inhibition could impair metabolic flexibility, which is the difference in the respiratory exchange ratio in fed and starved states. The conditional KO of HuR in skeletal muscle caused mild obesity in female but not male mice, due to impaired glucose tolerance, fat oxidation and palmitate oxidation. Thus, HuR is involved in the regulation of important skeletal muscle metabolism-related genes [58].

Although there is no evidence about the impact of small molecule HuR inhibition on the regulation of cell metabolism, targeting HuR could also affect cancer cell metabolism, and in particular their pro-survival response to glucose deprivation, as observed in HuR-deficient pancreatic cells.

4.4 Invasion and Metastasis

HuR extensively influences invasion and metastasis, two key aspects of cancer progression. As a representative example for the relevance of HuR in the progression of these hallmarks, HuR was observed to stabilize both *Snail* mRNA, whose protein is involved in the epithelial mesenchymal transition (EMT), and metalloproteases, involved in the degradation of the extracellular matrix [192]. HuR is involved in peroxide-induced cellular migration of mammary carcinoma cells, and HuR KO reduces this migration ability. HuR is overexpressed in pre-neoplastic lesions of invasive breast cancer, such as atypical ductal hyperplasia (ADH) and ductal carcinoma *in situ* (DCIS), when compared to healthy controls, confirming its role in the progression of invasion [193]. In addition, high levels of cytoplasmic HuR significantly correlate with metastasis in bladder cancer [194]. In clinical studies, HuR is also associated with lymph node metastasis in non-small lung carcinoma [109].

Due to its strong influence on invasion and metastatic ability of cancer cells, HuR activity was impaired through different approaches, using anti-HuR siRNA and small molecule HuR inhibitors. MCF7 and MDA-MB-

231 cells were transfected with a ribozyme transgene consisting of hammerhead ribozyme and HuR-specific antisense. The effect of HuR knockdown on human breast cancer cells reduced their growth and invasion and affected the expression of Cyclin D1 and MMP-9 [195]. Anti HuR siRNA conjugated with folate NPs (FNP) were designed and tested on human non-small-cell lung carcinoma (NSCLC), showing impairment in tumor cell migration [154]. *In vitro* scratch assays revealed the impairment of cellular migration also in an ovarian cancer cell model with altered HuR expression (OVCAR5-shHuRc257) [156].

Benzothiophene hydroxamate HuR inhibitor **11s** (**Table 4**) strongly reduced the invasiveness of breast cancer cells by inhibiting HuR-FOXQ1 interaction [150]. FOXQ1 is a transcription factor involved in breast cancer EMT, and its interaction with HuR contributes to cell invasion ability [150]. Finally, recently identified tanshinone mimic HuR inhibitors have been observed, through scratch assays, to remarkably reduce the migration ability of MDA and PANC-1 cells [142]. Tanshinone mimic **6ns** (**Table 3**), in addition to interfering with HuR-RNA interactions, efficiently blocked PANC-1 and MDA-MB-231 cell migration [142]. Tongue carcinoma HSC-3 and SAS cell lines were treated with polysulfonate **5s** (**Table 4**), and their ability to grow without adherence to the extracellular matrix and to neighbouring cells was investigated. Upon treatment with **5s**, attenuated motile and invasive properties were detected through *in vitro* wound healing and Matrigel invasion assays; the same happened in HuR knockdown HSC-3 cells [147].

Recently the role of HuR in malignant peripheral nerve sheath tumors (MPNST) was examined by us, finding a striking dependence of these cancer cells on HuR for their proliferation, survival and dissemination [171]. HuR was overexpressed in these highly aggressive sarcomas that originate in the peripheral nervous system, regulating the expression of numerous cancer-associated transcripts in human MPNST samples. A constitutive genetic inhibition of HuR in MPNST cells was sufficient to completely prevent tumor formation using xenograft models, whereas inducible ablation of HuR in pre-formed tumors led to their shrinkage. Remarkably, we found that HuR inhibition also prevented the formation and growth of metastatic nodules in lungs using a surrogate model of lung metastasis. This was particularly relevant since MPNSTs have a high metastatic potential, and up to 50% of patients develop metastatic disease, usually to the lung, which worsens the 5-year survival rates of patients [171]. Notably, pharmacological inhibition of HuR by chrysantone-like **1n** (**Table 2**) could reduce tumor growth in xenograft models as well as metastatic growth in lungs, highlighting the relevance of HuR as a potential therapeutic target for MPNSTs. Finally, using experimental and computational approaches, it was ascertained that HuR exerts such profound effects on these cancer cells because of its capacity to simultaneously regulate multiple essential oncogenic pathways, which converged on key transcriptional networks [171].

4.5 Elevation of local Angiogenesis

Tumor cells can promote vascular growth or angiogenesis through different mechanisms. Angiogenesis subsequently contributes to tumor growth and helps cancer cells enter the peripheral circulation [196].

Many clinical investigations have shown that HuR plays a critical role in promoting angiogenesis. In fact, cytoplasmic HuR accumulation stabilizes angiogenic factors, such as *VEGF-A*, interleukin-8 (*IL-8*), *HIF-α*, and *COX-2* [196-198]. Moreover, HuR acts by augmenting the hypoxic or inflammatory signal and promoting the proliferation and migration of endothelial cells [196,197].

In mesangial cells (hMCs) the vasoconstricting peptide angiotensin II (AngII) increases the capability of HuR to bind and stabilize *COX-2* mRNA. *COX-2* mRNA is a specific target of cytoplasmic HuR, and AngII stimulation is necessary for their interaction. Indeed, attenuated HuR expression mediated by siRNA affected the expression and function of COX-2 in cells after AngII treatment [118].

HIF-1 is a master regulator of tumor neovascularization. The heat shock transcription factor 1 (Hsf1) is overexpressed in a variety of tumors and plays a critical role in tumor progression by regulating numerous genes, including HuR. Upon Hsf1 knockdown, HuR levels decreased by about 70% in MCF7 and Hs578 cells, MCF7 xenografts and in Hsf1-KO mice, consequently affecting HIF-1 expression [199].

In myeloid cells HuR stabilizes different genes, including angiogenic regulators bearing ARE or U-rich sequences at their 3' UTR region. *VEGF-A* is a key angiogenic growth factor directly regulated by HuR and miR-200b in an antagonistic manner. After myeloid-specific deletion of HuR in mice (Elavl1Mø KO), bone marrow-derived macrophages presented a robust VEGF-A [68], VEGF and MMP-9 [200] downregulation. Furthermore, tumor slices from Lewis lung carcinoma (LLC) cells injected into Elavl1Mø KO mice displayed reduced vascular density and permeability, sprouting and branching, and contained vessels with dilated lumens. The same phenotype also occurred in zebrafish embryos through the injection of morpholino (MO)-targeted HuR, resulting in severe defects in subintestinal vein (SIV) vascular development [68]. Therefore, macrophages and monocytes are the primary sources of the inflammatory angiogenic process, and macrophage-specific HuR inhibition impairs the posttranscriptional mechanisms of angiogenic regulator genes [68,200].

Among HuR small molecule inhibitors, natural tanshinone **9n** (**Table 2**) is endowed with anti-tumor and anti-angiogenic activity both *in vitro* and *in vivo*. TNFα, besides being an important mediator of inflammation, also has proangiogenic effects. Compound **9n** has been observed to markedly reduce lipopolysaccharide (LPS)-induced *TNFα* mRNA expression in a dose dependent manner by increasing nuclear HuR localization, thus impairing HuR cytoplasmic translocation and, consequently, *TNFα* mRNA stabilization [132].

Tanshinone mimic **6ns** (**Table 3**) was tested for its capability to interfere with HuR-RNA binding in MCF7 cells. Compound **6ns** treatment revealed a decrease of *VEGF* mRNA copies and a reduction in its expression levels [142].

As previously mentioned, HuR interacts with *COX-2* mRNA, stabilizing its expression [118,196]. Moreover, chrysantone-like **1n** (**Table 2**) was found to interfere with HuR cytoplasmic localization and to impair its binding activity [123]. In Cajal-Retzius (CR) cells and in mice bearing HCT116 and HCA7 cell xenografts, **1n** inhibited HuR and decreased COX-2 expression in a dose dependent manner. Finally, HuR targeting by **1n** altered COX-2 expression and decreased angiogenesis both *in vitro* and *in vivo* [167].

HuR is associated with diabetic retinopathy (DR) [78], and is abundant in human retinal endothelial cells (HRECs) [151]. The aetiology of diabetic retinopathy is the onset of a diabetic macular oedema that causes retinal detachment and visual loss. Indoles **12s** and **13s** (**Table 4**) were selected as HuR inhibitors, presenting anti-inflammatory and anti-angiogenic properties. On the basis of MD experiments, these small molecules blocked HuR functions by competing with its mRNA binding site and modifying the structure of the HuR binding cleft. In addition to HuR impairment, *TNFα* and *VEGFA* expression were significantly decreased in HRECs treated with **12s** and **13s**, in high glucose media cultured conditions [151].

A matrigel tubule formation assay revealed Indoline sulfonamide **15s** (**Table 4**), and indoles **12s** and **13s** (**Table 4**) [151] as antiangiogenic HuR inhibitors, because their effects inhibited VEGF-induced migration and tube formation ability of human umbilical vein endothelial cells (HUVEC) [153] and of HRECs [151].

Eltrombopag is an approved oral drug used in clinics for the treatment of thrombocytopenia, severe aplastic anaemia as an agonist of the thrombopoietin receptor [201]. It is effective in disrupting the interaction between HuR and the ARE sequence of *VEGFA* mRNA. Furthermore, eltrombopag is a good anti-angiogenic drug, as demonstrated by its *in vivo* reduction of microvessels in tumor tissue [202].

4.6 Promotion of Cell Proliferation and Tumorigenesis

The CRISPR/Cas9 technology was used to define the role of HuR in PDA and colorectal cancer cells (CRCs) [175]. MiaPaca2, Hs766T, HCT116 and HCA7 cells lacking HuR displayed attenuated growth compared to the control and the HuR overexpression in HuR-null MiaPaCa2 cells restored a tumor growth phenotype. According to HuR function in promoting tumor cell growth, MIA.HuR-KO$^{(-/-)}$ and HsT.HuR-KO$^{(-/-)}$ cells were unable to grow into 3D cultures. Namely, cells with CRISPR/Cas9-mediated deletion of HuR only formed single cell sheets, and were unable to form substantial spheroids even after four weeks of plating, while positive

control cells easily formed spheroids in 3D cultures within a passage, continuing to grow and doubling in size [175].

As mentioned earlier, HuR was knocked down in four MPNST cell lines by lentiviral delivery of shRNAs [171]. HuR is highly aggressive in sarcomas originated from Schwann cells, and its downregulation produced a dramatic decrease of percentage in BrdU positive cells and in the ability of single cells to form colonies. After HuR silencing in MPNST cells, an analysis of β-galactosidase staining revealed a marked increase in cellular senescence. Therefore, HuR genetic inhibition has cytotoxic and cytostatic effects on tumor growth [171].

In normal intestinal epithelium, HuR has a predominant nuclear localization, while in adenocarcinomas it is mostly relocalized in the cytoplasm, where it is aberrantly regulating the stability of key pro-oncogenes responsible for cell proliferation, such as *cyclinA*, *cyclinB1* and *cFos*.

HuR-siRNA encapsulated in lipid NPs was delivered in human melanoma cell lines and by the Trypan blue exclusion assay method, cellular viability was assessed. HuR-NP treatment in combination with the kinase inhibitor UO126, generated a significant inhibitory effect in a human melanoma cell line overexpressing the microphthalmia-associated transcription factor (MITF) [203].

Several colorectal cancer cell lines overexpressing HuR (HCT116, HCA-7, RKO, HT-29, and SW480 cells) showed growth inhibition after treatment with chrysantone-like **1n** (**Table 2**) at different concentrations (1–100 μM), whereas only a weak effect was observed on normal epithelial cells [167]. Further studies highlighted the impact of **1n** on HuR-mediated colorectal tumorigenesis, discriminating among cells from different patient subtypes. Targeting HuR was used to prevent development of cancer in high-risk groups, such as those with familial adenomatous polyposis (FAP) or IBD. The level of HuR expression and localization was different in each condition, and to better address the question of how HuR expression is involved in each stage of tumor progression, azoxymethane/dextran sodium sulfate (AOM/DSS) and adenomatous polyposis coli $(APC)^{Min}$ mice models were used [13]. The *c-myc* gene, a known HuR target, was chosen as a biomarker to verify the functional inhibition of HuR upon **1n** treatment in intestinal tissue, due to its involvement in colorectal cancer development. As expected, both *c-myc* expression and the rate of tumorigenesis were reduced, as confirmed by the decrease of Ki67 positive cells among small intestinal crypt cells derived from the aforementioned mouse models [13].

Chrysantone-like **1n** was also tested against malignant glioma cells [188]. Treatment with **1n** (20 μM and 30 μM) strongly impaired the invasion rate of JX12 cells, and their $CD33^+$ subpopulation was even more sensible to **1n**, as shown by an increased inhibition of their invasivity at 10 μM. To assess the functional phenotype of brain tumor initiating cells, the formation of neurospheres was quantified by a limiting dilution assay. At 2.5 μM, **1n** attenuated the initiating stem cell frequency in forming neurospheres [188].

Compound **3ns** (**Table 3**) was tested for its potential antitumor activity in human lung cancer cells [189]. Several NSCLC cell lines (H1299, A549, HCC827, H1975) were treated for 24 and 48 hours with **3ns** (20 μM and 30 μM), and a consistent, dose dependent inhibition of tumor proliferation and induction of G1 cell cycle arrest was consistently observed. Conversely, limited or no effects were observed on normal epithelial cells.

The antitumor efficacy of **3ns** was evaluated in four thyroid cancer cell lines (SW1736, 8505 C, BCPAP and K1) [184]. Through a scratch assay, treatment with **3ns** (35 μM) reduced their invasion ability and impaired colony formation using an anchorage-independent assay in soft agar. This effect on colony formation and proliferation was ascribed to downregulation of mitochondrial associated protein MAD2, a HuR target overexpressed in thyroid cancer cells. MAD2 is involved in the regulation of cell division, in particular in the metaphase to anaphase transition, and treatment with **3ns** downregulated the expression of this gene *via* HuR inhibition, thus blocking tumorigenesis. This was confirmed by siRNA silencing of MAD2 and through a rescue experiment in which MAD2 was overexpressed after treatment with **3ns** [184].

Compound **3ns** was also tested in combination with the YAP inhibitor verteporfin (VP), and the CA3 and CDK4/6 inhibitor abemaciclib in PDAC cells. The combination of abemaciclib with **3ns** decreased the number

of PDAC colonies compared with both monotherapies, most likely due to their shared regulatory role of the cyclinD1 pathway [204].

Finally, HCT116 and HCT116 HuR-KO colorectal carcinoma cells were grown under anchorage- and serum-independent conditions. Cells lacking HuR were unable to form tumor spheroids and, after treatment with natural tanshinone **8n** (**Table 2**), HCT116 spheroids were significantly reduced [175].

According to these evidence, HuR modulators seem to be able to decrease cancer cell tumorigenesis at early stages, as evaluated by colony formation or through spheroids assays. Nevertheless, further data are needed to fully validate this hypothesis.

4.7 Resisting Apoptosis

Resistance to apoptosis is a key event in tumor development. HuR finely regulates the balance between cell survival and cell death by caspase-mediated apoptosis in response to lethal stress. Indeed, in normal conditions, HuR promotes cell survival by stabilizing and increasing the translation of mRNAs coding for antiapoptotic factors [18], whereas under such lethal stress HuR promotes apoptosis by increasing the expression of proapoptotic proteins [205].

Several studies have demonstrated that inhibition of HuR, by using either genetic approaches (gene deletion and siRNA) or small molecule inhibitors, promotes apoptosis [59,66,67]. For example, HuR acts in B cells as a key factor for a proper metabolic switch and cell growth during B cell maturation. In a B-cell precursor lineage, HuR deletion led to the induction of apoptosis [66,67]. Moreover, annexin V staining of siHuR MiaPaCa2 cells exposed to death receptor 5 (DR5) showed an increase in the apoptotic signal [206].

Among several small molecule HuR inhibitors, natural tanshinone **8n** (**Table 2**) showed anti-tumor effects in different human breast [207] and colon cancer cell lines [126] by inducing apoptosis. Induction of apoptosis was also observed with compound **1n** (**Table 2**) in colon cancer [167], in glioblastoma cells [188] and in MPNST cells [171]. Finally, apoptosis induction was observed in thyroid cancer cells treated with coumarin-like **3ns** (**Table 3**) [184].

Interestingly, microtubule inhibitor **15s** (**Table 4**) led to a concentration-dependent increment of TUNEL-positive A549 cells population, by inhibiting the expression of *HIF-1α* through reduced translocation of HuR to the cytoplasm [153]. Finally, in UCB, pyrvinium pamoate **17s** (**Table 4**), in combination with genotoxic agents, increased apoptosis by triggering DNA damage in 5637 cells [111].

4.8 Evasion of Immune Recognition and Tumor invasion promotion

Cancer onset and progression are strongly determined by tumor capacity of evading the immune response, with subsequent promotion of inflammation [121]. The tumor microenvironment is preserved by either inflammatory, stroma and tumor cells, often exploiting signalling molecules, such as cytokines, chemokines, to promote invasion, migration and metastasis [91]. Considering that ≈90% of mRNAs coding cytokines and chemokines contain repeated ARE sequences in their 3' UTR structure, HuR probably regulates these unstable transcripts in competition with stabilizing and destabilizing trans-factors, such as other RBPs like tristetraprolin (TTP) and T cell-restricted intracellular antigen 1 (TIA1). This gives rise to a rapid degradation and turnover of these mRNAs in response to changes in cells' or tissues' environments. Consequently, HuR plays an important role in innate, adaptive immunity and inflammatory pathways, in physiological and pathological condition [67,69,169,208,209]. Indeed, cancer-driven immune escape still represents an issue for anticancer therapy [210]. In this context, the role of HuR remains controversial, as indeed its deletion or inhibition leads to different outcomes. Co-culture of MCF-7 tumor-spheroids with primary human $CD14^+$ monocytes attracted and retained macrophages in the 3D tumor spheroids [211]. Tumor progression is strongly affected by infiltrating immune cells, and environmental changes may affect the activity of HuR. In fact, when HuR was knocked down by shRNAs, the expression level of C-C motif chemokine ligand 5 (CCL5) increased concomitantly to the infiltration of macrophages in the tumor spheroid. Moreover, a model of 3D

breast cancer showed a decrease in size upon HuR depletion, supporting a role for HuR in enhancing cancer proliferation [211].

Recently, in glioblastoma, HuR deletion has been associated with a decrease of tumor growth and proliferation, as in particular a reduction in the number of tumor associated macrophages (TAMs), showing M1-like increased polarization. In fact, HuR KO mice were characterized by substantial changes for key parameters determining cancer progression, such as migratory and chemoattractive capabilities, with substantial rearrangements of chemokine and cytokine production, modifying microenvironment conditions and reducing tumor growth [72]. This demonstrated the importance of HuR as a valuable target for therapy in this field. To this purpose, chrysantone-like **1n** (**Table 2**), whose activity has been previously described to downregulate different mRNA cytokines in cellular contexts such as GBM xeno-lines [72,123,167], has been tested *in vivo* in AOM/DSS mice as earlier described. However, in this model the treatment with **1n** induced an attenuation of eosinophils associated with tumor and a decrease of pro-inflammatory molecules causing an exacerbation of tumor development and invasiveness, therefore worseninig the outcome of the treatment. On the other side, using **1n** in an APC^{Min} model of FAP and colon cancer partially ameliorated their carcinogenic conditions [13]. These results are in line with genetic models in which the ablation of HuR in the colon tissue protects from tumor burden [59], while myeloid cell–specific deletion of HuR exacerbated chemically induced colitis [64].

In other words, small molecule HuR inhibitors are validated agents for immune-restoring therapy, although the complexity of the HuR regulatory functions presented so far must be kept in mind.

In conclusion, after having substantiated the claim of HuR influencing the whole Hallmarks of Cancer panel, we must also say that its emerging role in inflammatory processes and diseases is of significant relevance as well, also in terms of its pharmacological-small molecule targeting. Thus, in the next Chapter we provide a somewhat detailed overview of this fast growing field, commenting upon multiple and sometimes contrasting opinions.

5 A controversial role for HuR in immunity

5.1 HuR in immunity

HuR determines the development of a pro-inflammatory response to agents such as LPS, since it prevents degradation of toll-like receptor 4 (TRL4) mRNA, giving rise to an upregulation of inflammation processes in models of vascular inflammation and atherogenesis [212]. Furthermore, HuR stabilizes several inducible transcripts, including *interferon-γ* (IFN-γ)*, TNF-α, IL-6; IL-8, IL-3, IL-1β* and the *urokinase-type plasminogen activator* (uPA), that are key mediators of the inflammatory and immune responses [166,213-219]. CX3CL1/fractalkine is a chemokine ligand specific for natural killer (NK) cells and monocytes maturation It contains ARE elements in its 3' UTR and can be post-transcriptionally regulated by HuR. This suggests that HuR can modulate the development of two among the major players in the innate immunity system [220]. Recently, sequential photoactivatable ribonucleoside-enhanced crosslinking and immunoprecipitation (PAR-CLIP) experiments on normal and HuR KO bone-marrow derived macrophages (BMDMs) showed that the expression of lineage specific genes involved in vascular development and angiogenesis is post-transcriptionally determined by an intricate interplay between HuR and various mRNAs [221]. Moreover, similar PAR--CLIP experiments were carried out in primary macrophages under LPS stimuli, proving the existence of a complex post-transcriptional landscape driven by balancing activities of several RBPs, in particular HuR and TTP [222]. In fact, groups of transcripts bound and modulated exclusively by TTP or HuR were identified; another group, including mRNAs for *TNFα* or *CXCL2*, can interact and bind simultaneously to both TTP and HuR, establishing a competition between stabilizing and destabilizing effects that is reflected in the insurgence of a vulnerable and tightly regulated post-transcriptional pattern influenced by both TTP and HuR [222,223]. Moreover, another molecular mechanism has been proposed to explain the HuR-mediated increase of different cytokines and chemokines (e.g. CXCL2) in macrophages, due to stabilization of their mRNAs. Such mechanism entails a PTM carried out by poly(ADP-ribose)polymerase 1 (PARP1) to HuR, known as a PAR-ylation. This modification of HuR influences its shuttling to the cytoplasm, and increases its interaction with different pro-inflammatory mRNAs [120]. Moreover, in mammalian macrophages, HuR behaves as a mRNAs sponge and derepresses inflammatory agents by counteracting the induction of anti-inflammatory response driven by infection of pathogens, such as *Leishmania donovani* [224].

Despite these evidences, the role of HuR in immunity remains controversial. For example, HuR deficiency in macrophages derived from mice lacking HuR in the myeloid-lineage cells, has been linked with an increase of pro-inflammatory cytokines. HuR KO cells presented an increased CCR2-mediated chemotaxis and enhancements in the expression of inflammatory mRNAs (including *Tnf, Tgfb, Il10, Ccr2*, and *Ccl2*), due to mis-regulation in their translational and stability levels. This caused an increased susceptibility of these mice to colitis-associated cancer [69]. Furthermore, experiments performed in a co-culture model of primary human macrophages and MCF7 breast cancer cell lines, demonstrated that HuR suppresses the production of the leukocyte attracting chemokine *CCL5*, reducing the infiltration of pathogenic macrophages in the tumor site, and preventing the exacerbation of inflammation processes [211]. This confirms for HuR a regulatory role for both pro-inflammatory and anti-inflammatory agents, with contrasting, strongly cell lineage-dependent effects whose overall results are difficult to be precisely predicted and determined. Accordingly, exploiting intestinal inflammation mice models in which HuR has been deleted in Intestinal epithelial cells (IECs) and in myeloid-derived immune compartments, HuR KO increased in cell death, confirming that HuR has a role in the maintenance of the intestinal barrier integrity and homeostasis. In parallel, though, the lack of HuR in myeloid lineages fueled the inflammation process, thus exacerbating a pathological condition [225]. Conversely, a conditional HuR KO mice model confirmed that HuR is pivotal for the maintenance of hematopoietic stem cells during hematopoiesis, but also its essential role for B cell development and a homeostatic balance between T and B cells [46,67,71,226]. In particular, using a Cre-LoxP system, HuR deletion in thymocyte development resulted in a loss of peripheral T cells, highlighting its

importance in controlling thymocyte maturation and trafficking [71]. Nevertheless, in T cells HuR modulates maturation and polarization of Th2 and Th17 cell lineages, enhancing the stability of IL-2 and IL-17 by binding to their 3' UTRs [64,65].

5.2 HuR targeting in inflammatory diseases

In a pathologic context, HuR deletion in distal lung epithelium decreased neutrophilia and pulmonary inflammation levels induced by IL-17, through increasing mRNA decay of chemokines such as CXCL1 and CXCL5 [227]. Th17 cells are the major mediators of the generation of inflammatory infiltrations in the central nervous system (CNS) during neuroinflammation. Functional studies and HuR conditional KO in $CD4^+$ T cells of a mouse strain, determined that in an experimental autoimmune encephalomyelitis (EAE) model, HuR binds directly to the 3' UTR of the C-C chemokine receptor 6 (CCR6). CCR6 is a surface receptor on Th17 cells, and is responsive to migration stimuli exerted by the release of chemokine ligand 20 (CCL20), which is constitutively secreted by choroid-plexus epithelial cells at the site of inflammation. A complete ablation of HuR significantly decreases the production of CCR6, thus lowering the number of migrating Th17 cells, ameliorating the pathogenic neuroinflammation processes in the CNS, and the EAE outcome in mice [228].

Consequently, there is emerging evidence concerning the targeting of HuR with small molecule inhibitors in order to ameliorate disease onset and reduce autoimmune inflammation [63]. In fact, administration of natural tanshinone **8n** (**Table 2**) in EAE mice models led to a reduction of the aggressiveness, and to a delayed onset of the ALS-like pathology. Compound **8n**, injected at 10 mg/kg every 48 hours from day 5 to day 15 of disease induction, caused a decreasing number of infiltrates in CNS and lower demyelination. Furthermore, through flow cytometry assays the number of $CD4^+$ T cells producing IFN-γ and IL17 in mice spleen was significantly reduced, as was the number of $CD11b^+$ myeloid cells present in their spinal cords. Moreover, signals of pro-inflammatory mRNAs (e.g. IL17, IFN-γ) and adhesion factors (Vla-4) were lowered in CNS after treatment with **8n**. Nevertheless, no alteration of the balance between CD4, CD8 T cells, B cells and macrophages was observed in the spleen of treated mice compared with control, suggesting that compound **8n** did not cause systemic toxicity *in vivo* [63]. Lastly, a HuR targeting strategy has been applied to reduce inflammatory contributions during the progression of chronic kidney disease (CKD), that is characterized by sustained inflammation and fibrosis development, leading to final end-stage renal disease (ESRD). In particular, benzothiophene hydroxamate HuR inhibitor **11s** (**Table 4**) was tested at 50 mg/kg daily for 5 days in an experimental anti-Thy 1.1 nephritis rat model, characterized by high level of glomerular HuR. Injections of **11s** showed no side effects (e.g. peritonitis insurgence), but caused the lowering of urea levels in the serum and of protein content in urines when compared with untreated rats. Moreover, the analysis of histological renal sections staining showed a compound **11s**-dependent reduction of glomerulosclerosis, followed by a reduction in mRNA expression and protein production of profibrotic markers such as TGFβ1, plasminogen activation inhibitor 1 (PAI-1) and fibronectin. Finally, **11s** treatments decreased the number of monocytes and macrophages invading glomerulal sites, and stimulated M2 macrophage activation and renal production of NF-κB-p65, that promotes HuR transcription during tumor development [229] and induces glomerular HuR transcription and shuttling to the cytoplasm [230].

6. Conclusion

In this review, we focussed mostly on the therapeutical potential of HuR inhibition in cancer. Considering that overexpression of HuR, or its mis-localization along with accumulation either in the nucleus or in the cytoplasm correlates with tumor development and progression, HuR modulators – in particular small molecule inhibitors - have been repeatedly used in multiple *in vitro* and *in vivo* models, providing promising preliminary results against cancerogenic traits also known as Hallmarks of Cancer (**Figure 6**). Thus, they represent a suitable and prospective option in cancer therapy.

Although their effects in multiple cancer cellular contexts were described, their *in vivo* evaluation remains limited, and should significantly increase in the next years. Being HuR ubiquitously expressed, and regulating a variety of different key mRNAs, the development of suitable, HuR-centered *in vivo* models is still controversial. Indeed, HuR KO in complex systems usually leads to the exacerbation of the disease [224], and similar results were obtained when treating with chrysantone-like **1n** (**Table 2**) in similar models [13], suggesting that targeting the pleiotropic functions of HuR could arise some undesired effects.

Another concern, mentioned in **Paragraph 1.5**, regards the specificity of these compounds versus other members of the ELAVL family of proteins, with shared structural properties with respect to HuR. Indeed, available data in this regard are not yet exhaustive for HuR inhibitors tested either in *vitro* or *in vivo*. Nevertheless, the scenario described in this review is strongly supporting the rational design, synthesis and structural optimization of synthetic HuR inhibitors, to overcome the limitations of current, mostly naturally occurring HuR modulators in terms of bioavailability (e.g. solubility) and specificity. When properly optimized for HuR selectivity, safety and *in vivo* efficacy, such small molecule HuR inhibitors could on one side minimize the possible insurgence of side effects, and on the other side become potent and bioavailable enough to foresee the identification of one or more HuR-targeted clinical candidate as anticancer agents in the next years.

7. Acknowledgments

This work was supported by Associazione Italiana Ricerca sul Cancro (IG grant # 21548 to A.P.), by AEI/FEDER, EU (RTI2018-097503-B-I00 and SAF2015-62588-ERC to A.W.), Severo Ochoa Excellence Accreditation (SEV-2016-0644 to A.W.), Fundación BBVA (Becas Leonardo to A.W. and M.V.R.), Gilead Sciences International Research Scholars Program in Liver Disease to M.V.R, the European Community's H2020 Framework Program ERC Consolidator Grant (865157-MYERIBO to A.W.)**,** MIUR-PRIN 2017 (Grant PHRC8X to L.M. and V.L.P.) and Regione Campania-POR Campania FESR 2014/2020 (Project N. B61G18000470007 to L.M. and V.L.P.).

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