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Synthesis and Preliminary Evaluation of Tanshinone Mimic Conjugates for Mechanism of Action Studies

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Human antigen R (HuR) is an RNA binding protein (RBP) belonging to the ELAV (Embryonic Lethal Abnormal Vision) family, which stabilizes mRNAs and regulates the expression of multiple genes. Its altered expression or localization is related to pathological features such as cancer or inflammation. Dihydrotanshinone I (DHTS I) is a naturally occurring, tetracyclic ortho-quinone inhibitor of the HuR-mRNA interaction. Our earlier efforts led to the identification of a synthetic Tanshinone Mimic (TM) **2** with improved affinity for HuR. Here we report five new TM probes **3–5** bearing a detection-promoting moiety (either photo affinity probe - PAP or biotin) as a para-substituent on the phenyl-sulphonamide for mechanism of action (MoA) studies. Biological and biochemical assays were used to characterize the novel TM conjugates **3–5**. They showed similar toxic activity in HuR-expressing triple-negative breast cancer MDA-MB-231 cells, with micromolar CC_{50}s. REMSAs revealed that photoactivatable groups (**4a** and **4b**), but not biotin (**5a** and **5b**), prevented conjugates' ability to disrupt rHuR-RNA complexes. Further biochemical studies confirmed that biotinylated probes, in particular **5a**, can be used to isolate rM1 M2 from solutions, taking advantage of streptavidin-coated magnetic beads, thus being the most promising HuR inhibitor to be used for further MoA studies in cell lysates.

Introduction

Human antigen R (HuR)[1] is an RNA-binding protein (RBP) member of the embryonic lethal abnormal vision (ELAV) family. As HuR is involved in the regulation of post-transcriptional gene expression for several AU-rich mRNAs encoding for relevant proteins, its altered expression or localization could lead to multiple pathologic phenotypes, including cancer and inflammation.[2–4]

Small molecule HuR inhibitors include natural and synthetic compounds that were discovered either from high throughput screenings (HTS) or from rational drug design.[3] Among natural ones, Tanshinones – and Dihydrotanshinone I **1** (DHTS I, Figure 1) in particular – were identified as hit compounds by our research group; using them as a model, we developed a fully synthetic class of bicyclic orthoquinone indole-sulfonamide small molecules, called Tanshinone Mimics (TMs).[5–7] Among them, TM **2** showed a higher ability to disrupt HuR-RNA interaction than natural Tanshinones and was selected as an early lead.[7]

Starting from early lead **2**, a library of synthetic analogs of Tanshinones has been expanded,[8,9] leading to new derivatives endowed with improved affinity and solubility that were thoroughly characterized *in vitro*, providing extensive structure-activity relationships (SARs), and *in vivo*,[8] validating the relevance of TMs as HuR-targeted treatments against cancer and inflammation.

Structural details on the interaction mode of TMs with HuR,[7–9] elucidated through a combined approach using NMR

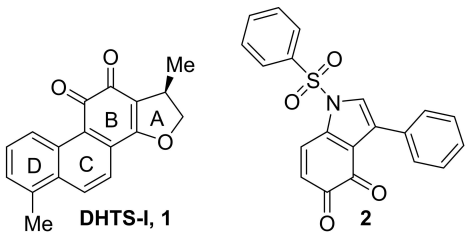

Figure 1. Structures of natural DHTS I **1**, and TM lead **2**.

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and MD simulations, coherently indicate that these small molecules bind to the region between the interconnecting linker and the β-platform of both RNA recognition motifs (RRMs) RRM1 and RRM2 in HuR, and that binding to TMs alters their conformational freedom and reciprocal reorientation. This stabilizes an unproductive, "closed" conformation that hampers target mRNA binding.[7]

Nevertheless, further elucidation of the mechanism of action (MoA) of TMs, as well as information on target selectivity over other RBPs would be crucial in the rational design and synthesis of new HuR ligands.[10] Thus, to acquire information on the selectivity and MoA of TMs, we aimed to introduce additional affinity chromatography or photoactivatable groups on TMs (i.e., biotin, azide, or trifluoromethylphenyl diazirine).[10,11]

The use of biotinylated probes is among the most common techniques for target identification studies. Biotin (vitamin B7) binds the proteins avidin and streptavidin with high affinity (K_d from 10^{-14} to 10^{-15} mol/L) and specificity, establishing one of the strongest noncovalent protein-ligand interactions known so far. Thus, biotinylated probes complexed with their molecular target(s) after incubation in a cell lysate can be isolated from the sample by exploiting this highly stable noncovalent interaction, using avidin- or streptavidin-decorated supports (pull-down assay).[12]

Conversely, photoaffinity labeling (PAL) covalently links hits to their target proteins. Arylazides and diazirines are linked to hits in so-called photoaffinity probes (PAPs). PAPs are stable compounds that can be selectively photo-activated to yield highly reactive intermediates (singlet nitrenes from azides at <300 nm, singlet carbenes from diazirines at 350–380 nm).[13,14] Such intermediates immediately react with neighbouring residues on the target protein, allowing its identification through MS analysis.

Thus, following computational suggestions and chemical feasibility assessments, we tackled the synthesis of ortho-quinonic TM chemical probes bearing a probe moiety as a para-substituent on the phenyl-sulphonamide. The structure of PAPs **3**, **4a**,**b** and biotinylated probes **5a**,**b** is reported in Figure 2.

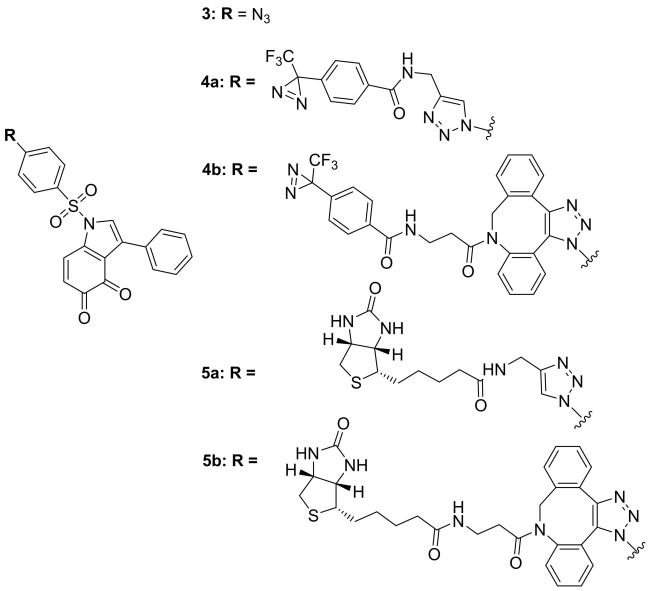


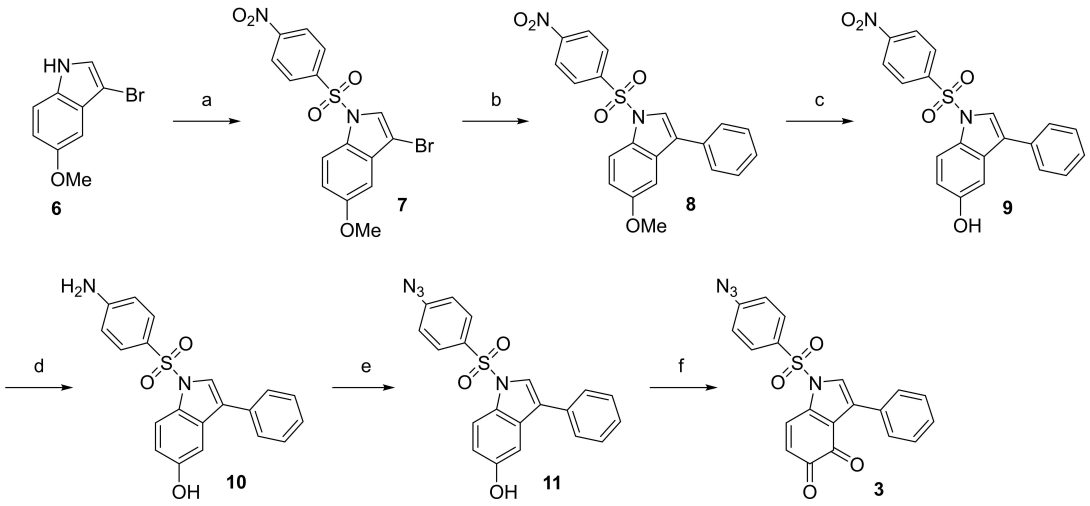
Figure 2. Structure of target TM-based chemical probes **3**–**5**.

Results and Discussion

Key Intermediate-PAP TM-Phenylazide 3

Compound **3** (Figure 3) may either be used per se as PAP, or may act as a key intermediate for the synthesis of target compounds **4**–**5** through a click reaction with suitable alkynyl-diazirine or -biotin constructs.

For the synthesis of phenylazide **3** (Scheme 1), a precedented procedure[7] was optimized, taking into consideration



Scheme 1. Synthesis of phenylazide TM **3**. a) p-NO_2-$PhSO_2Cl$, 50% KOH(aq), nBu_4HSO_4, DCM, r.t., 30′, 93%; b) $PhB(OH)_2$, Pd-tetrakis, 2 M K_2CO_3, 4:1 dry DME/EtOH, N_2, 7 h, 100 °C, then 12 h, r.t., 60%; c) 1 M BBr_3 in dry DCM, dry DCM, −78 °C to 10 °C, N_2, 82%; d) $SnCl_2 \cdot 2H_2O$, 1:1 THF/MeOH, 80 °C, 2 h, >99%; e) $NaNO_2$(aq), then NaN_3(aq), 1:1 HCl/AcOH, 0 °C to r.t., 1 h, >99%; f) IBX, DMF, r.t., 2 h, 60%.

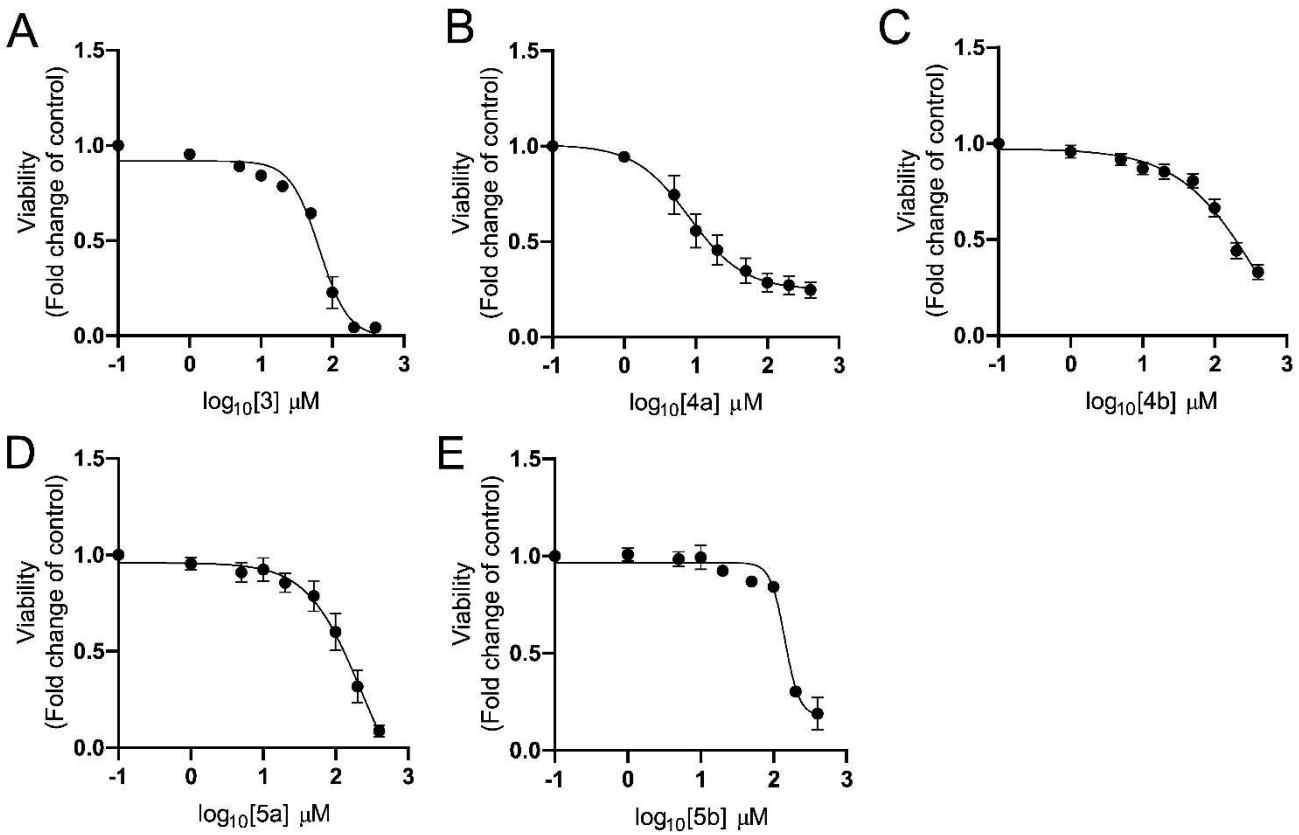


Figure 3. Dose-response viability curves of MDA cells treated with TM probes. MDA-MB-231 cells treated with concentration up to 500 μM of **3**, **4a**, **4b**, **5a** and **5b**. Viability assessed with the Ozblue viability kit.

the orthogonal reactivity and stability of each chemical moiety, and in particular of the ortho-quinone and phenylazide groups.

Intermediate bromoindole **6**[7] was reacted with p-NO_2-phenylsulfonyl chloride in the presence of a strong base (KOH) and nBu_4HSO_4 as phase transfer catalyst (step a, Scheme 1), providing intermediate **7** in good yields after multiple crystallizations from n-hexane and DCM. Suzuki coupling with commercial phenyl-boronic acid, palladium(0)tetrakis as catalyst and 2 M K_2CO_3 as base allowed the synthesis of 1-(*p*-NO_2-phenylsulfonamido)-3-phenyl-5-methoxyindole **8** (step b, Scheme 1) that was then submitted to methylether cleavage with 1 M BBr_3 in dry DCM (step c, Scheme 1) followed by nitro-reduction with $SnCl_2$ (step d, Scheme 1). Intermediate **10** was then transformed into the corresponding phenylazide **11** through a diazotization reaction using an aqueous solution of $NaNO_2$ in 1:1 acetic acid and hydrochloric acid, followed by reaction with sodium azide (step e, Scheme 1). The reaction was started at 0 °C and slowly allowed to warm to room temperature, to avoid decomposition of intermediate nitrosonium ion; quantitative yields were obtained. Lastly, oxidation of the phenol was performed under standard IBX oxidation conditions (step f, Scheme 1), yielding target TM-phenylazide **3** in overall good yields.

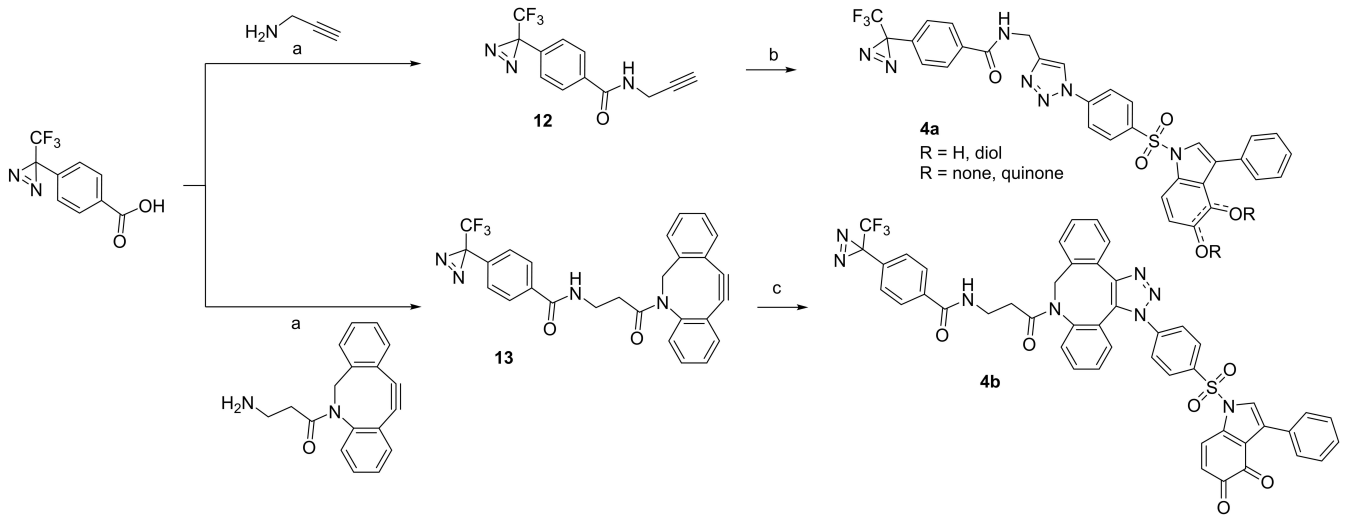
TM-phenylazide **3** was employed as a common intermediate to obtain diazirine- and biotin-containing TM chemical probes **4**–**5**, and was also submitted to biological characterization as a putative PAP.

Diazirine-Based TM Affinity Probes 4a,b

To further expand the library of TM probes, we envisaged the synthesis of triazole-connected TM diazirine probes **4a**,**b**.

Both target compounds would be obtained by coupling key intermediate TM-phenylazide **3** with diazirine amides **12** and **13** respectively, through either a classic Copper-Catalyzed (CuAAC) or a Strain-Promoted Azide-Alkyne Cycloaddition (SPAAC) (step a, Scheme 2). Even though the former has been widely used as a bio-orthogonal reaction in life science research, the latter has ensured the successful application of click reactions to living cells without copper-induced cytotoxicity.[15] Both amides **12** and **13** could derive from standard amidation of commercial trifluoromethylphenyl diazirine carboxylate with propargyl- and dibenzocyclooctyne (DBCO)-amine respectively, using DCC as coupling reagent (step a, Scheme 2).

Intermediates **12** and **13** were then submitted, respectively, to CuAAC or SPAAC protocols to be coupled with **3** (steps b and c respectively, Scheme 2). In the CuAA cycloaddition, an excess of sodium ascorbate was needed to complete the catalytic cycle, as it was also consumed by the reduction of the quinone to diol. Target probe **4a** was obtained in acceptable

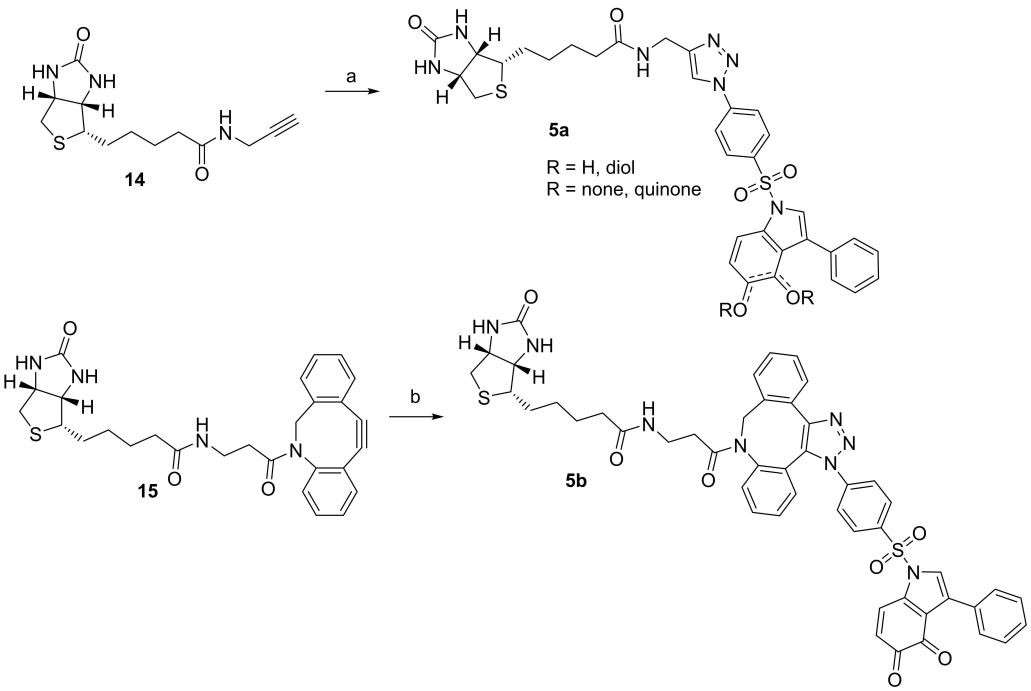


Scheme 2. Synthesis of diazirine-based TM PAPs **4a**,**b**. a) DCC, HOBt, DIPEA, N_2, dry DCM, r.t., 3 h, 87% (**12**), 60% (**13**); b) **3**, Na ascorbate (1.25 eq), $CuSO_4.5H_2O$ (0.05 eq), 3:1 DMF/H_2O, r.t., 6 h, 44%; c) **3**, DCM, r.t., 3 h, 68%.

yields in its reduced diol form, which slowly equilibrates to the more stable quinone. Conversely, since no catalytic cycle is involved in the SPAA Cycloaddition, the desired probe **4b** was obtained in moderate yields in its oxidized quinone form as a stereoisomeric mixture (3:2 ratio, determined by ^{1}H NMR), due to the asymmetry of DBCO amine **13** (for simplicity, only one isomer is depicted). As the SPAA Cycloaddition was tested with the aim to assess it for in cellulo execution[16] where the separation of the two isomers would not be feasible, we did not isolate the two stereoisomers.

Biotin-Based TM Probes 5a,b

Biotin-based TM affinity probes **5a**, **b** were conceived through a similar synthetic strategy as in Scheme 2. Thus, TM-phenylazide **3** was to be coupled to an alkynyl biotinamide either through a CuAAC (target **5a**), or a SPAAC protocol (target **5b**). As reported in Scheme 3, biotin-propargylamine **14**[17] and biotin-DBCO **15**[18] were submitted, respectively, to CuAA or SPAA Cycloaddition onto phenylazide **3**. As already explained, an excess of sodium ascorbate was needed in the CuAA-cycloaddition to



Scheme 3. Synthesis of biotin-based TM probes **5a**,**b** a) **3**, Na ascorbate (1.25 eq), $CuSO_4.5H_2O$ (0.05 eq), 3:1 DMF/H_2O, r.t., 6 h, 61%; b) **3**, 1:1 MeOH/DMF, r.t., 5 h, 44%.

obtain **5a** in its diol form (step a), which slowly equilibrates to the more stable quinone. Target probe **5b** was obtained as a quinone in moderate yields through an SPAA Cycloaddition (step b, Scheme 3). Once again, a stereoisomeric mixture (3:2 ratio determined from ^{1}H NMR) was obtained due to the asymmetry of DBCO amine **15** and the two stereoisomers were not separated.

TM Probes Maintain Cytotoxic Activity Against Breast Cancer Cells

TM probes **3**–**5** were tested both in biological and biochemical assays. Firstly, we aimed to understand whether these compounds might present a biological activity, as previously described for TMs.[9] Since breast cancer is among the most HuR-upregulated tumor types,[19] we used a model of triple-negative breast cancer (MDA-MB-231) to test them. One day after seeding, cells were exposed to increasing concentrations of compounds **3**, **4a**, **4b**, **5a** and **5b** for 48 hours, after which cellular viability was assessed using the metabolic OzBlue assay. All compounds decreased cellular viability at the highest doses, however the CC_{50} values (concentration of compound that is cytotoxic to 50% of the cells population) could be determined only for **3** (65.54 μM) and **4a** (8.092 μM) (Figure 3 A–E and Supplementary Table S1). The lack of solubility of the compounds above 100 μM prevented their test at higher concentrations, and the precise calculation of the CC_{50} for **4b**, **5a** and **5b** (Supplementary Table S1).

REMSA Confirms Biotinylated Compound's Ability to Disrupt Protein-RNA Interaction

To evaluate whether PAPs and biotinylated TMs could still displace HuR from its target RNA, we performed an RNA Electrophoretic Mobility Shift Assay (REMSA). This technique is based on the principle that a protein-RNA complex migrates less in a polyacrylamide gel than free RNA, which is displaced from the protein-RNA complex by the small molecules. We produced the recombinant HuR protein, containing the first two of the three RNA recognition motifs (rM1 M2), which was incubated with an AU-rich, 26-nucleotide probe linked to an infrared fluorophore (DY681) to allow the detection of both the protein-RNA complex and the free probe. TM probes **3**–**5** were individually added, at increasing concentrations ranging from 1 to 100 μM (maximum solubility of the compounds), to the reaction mixtures containing the rM1 M2 and the AU-rich RNA probe; then, the reaction mixture was run on a polyacrylamide gel. Free RNA, not incubated with rM1 M2, was used as a control, and lead compound **2** (TM6a), which had been previously identified as TM with HuR inhibitory activity,[7] was added as a reference. As expected, compound **2** presented the greatest inhibitory effect on HuR-RNA complex formation, with a half-maximal inhibitory concentration (IC_{50}) of 15.73 μM (Figure 4A).

Compound **3**, which was used as an intermediate for the synthesis of both PAPs and biotin-labeled TMs, presented a decreased ability to disrupt the protein-RNA complex compared with compound **2** (Figure 4B). Instead, TMs presenting the PAP moiety (**4a** and **4b**), completely lost the ability to disrupt the rM1 M2-RNA complex (Figure 4C–D). On the other hand,

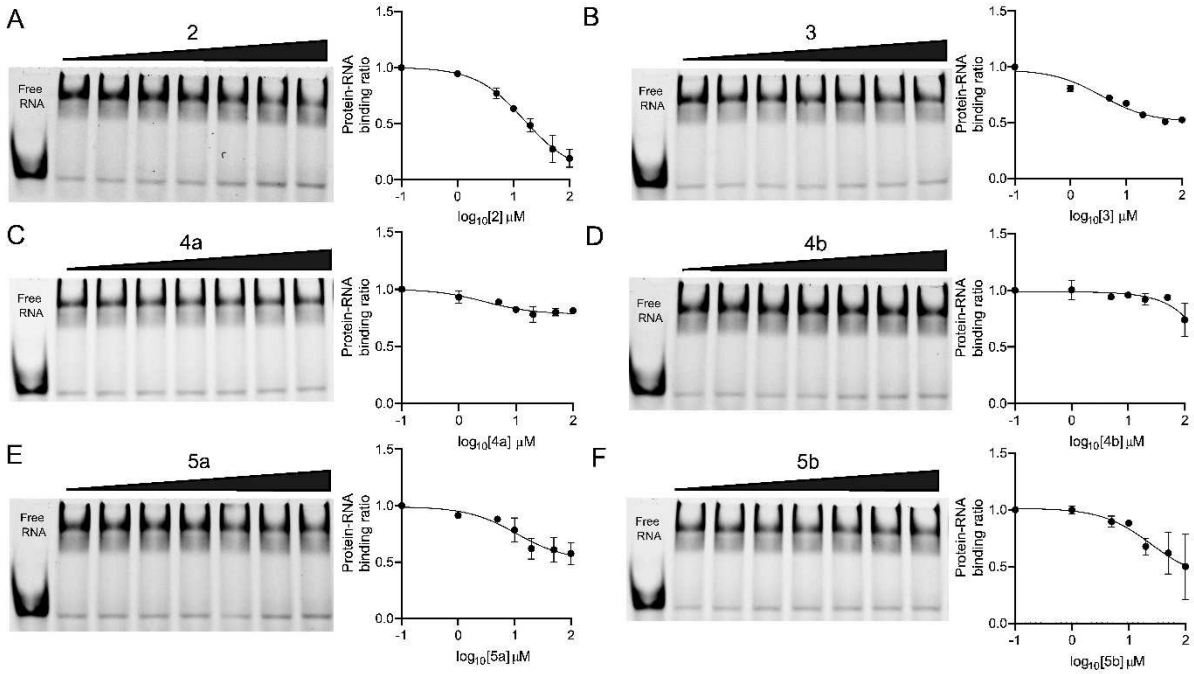


Figure 4. REMSAs of TM probes **3**–**5**. The rM1 M2-RNA complex was incubated with 0, 1, 5, 10, 20, 50, 100 μM (from left to right) of each TM. The AU-rich RNA probe was used as a negative control (free RNA). The upper rM1 M2-RNA complex bands were quantified using ImageJ and plotted in function of the logarithm of TMs concentration.

biotinylated probes **5a** and **5b** maintained inhibitory activity, although lower than compound **2**, and compound **5a** was the most active among the TM probes (Figure 4D, E). Thus, biotinylated compounds **5a** and **5b** were the most promising candidates for mechanistic studies. No further studies were performed on TM conjugates **4a** and **4b**.

Compound 5a Successfully Pulls Down Recombinant HuR in vitro

After confirming the ability of biotinylated compounds **5a** and **5b** to decrease rM1 M2-RNA binding, we investigated whether we could use them to pull down the rM1 M2 protein. Compounds **5a** and **5b** were incubated with both streptavidin-coated magnetic beads and the rM1 M2 protein, after which the expected rM1 M2-TM complexes were isolated from the reaction mixture using a magnetic rack. After denaturation, samples were run in a polyacrylamide gel and blotted with an anti-HuR antibody. Several controls were used, including the individual loading of the magnetic beads (beads only) and rM1 M2 (rM1 M2), as well as beads incubated with biotin, with (Beads, biotin, rM1 M2) or without (Beads, biotin) the rM1 M2 protein. Controls incubated with biotin were used to detect any non-specific interaction between streptavidin and rM1 M2. Western blot analysis allowed us to conclude that both **5a** and **5b** can be used to pull-down the rM1 M2 protein *in vitro.* An increased amount of pulled-down rM1 M2 was observed using **5a** (Figure 5), in agreement with its higher ability to displace protein/RNA interaction. As expected, rM1 M2 was not detected using either beads alone or beads incubated with biotin, and was only minimally detected in samples containing beads, biotin, and rM1 M2 (Figure 5).

Taken together, these data confirmed the ability of both biotinylated compounds to bind to rM1 M2 and to isolate it from a biological sample; among them, **5a** was the most effective.

Conclusions

We report the synthesis of five new TM probes **3**–**5** bearing a probe moiety as a para-substituent on the phenyl-sulphonamide. The synthetic procedure involves the introduction of an azide group on the phenyl-sulphonamide of lead compound TM **2** (originating TM **3**, which can also be used per se as a PAP) and further conjugation through a CuAA or SPAA click reaction with suitable alkynyl-diazirine or -biotin constructs to give TM probes **4**–**5** in discrete to moderate yields. Their biological characterization confirmed that all TM probes decrease the viability of triple-negative breast cancer MDA-MB-231 cells. Their CC_{50}s are in the micromolar range, as previously reported for other TMs from our laboratory[7,9] and in literature.[20] REMSAs revealed that novel TM probes **3**–**5** presented a lower ability to dissociate the rM1 M2-RNA, compared with the original lead compound **2**. This can be explained by the spatial constraints deriving from the moieties added to compound **2**, which could partially hinder their binding to the recombinant protein, and consequently the displacement of mRNA from the complex. The lack of solubility of the compounds above 100 μM prevented their test at higher concentrations, and the correct calculation of the CC_{50} (for the viability curves) or IC_{50} (REMSAs) vales for compounds **4b**, **5a** and **5b**. Since biotinylated TM probes **5a**,**b** retained a higher HuR inhibitory ability than PAP probes **4a**,**b**, we focused on their application in pull-down experiments to take advantage of the streptavidin-biotin binding affinity aiming to isolate rM1 M2 from biological media. In detail, we observed that both biotinylated probes, and **5a** with stronger potency, could bind to both streptavidin magnetic beads and rM1 M2, allowing the isolation of the target protein from solutions. This result was in line with data obtained by REMSAs, in which **5a** presented a higher ability to bind to rM1 M2 and disrupt the rM1 M2-RNA binding. In conclusion, we managed to synthesize and characterize the HuR-targeting, biotinylated TM probe **5a**, which is effective both at the biological and biochemical levels, as a candidate small molecule for further in vitro MoA studies.

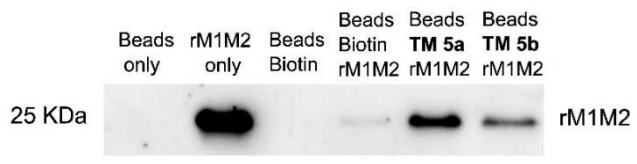


Figure 5. Pull-down assay using TM probes **5a** and **5b**. Streptavidin magnetic beads were used to pull down the **5a**/**5b**–rM1 M2 complex. Probes **5a** and **5b** as well as biotin were used at 500 μM, while 50 ng of rM1 M2 was added to the respective samples. rM1 M2 was used as a positive control (5 ng), while the conditions "Beads only", "Beads Biotin" and "Beads Biotin rM1 M2" were used as negative controls.

Experimental Section

Synthetic Procedure

General

Reagent-grade chemicals and solvents were purchased from Sigma-Aldrich or FluoroChem, and were used without any further purification. Dry solvents were purchased from Sigma-Aldrich, and reactions in anhydrous conditions were performed under nitrogen atmosphere, using a dry nitrogen flux (passage through Drierite (Ca_2SO_4) trap as a drying agent). Reactions were monitored by analytical thin layer chromatography (TLC), using silica gel 60 F_{254} pre-coated glass plates (0.25 mm thickness). Visualization was accomplished by irradiation with a UV lamp and/or staining with alcoholic ninhydrin solution or cerium/molybdate reagent. Purifications were carried out by direct phase flash chromatography on Macherey-Nagel silica gel (particle size 60 μm, 230–400 mesh) or through an automated BiotageTM chromatography system. NMR spectra were recorded on a Bruker Advance 400 instrument in deuterated solvents either at 400 MHz (^{1}H-NMR) or at 101 MHz (^{13}C NMR). Chemical shifts are expressed in δ (ppm) with tetramethylsilane (TMS) employed as an internal standard. Coupling constants are given in Hertz and rounded to the nearest 0.1 Hz. The following

abbreviations are used to describe spin multiplicity: s = singlet, d = doublet, t = triplet, q = quartet, m = multiplet, bs = broad signal, dd = doublet of doublets. LC–MS data were collected with a Waters AcquityTM Ultra performance LC (UPLC)-MS System equipped with a tunable UV (TUV) detector, a single quadrupole (SQD) mass spectrometer and ACQUITY UPLC BEH SHIELD RP18 columns (2.1x100 mm, id = 1.7 μm). HPLC compounds' purity was determined by monitoring at 220 nm using H2O (HiPerSolv Chromanorm Water VWR for HPLC-MS) + 0.05 % trifluoroacetic acid (spectroscopic grade) as phase A, and acetonitrile (HiPerSolv Chromanorm Acetonitrile SuperGradient VWR) + 0.05 % trifluoroacetic acid (spectroscopic grade) as phase B, and a gradient from 5 % B to 100 % B in 5 min, followed by 100 % B for 1 min; a 0.5 mL/min flow was used, with 2 min equilibration time.

Key Intermediate and Photoaffinity Probe TM-Phenylazide 3

Synthesis of 3-bromo-5-methoxy-1-((4-nitrophenyl) sulfonyl)-1H-indole 7

An aqueous solution of 50 % KOH (8.2 mL, 8 eq) was added to a mixture of **6** (2.643 g, 11.69 mmol, 1 eq) and n-Bu_4NHSO_4 (399 mg, 1.174 mmol, 0.1 eq) in DCM (118 mL). The reaction was stirred vigorously at room temperature for 10 minutes, then a solution of p-nitro-benzenesulfonyl chloride (4.404 g, 19.87 mmol, 1.7 eq) in DCM (118 mL) was added. The mixture turned to yellow. The reaction was stirred at room temperature and monitored by TLC (eluent mixture: 9:1 n-hexane/EtOAc, developed in molybdic reagent). After 2 hours, distilled water (130 mL) was added, and the aqueous phase was extracted with DCM (3×70 mL). The collected organic phases were washed with distilled water (3×100 mL), dried over Na_2SO_4, filtered and the solvent was evaporated under reduced pressure. The crude was purified through two crystallizations from 1:1.2 n-hexane/DCM, affording 4.159 g of pure compound **7** as a yellow solid (10.9 mmol, 93 % yield). MS (ESI+): the compound couldn't be detected. Calculated MS for $C_{15}H_{11}BrN_2O_5S$: 409.96. ^{1}H NMR (400 MHz, acetone-d6) δ (ppm) 8.41 (d, J = 9.2 Hz, 2H, CH phenyl-sulfonamide), 8.31 (d, J = 9.2 Hz, 2H, CH phenyl-sulfonamide), 7.98 (d, J = 9.1 Hz, 1H, H7 indole), 7.92 (s, 1H, H2 indole), 7.08 (dd, J = 9.1, 2.5 Hz, 1H, H6 indole), 6.96 (d, J = 2.4 Hz, 1H, H4 indole), 3.86 (s, 3H, OCH_3). ^{13}C NMR (101 MHz, acetone-d6) δ (ppm) 158.7, 143.2, 131.9, 129.5, 126.8, 125.8, 116.6, 115.7, 102.9, 101.4, 56.1.

Synthesis of 5-methoxy-1-((4-nitrophenyl)sulfonyl)-3-phenyl-1H-indole 8

In a two-necked round-bottom flask, equipped with a valve on the lateral neck and with a rubber septum on the main-middle neck, bromo-methoxyindole **7** (1.803 g, 4.72 mmol, 1 eq) and phenyl-boronic acid (678 mg, 5.52 mmol, 1.17 eq) were added, then the flask was flushed with nitrogen to remove any trace of oxygen. Subsequently, dry and previously deareated 4:1 DME/EtOH and 2 M aqueous K_2CO_3 solution (1.29 eq) were added. Finally, $Pd(PPh_3)_4$ (273 mg, 0.24 mmol, 0.05 eq) was added under nitrogen flushing. The rubber septum was removed, and a condenser equipped with a valve was fitted onto the main-middle neck. The solution was stirred under N_2 at reflux (100 °C) for 7 hours, then at room temperature for additional 12 hours monitoring by TLC (eluent mixture: 9:1 n-hexane/EtOAc, developed in molybdic reagent). Finally, the reaction was diluted with a saturated NH_4Cl solution (100 mL) and extracted with EtOAc (3×100 mL). The collected organic phases were washed with brine (300 mL), dried over Na_2SO_4, filtered and the solvent was evaporated under reduced pressure. The crude was purified through two crystallizations from 1:1.2 n-hexane/DCM, affording 1.160 g of pure compound **9** as a yellow solid (2.79 mmol, 60 % yield). ^{1}H NMR (400 MHz, acetone-d6) δ (ppm) 8.40–8.33 (4H, m, CH phenyl-sulfonamide), 8.02 (d, J = 9.5 Hz, 1H H7 indole), 7.91 (s, 1H, H2 indole), 7.72–7.70 (m, 2H, CH phenyl), 7.52–7.48 (2H, m, CH phenyl), 7.41–7.39 (m, 1H, CH phenyl), 7.28 (d, J = 2.9 Hz, 1H, H4 indole), 7.05 (dd, J = 9.5, 2.9 Hz, 1H, H6 indole), 3.83 (s, 3H, OCH_3). The ^{1}H-NMR spectrum was consistent with that reported in literature.[7]

Synthesis of 1-((4-nitrophenyl)sulfonyl)-3-phenyl-1H-indol-5-ol 9

A 1 M solution of BBr_3 in dry DCM (17 mL, 6 eq) was slowly added under stirring to a solution of methoxyindole **8** (1.160 g, 2.84 mmol, 1 eq) in dry DCM (28.4 mL), under nitrogen atmosphere and at −78 °C. The temperature was slowly increased to 0 °C while monitoring the reaction by TLC (eluent mixture: 7:3 n-hexane/EtOAc, developed in molybdic reagent). Still at 0 °C, the solution was diluted with distilled water (100 mL) and neutralized with a saturated $NaHCO_3$ solution. The reaction mixture was extracted with DCM (3×100 mL) and the collected organic layers were washed with brine, dried over sodium sulfate, and filtered. The solvent was removed under reduced pressure and the crude was purified by column chromatography (eluent mixture: 7:3 n-hexane/EtOAc), affording 921 mg of pure compound **9** as a yellow solid (2.34 mmol, 82 % yield). ^{1}H NMR (400 MHz, acetone-d6) δ (ppm) 8.40–8.31 (4H, m, CH phenyl-sulfonamide), 7.94 (d, J = 8.7 Hz, 1H H7 indole), 7.87 (s, 1H, H2 indole), 7.67–7.65 (m, 2H, CH phenyl), 7.51–7.47 (2H, m, CH phenyl), 7.41–7.37 (m, 1H, CH phenyl), 7.21 (d, J = 2.2 Hz, 1H, H4 indole), 6.98 (dd, J = 9.0, 2.4 Hz, 1H, H6 indole). The ^{1}H-NMR spectrum was consistent with that reported in literature.[7]

Synthesis of 1-((4-aminophenyl)sulfonyl)-3-phenyl-1H-indol-5-ol 10

Nitro-compound **9** (1.038 g, 2.63 mmol, 1 eq) and $SnCl_2 \cdot 2H_2O$ (2.971 g, 13.7 mmol, 5 eq) were dissolved in 1:1 THF/MeOH (22 mL). The solution was refluxed at 80 °C for 2 hours, monitoring by TLC (eluent mixture: 7:3 n-hexane/EtOAc). After reaction completion, the solvent was evaporated under reduced pressure. A saturated $NaHCO_3$ solution was added until neutralization to the residue. The crude was extracted with EtOAc (50 mL). The resultant suspension was filtered through a pad of celite, and the collected organic layers were washed with brine (50 mL), dried over sodium sulfate, and evaporated, yielding 957 mg of crude product **10** that was used without any further purification (2.63 mmol, quantitative). MS (ESI+): m/z 365.20 $[M+H]^+$. Calculated MS for $C_{20}H_{16}N_2O_3S$: 364.09. ^{1}H NMR (400 MHz, acetone-d6) δ (ppm) 8.21 (s, 1H, OH), 7.89 (d, J = 8.9 Hz, 1H, H7 indole), 7.76 (s, 1H, H2 indole), 7.74–7.60 (m, 4H, CH phenyl, CH phenyl-sulfonamide), 7.52–7.42 (m, 2H, CH phenyl), 7.40–7.31 (m, 1H, CH phenyl), 7.21 (d, J = 2.3 Hz, 1H, H4 indole), 6.93 (dd, J = 8.9, 2.4 Hz, 1H, H6 indole), 6.72–6.64 (m, 2H, CH phenyl-sulfonamide), 5.69 (bs, 2H, NH_2). ^{13}C NMR (101 MHz, acetone-d6) δ (ppm) 155.0, 154.9, 134.4, 131.1, 130.6, 130.1, 129.7, 128.4, 128.0, 125.0, 123.5, 115.6, 114.6, 114.0, 105.6.

Synthesis of 1-((4-azidophenyl)sulfonyl)-3-phenyl-1H-indol-5-ol 11

Intermediate aniline **10** was dissolved in a 1:1 mixture of glacial acetic acid and concentrated HCl (5.2 mL), and the solution was cooled to 0 °C. Then, a 1.8 M $NaNO_2$ aqueous solution (4.4 mL) was slowly added, and a brownish solid was immediately formed. The mixture was vigorously stirred at 0 °C for 15 minutes. Then, a 1.8 M NaN_3 aqueous solution (4.4 mL) was slowly added and a yellowish

solid immediately formed. The mixture was stirred at room temperature for 1 hour, monitoring by TLC (eluent mixture: 7:3 n-hexane/EtOAc). After reaction completion, distilled water was added to the mixture (20 mL) and the crude was extracted with DCM (3×20 mL). The collected organic layers were washed with brine (50 mL), dried over sodium sulfate and evaporated, yielding 1.025 g of pure azide **11** that was used without further purification (2.63 mmol, quantitative). MS (ESI+): m/z 391.23 $[M+H]^+$. Calculated MS for $C_{20}H_{14}N_4O_3S$: 390.08. ^{1}H NMR (400 MHz, acetone-*d6*) δ (ppm) 8.33 (bs, 1H, OH), 8.10–8.00 (m, 2H, CH phenyl-sulfonamide), 7.92 (d, J = 8.9 Hz, 1H, H7 indole), 7.82 (s, 1H, H2 indole), 7.70–7.60 (m, 2H, CH phenyl), 7.53–7.44 (m, 2H, CH phenyl), 7.42–7.33 (m, 1H, CH phenyl), 7.27–7.18 (m, 3H, CH phenyl-sulfonamide, H4 indole), 6.96 (dd, J = 8.9, 2.4 Hz, 1H, H6 indole). ^{13}C NMR (101 MHz, acetone-*d6*) δ (ppm) 147.2, 134.5, 134.0, 131.5, 130.5, 129.9, 129.8, 128.5, 128.4, 124.9, 120.8, 115.6, 106.0.

Synthesis of 1-((4-azidophenyl)sulfonyl)-3-phenyl-1H-indole-4,5-dione 3

IBX (883 mg, 3.16 mmol, 1.2 eq) was added to a solution of methoxy phenol intermediate **11** (1.025 g, 2.63 mmol, 1 eq) in DMF (15 mL). The reaction mixture was stirred at room temperature and monitored by TLC (eluent mixture: 7:3 n-hexane/EtOAc, developed in molybdic reagent). After reaction completion, the mixture was diluted with distilled water (100 mL). The aqueous phase was extracted with EtOAc (3×100 mL). The collected organic phases were washed with brine (300 mL), dried over sodium sulfate, and filtered. The crude was purified by flash chromatography (eluent mixture: 7:3 n-hexane/EtOAc), affording 646.7 mg of pure compound **3** as a red solid (1.58 mmol, 60%). MS (ESI+): m/z 405.05 $[M+H]^+$. Calculated MS for $C_{20}H_{12}N_4O_4S$: 404.06. ^{1}H NMR (400 MHz, acetone-*d6*) δ (ppm) 8.29–8.17 (m, 2H, CH phenyl-sulfonamide), 8.08 (d, J = 10.5 Hz, 1H, H7 indole), 7.75 (s, 1H, H2 indole), 7.70–7.63 (m, 2H, CH phenyl), 7.45–7.31 (m, 5H, CH phenyl-sulfonamide, CH phenyl), 6.21 (d, J = 10.5 Hz, 1H, H6 indole). ^{13}C NMR (101 MHz, acetone-*d6*) δ (ppm) 182.3, 174.8, 148.8, 137.8, 133.8, 132.1, 131.5, 130.7, 130.5, 129.6, 129.1, 128.9, 127.1, 124.6, 121.6.

TM-Based Chemical Probes

N-(prop-2-yn-1-yl)-4-(3-(trifluoromethyl)-3H-diazirin-3-yl)benzamide 12

A solution of propargyl amine (0.033 mL, 0.52 mmol, 1.2 eq) and DIPEA (0.091 mL, 0.52 mmol, 1.2 eq) in dry DCM (0.7 mL) was added to a solution of 4-(3-(trifluoromethyl)-3H-diazirin-3-yl)benzoic acid (98.0 mg, 0.43 mmol, 1 eq), HOBt (72.2 mg, 0.52 mmol, 1.2 eq) and DCC (111.0 mg, 0.52 mmol, 1.2 eq) in dry DCM (1 mL) under nitrogen atmosphere at room temperature. The reaction mixture was stirred at room temperature for 3 hours. After reaction completion (TLC monitoring, eluent mixture: 9:1 DCM/MeOH, developed in molybdic reagent), the solvent was removed under reduced pressure. The crude was purified by flash chromatography (eluent mixture: 99:1 DCM/acetone) to afford 100.6 mg of pure compound **12** as a white solid (0.376 mmol, 87% yield). MS (ESI+): m/z 268.13 $[M+H]^+$. Calculated MS for $C_{12}H_8F_3N_3O$: 267.06. ^{1}H NMR (400 MHz, $CDCl_3$) δ (ppm) 7.85–7.77 (m, 2H, CH phenyl), 7.30–7.22 (m, 2H, CH phenyl), 6.28 (bs, 1H, NH), 4.26 (dd, J = 5.2, 2.6 Hz, 2H, CH2), 2.30 (t, J = 2.6 Hz, 1H, C≡CH). ^{13}C NMR (101 MHz, $CDCl_3$) δ (ppm) 166.0, 134.9, 132.9, 127.6, 126.9, 126.8, 123.4, 120.7, 79.2, 72.4, 30.1.

Synthesis of DBCO-diazirine 13

A solution of dibenzocyclooctyne-amine (75.1 mg, 0.27 mmol, 1 eq) and DIPEA (0.057 mL, 0.33 mmol, 1.2 eq) in dry DCM (0.5 mL) was added to a solution of 4-(3-(trifluoromethyl)-3H-diazirin-3-yl)benzoic acid (65.0 mg, 0.27 mmol, 1 eq), HOBt (44.6 mg, 0.33 mmol, 1.2 eq) and DCC (68.1 mg, 0.33 mmol, 1.2 eq) in dry DCM (1 mL) under nitrogen atmosphere, at room temperature. The reaction mixture was stirred at room temperature for 3 hours. After reaction completion (TLC monitoring, eluent mixture: 9:1 DCM/MeOH, developed in molybdic reagent), the solvent was removed under reduced pressure. The crude was purified by flash chromatography (eluent mixture: 95:5 DCM/MeOH) to afford 79.1 mg of pure compound **13** as a white solid (0.162 mmol, 60% yield). MS (ESI+): m/z 489.24 $[M+H]^+$. Calculated MS for $C_{27}H_{19}F_3N_4O_2$: 488.15. ^{1}H NMR (400 MHz, $CDCl_3$) δ (ppm) 8.42 (t, J = 5.5 Hz, 1H, NH), 7.76 (d, J = 8.6 Hz, 2H, CH phenyl-diazirine), 7.65–7.63 (m, 1H, CH phenyl-cyclooctyne), 7.61–7.57 (m, 1H, CH phenyl-cyclooctyne), 7.52–7.33 (m, 5H, CH phenyl-cyclooctyne), 7.31 (d, J = 8.2 Hz, 2H, CH phenyl-diazirine), 7.24–7.21 (m, 1H, CH phenyl-cyclooctyne), 5.05 (d, J = 14.0 Hz, 1H, CH_2 cyclooctyne), 3.63 (d, J = 14.0 Hz, 1H, CH_2 cyclooctyne), 3.14 (m, 1H, CH_2), 2.60–2.51 (m, 2H, CH_2), 1.99 (m, 1H, CH_2). ^{13}C NMR (101 MHz, $CDCl_3$) δ (ppm) 170.1, 164.9, 151.4, 148.3, 135.8, 132.5, 129.5, 128.9, 128.2, 128.0, 127.7, 126.8, 126.3, 125.1, 122.4, 121.4, 114.3, 108.1, 54.8, 35.9, 33.9.

Synthesis of Diazirine-Containing TM Chemical Probe 4a

A freshly prepared 0.075 M aqueous solution of $CuSO_4 \cdot 5H_2O$ (0.165 mL, 0.1 eq) and 0.5 M aqueous solution of sodium ascorbate (0.310 mL, 1.25 eq) were added to a stirred solution of alkyne-diazirine **12** (31.9 mg, 0.124 mmol, 1 eq) and azide **3** (49.9 mg, 0.124 mmol, 1 eq) in 3:1 DMF/H_2O (1.5 mL). The resulting mixture was stirred at room temperature for 4 hours and monitored by TLC (eluent mixture: 98:2 DCM/MeOH, developed in molybdic reagent). After reaction completion, the solvent was evaporated under reduced pressure. The crude was purified by Biotage™ reverse phase chromatography (eluent mixture: CH_3CN/H_2O, gradient from 0% to 100% CH_3CN) affording 36.4 mg of pure compound **4a** as a brown solid (0.054 mmol, 44% yield). MS (ESI+): m/z 674.54 $[M+H]^+$. Calculated MS for $C_{32}H_{22}F_3N_7O_5S$: 673.14. Purity >90%. ^{1}H NMR (400 MHz, acetone-*d6*) δ (ppm) 8.57 (s, 1H, CH triazole), 8.21 (d, J = 8.9 Hz, 2H, CH phenyl-sulfonamide), 8.10 (d, J = 8.9 Hz, 2H, CH phenyl-sulfonamide), 8.03 (d, J = 8.5 Hz, 2H, CH phenyl diazirine), 7.70–7.63 (m, 2H, CH phenyl indole), 7.57 (s, 1H, H2 indole), 7.46 (d, J = 8.7 Hz, 1H, H7 indole), 7.41–7.27 (m, 5H, CH phenyl diazirine, CH phenyl indole), 7.00 (d, J = 8.7 Hz, 1H, H6 indole), 4.70 (s, 2H, CH_2). ^{13}C NMR (101 MHz, acetone-*d6*) δ (ppm) 166.3, 141.9, 137.8, 137.0, 134.6, 132.0, 130.4, 129.8, 129.0, 128.5, 127.9, 127.4, 124.6, 121.4, 115.1, 105.4, 36.0.

Synthesis of Diazirine-Containing TM Chemical Probe 4b

Azide **3** (29.3 mg, 0.074 mmol, 1 eq) and DBCO-diazirine **13** (36.2 mg, 0.074 mmol, 1 eq) were dissolved in DCM (1.0 mL). The solution was stirred at room temperature for 3 hours, monitoring by TLC (95:5 DCM/MeOH, developed in molybdic reagent). The crude was purified by column chromatography (eluent mixture: 98:2 DCM/MeOH), affording 44.8 mg of pure compound **4b** as a red solid (0.050 mmol, 68%). MS (ESI+): m/z 893.18 $[M+H]^+$. Calculated MS for $C_{47}H_{31}F_3N_8O_6S$: 892.20. Purity >90%. ^{1}H NMR (400 MHz, DMSO-*d6*) (2:3 isomer ratio:) δ (ppm) 8.47–8.35 (m, 3H, aromatic H), 8.00–7.50 (m, 11H, aromatic H), 7.43–7.26 (m, 7H, aromatic H), 7.08–7.00 (m, 1H, aromatic H), 6.78–6.75 (m, 1H, aromatic H), 6.22–6.19 (m, 1H, aromatic H), 6.04 (d, J = 17.4 Hz, 0.6H CH_2 cyclooctyne isomer A), 5.70 (d, J = 18.9 Hz, 0.4H, CH_2 cyclo-

octyne isomer B), 5.07 (d, J = 18.9 Hz, 0.4H, CH_2 cyclooctyne isomer B), 4.59 (d, J = 17.4 Hz, 0.6H, CH_2 cyclooctyne isomer A), 3.36–3.28 (m, 0.4H, CH_2 isomer B), 3.26–3.16 (m, 1.2H, CH_2 isomer A), 3.16–2.97 (m, 0.4H, CH_2 isomer B), 2.94–2.75 (m, 0.4H, CH_2 isomer B), 2.41–2.24 (m, 0.4H, CH_2 isomer B), 2.13–2.01 (m, 0.6H CH_2 isomer A), 1.65–1.53 (m, 0.6H, CH_2 isomer A). ^{13}C NMR (400 MHz, DMSO-*d6*, some signals are split due to the presence of two isomers) δ (ppm) 181.3, 174.0, 171.2, 166.0, 143.2, 140.5, 137.2, 136.8, 135.9, 134.3, 134.2, 133.3, 132.9, 131.9, 130.5, 130.2, 129.9, 129.5, 129.3, 128.9, 128.6, 128.4, 128.3, 127.0, 126.6, 123.3, 122.9, 120.6, 54.7, 51.4, 35.8, 33.7.

Synthesis of Biotin-Containig TM Chemical Probe 5 a

A freshly prepared 0.075 M aqueous solution of 0.075 M $CuSO_4 \cdot 5H_2O$ (0.098 mL, 0.1 eq) and 0.5 M aqueous solution of sodium ascorbate (0.185 mL, 1.25 eq) were added to a stirred solution of alkyne-biotin **14** (21.5 mg, 0.074 mmol, 1 eq) and azide **3** (30.3 mg, 0.074 mmol, 1 eq) in 3:1 DMF/H2O (1.0 mL). The resulting mixture was stirred at room temperature for 4 hours and monitored by TLC (eluent mixture: 9:1 DCM/MeOH, developed in molybdic reagent). After reaction completion, the solvent was evaporated under reduced pressure. The crude was purified by Biotage™ reverse phase chromatography (eluent mixture: CH_3CN/H_2O, gradient from 0% to 100% CH_3CN) affording 31.1 mg of pure compound **5 a** as a brown solid (0.045 mmol, 61% yield). MS (ESI +): m/z 674.54 $[M+H]^+$. Calculated MS for $C_{33}H_{33}N_7O_6S_2$: 688.30. Purity >85%. ^1H NMR (400 MHz, MeOD) δ (ppm) 8.39 (s, 1H, H triazole), 8.10 (d, J = 8.9 Hz, 2H, H phenyl-sulfonamide), 7.98 (d, J = 8.8 Hz, 2H, H phenyl-sulfonamide), 7.67–7.52 (m, 2H, H phenyl), 7.48 (s, 1H, H2 indole), 7.42 (d, J = 8.7 Hz, 1H, H7 indole), 7.37–7.31 (m, 2H, H phenyl), 7.31–7.25 (m, 1H, H phenyl), 6.91 (d, J = 8.7 Hz, 1H, H6 indole), 4.54–4.41 (m, 2H, CH_2-triazole), 4.29 (dd, J = 7.8, 4.6 Hz, 1H, CH biotin), 4.17 (dd, J = 7.9, 4.4 Hz, 1H, CH biotin), 3.06 (dt, J = 10.1, 5.7 Hz, 1H, CH biotin), 2.66 (dd, J = 12.8, 4.9 Hz, 1H, CH_2 biotin), 2.40 (d, J = 12.7 Hz, 1H, CH_2 biotin), 2.23 (t, J = 7.1 Hz, 2H, CH_2 biotin), 1.71–1.58 (m, 3H, CH_2 biotin), 1.58–1.45 (m, 1H, CH_2 biotin), 1.39–1.27 (m, 2H, CH_2 biotin). ^{13}C NMR (101 MHz, MeOD) δ (ppm) 176.6, 147.7, 142.0, 138.6, 134.9, 132.2, 130.6, 129.9, 129.2, 129.1, 128.7, 128.1, 127.1, 124.7, 122.5, 121.8, 120.0, 115.3, 105.7, 98.7, 63.2, 61.5, 56.9, 49.9, 40.9, 36.5, 35.4, 29.5, 29.4, 26.7.

Synthesis of Biotin-Containig TM Chemical Probe 5 b

Azide **3** (17.4 mg, 0.043 mmol, 1 eq) and DBCO-biotin **15** (21.6 mg, 0.043 mmol, 1 eq) were dissolved in 1:1 MeOH/DMF (0.5 mL). The solution was stirred at room temperature for 3 hours, monitoring by TLC (9:1 DCM/MeOH, developed in molybdic reagent). The crude was purified by column chromatography (eluent mixture: 93:7 DCM/MeOH), affording 17.0 mg of pure compound **5 b** as a red solid (0.019 mmol, 44% yield). MS (ESI +): m/z 907.86 $[M+H]^+$. Calculated MS for $C_{48}H_{42}N_8O_7S_2$: 906.26. Purity >85%. ^1H NMR (400 MHz, DMSO-*d6*) δ (ppm) (2:3 isomer ratio) 8.48–8.40 (m, 2H, aromatic H), 8.04–7.86 (m, 3H, aromatic H), 7.78–7.70 (m, 1H, aromatic H), 7.68–7.54 (m, 5H, aromatic H), 7.46–7.24 (m, 6H, aromatic H), 7.10–7.00 (m, 1H, aromatic H), 6.81–6.73 (m, 1H, aromatic H), 6.38 (m, 2H, NH), 6.21 (d, J = 10.4 Hz, 1H, H7 indole), 6.03 (d, J = 17.5 Hz, 0.60H, CH_2 cyclooctyne isomer A), 5.66 (d, J = 18.8 Hz, 0.40H, CH2 cyclooctyne isomer B), 5.05 (d, J = 18.8 Hz, 0.40H, CH_2 cyclooctyne isomer B), 4.58 (d, J = 17.5 Hz, 0.60H, CH_2 cyclooctyne isomer A), 4.30–4.28 (m, 1H, CH), 4.11–4.07 (m, 1H, CH), 3.06–2.94 (m, 2H, CH_2), 2.88–2.67 (m, 3H, CH_2, CH), 2.58–2.55 (m, 1H, CH_2), 2.24–2.16 (m, 1H, CH_2), 1.93–1.89 (m, 3H, CH2), 1.58–1.50 (m, 1H, CH_2), 1.46–1.34 (m, 3H, CH_2), 1.23–1.15 (m, 2H, CH_2). ^{13}C NMR (400 MHz, DMSO-*d6*, some signals are split due to the presence of two isomers) δ (ppm) 163.3, 146.2, 140.8, 140.3, 139.1, 133.9, 133.3, 132.4, 131.6, 129.5, 129.0, 128.6, 128.4, 128.1, 127.6, 126.7, 125.0, 124.4, 123.9, 119.0, 118.4, 114.6, 103.8, 61.1, 60.6, 59.2, 55.4, 54.5, 51.1, 41.2, 34.9, 33.7, 32.3, 28.1, 28.1, 24.2.

Biological Evaluation

Cell Culture

Triple negative breast cancer cells MDA-MB-231 (MDA) were purchased from American Type Culture Collection (ATCC). Cells were grown in DMEM supplemented with 10% fetal bovine serum (FBS), 1% glutamine and 1% penicillin/streptomycin cocktail (all from Gibco); and kept inside a humidified incubator at 37 °C and 5% CO_2.

Cell Treatment and Viability Assay

Viability was assessed using the metabolic OzBlue assay (OzBlue Biosciences). MDA cells were seeded at a confluence of 5000 cells per well, in a 96 well plate, and left to adhere to its surface. The following day, cells were treated with increasing concentrations of **3**, **4 a**, **4 b**, **5 a** and **5 b** up to 500 μM. Each concentration was tested in triplicate, and DMSO (0.1%) was used as the vehicle control. Following 48 hours of treatment, 8 μL of OzBlue reagent was added to each well and the plate was incubated for 2 hours, after which the fluorescence at 460/490 nm was measured using a plate reader (Varioskan Lux). The fluorescence signal was proportional to cell viability and was normalized to the signal of the vehicle control. CC_{50} values and Hill coefficients for the curve fitting of each compound were calculated with GraphPad Prism v10 using the log(inhibitor) vs response–Variable slope (four parameters)

RNA Electro Mobility Shift Assay (REMSA)

The HuR RNA recognition motifs M1 M2 (rM1 M2) recombinant protein was purified as previously described.[5,6,21] For REMSA, the protein was incubated for 90 minutes at room temperature with DMSO (control), TM2 or TM probes **3**–**5** at concentrations ranging from 1 to 100 μM. Afterward, the mixture was incubated with the 5′-DY681-labeled AU-rich RNA probe (0.25 nM) for 10 minutes at room temperature. Samples and controls were loaded on a 12% native polyacrylamide gel which ran for 90 minutes at 80 V. Images were developed with a Typhoon Trio scanner (GE Healthcare) at optimal exposure.

Pull-Down Assay for Protein-TM Probe Interaction

Dynabeads MyOne streptavidin-coated magnetic beads (Life Technologies, 65601) were used for pull-down assays. Beads were firstly washed five times with 700 μL TENT buffer 1x (100 mM Tris-HCl pH 8, 1 mM EDTA, 250 mM NaCl, and 0.5% Triton X-100) and resuspended in 10 μL (per condition) of TENT 1x buffer. Beads were then incubated with **5 a**, **5 b** or biotin (200 μM) in a reaction volume of 40 μL for 1 hour, at 4 °C in a thermoshaker (shaking at 500 rpm). Afterwards, reaction mixtures were incubated with rM1 M2 (50 ng), and incubated for further 1 hour in the same conditions. The control conditions were: (i) streptavidin magnetic beads, (ii) rM1 M2 (iii) streptavidin magnetic beads incubated with biotin, and (iii) streptavidin magnetic beads incubated with biotin and rM1 M2. Following incubation with rM1 M2, reaction mixtures containing the magnetic beads (all apart from ii, rM1 M2 only) were washed five times with 700 μL TENT buffer 1x. Beads were resuspended in 18 μL of TENT buffer 1x and 6 μL of sample buffer 4x (240 mM Tris-

HCl pH 6,8, 400 mM DTT, 8% SDS, 40% glycerol, 0.4% bromophenol blue), and incubated at 98 °C for 10 minutes. Beads were removed from the reaction mixture, while the remaining solution was loaded into a 15% polyacrylamide gel. For the input control (condition ii, rM1 M2 only) only one tenth of the sample was loaded, corresponding to 5 ng of rM1 M2. Samples ran at 120 V for 2 hours, and proteins were transferred into a PVDF membrane for 80 minutes at 330 mA. The membrane was then blocked with 5% milk solution and incubated with a primary anti-HuR antibody (Santa Cruz Biotechnologies, sc-71290, mouse, 1:1000) for 90 minutes at room temperature, followed by 1 hour incubation with an HRP-conjugated anti-mouse secondary antibody (Jackson Immunoresearch Laboratories, 111–035-003, 1:5000). The chemoluminescent signal was detected with Amersham ECL Select from GE Healthcare at ChemiDoc (BioRad).

Supporting Information

The authors have cited additional references within the Supporting Information (Ref. [30,31]).

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Conflict of Interests

The authors declare no conflict of interest.

Data Availability Statement

The data that support the findings of this study are available from the corresponding author upon reasonable request.

Keywords: RNA Binding Proteins · HuR · Tanshinone Mimics · photoaffinity probes · affinity chromatography

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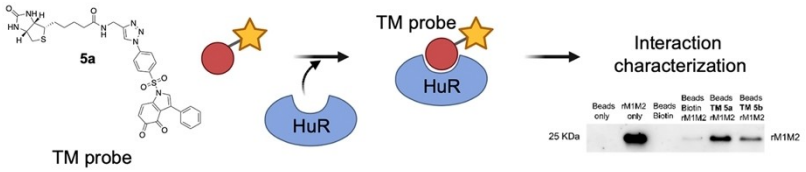

A small array of Tanshinone Mimics (TM) probes with a detection-promoting moiety (either a photoaffinity probe - PAP or biotin) has been synthesized for mechanism of action (MoA) studies on Human antigen R (HuR). Biological and biochemical assays were used to characterize the novel TM conjugates. Among these, biotinylated probe **5a** proved to be the most promising compound.

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1 – 11

Synthesis and Preliminary Evaluation of Tanshinone Mimic Conjugates for Mechanism of Action Studies