Identification and validation of novel small molecule disruptors of HuR-mRNA interaction

Xiaoqing Wu, Lan Lan, David Michael Wilson, Rebecca T. Marquez, Wei-chung Tsao, Philip Gao, Anuradha Roy, Benjamin Andrew Turner, Peter McDonald, Jon A Tunge, Steven A Rogers, Dan A. Dixon, Jeffrey Aubé, and Liang Xu

Supplementary methods

Reagents: HuR antibody was purchased from Santa Cruz Biotechnology, Bcl-2, Msi1, PARP, capase-3 and LC3 antibodies were purchased from Cell Signaling Technology, α-Tubulin and XIAP antibodies were purchased from Sigma-Aldrich and BD Biosciences, respectively.

Protein expression and purification. pTBSG-HuR and pTBSG-RRM1/2 plasmids encoding full-length HuR and the RNA recognition motifs 1 and 2 (RRM1/2, residues G18−N186) of HuR, respectively, were constructed by KU COBRE-PSF Protein Purification Group using human HuR cDNA (NM_001419) and expressed using a T7 promoter. His6-tagged HuR and RRM1/2 proteins were expressed in *E. coli* and purified as described previously. Bcl-xL protein was also produced by KU COBRE-PSF Protein Purification Group using human Bcl-xL cDNA (NM_138578).

AlphaLISA assay. 3'-biotin modified RNA oligos with same sequence as those used in FP assay were purchased from Dharmacon and also same pretreated before binding analysis. The assays were performed in 96-well white 1/2 area plates (Perkin Elmer) with a final volume of 50 μL. For the optimization and determination of K_d, a series concentrations of HuR RRM1/2 were incubated with different concentrations of biotinylated RNA or 25 nM biotinylated RNA in the assay. Subsequently, streptavdin-donor beads and nickel chelate-acceptor beads (Perkin Elmer, 20 μg/mL final concentration) were added and the reaction was placed at RT for 60 min to reach equilibrium. For compound competition assay, multiple doses of compounds were added to the wells first, followed by pre-formed RRM1/2−ARE ^{Msi1} complex (100 nM RRM1/2

protein and 25 nM Msi1 RNA), donor beads and acceptor beads. Measurements were taken after incubation at RT for 2 h. K_d, % of inhibition, IC_{50} and K_i were determined as described in FP assay. In the false positive test, compounds (20 μM final concentration) were incubated with streptavdin-donor beads and biotinylated BSA-acceptor beads from AlphaLISA TruHits kit for 2 h, % of inhibition was calculated by comparing to the DMSO control.

Surface plasmon resonance. The SPR experiments were performed using a BIACORE 3000 (GE Healthcare) equipped with a CM5 sensor chip. The ligands (HuR, 39 kDa; RRM1/2, 21.5 kDa) were immobilized using amine-coupling chemistry. The surfaces of flow cells on the sensor chip were activated for 7 min with a 1:1 mixture of 0.1 M NHS (N-hydroxysuccinimide) and 0.1 M EDC (3-(N, N-dimethylamino) propyl-N-ethylcarbodiimide) at a flow rate of 5 μL/min. Full-length HuR protein and RRM1/2 protein were then applied to the flow cells in 10 mM sodium acetate, pH 4.5, and immobilized to a density of 7200 RU and 3800 RU (response units), respectively. An adjacent flow cell was left blank to serve as a reference surface. All the surfaces were blocked with a 7-minute injection of 1 M ethanolamine, pH 8.0. To collect kinetic binding data, the testing compounds in 20 mM HEPES pH 7.4, 150 mM NaCl, 3 mM EDTA, 0.05% p20 (v/v), 5% DMSO (v/v), were injected over the flow cells at the indicated concentrations at a flow rate of 60 μL/min and at 20°C. The complex was allowed to associate for 4 min and dissociate for 3 min. The curves were calculated from the experimentally observed curves by successive subtractions of signals obtained for the reference surface and signals for the running buffer injected under the same conditions as testing compound.

mRNA stability assay. HCT-116 cells were seeded in six-well plates and treated with 5 μg/mL actnomycin D (Sigma-Aldrich), together with 20 μM CMLD-1, CMLD-2, NC-3, or DMSO control, respectively. Total RNA was isolated at the indicated time points and qRT-PCR was performed as we reported previously using the primers listed in Supporting Information Table 2. Half-life ($t_{1/2}$) was determined from nonlinear regression fit of the data according to a one-phase decay model using Prism 5.0.

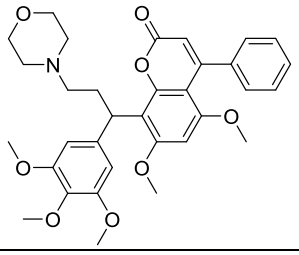
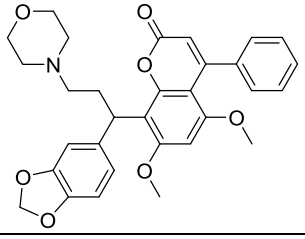
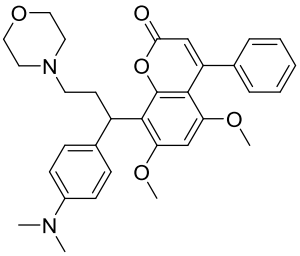
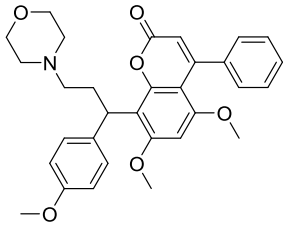
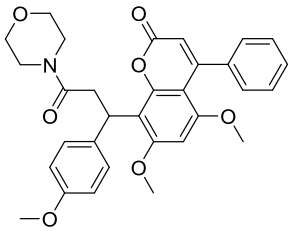
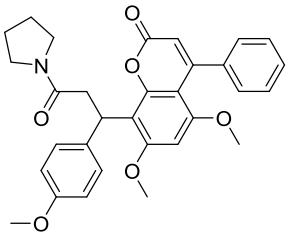
Supplementary figure legends

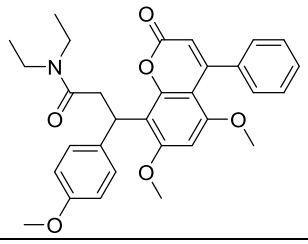
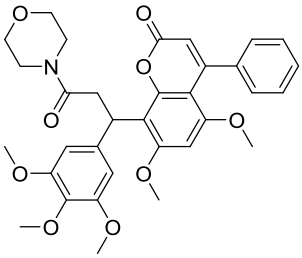
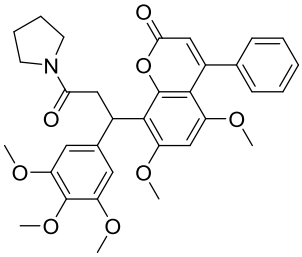
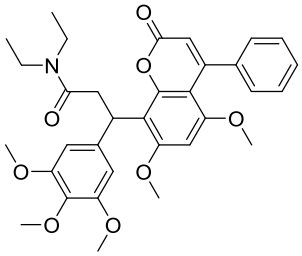
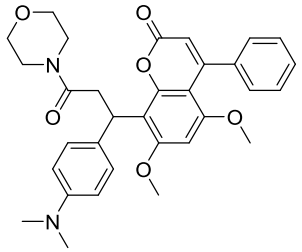
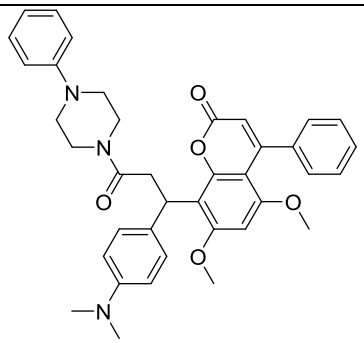
Figure S1. AlphaLISA assay optimization. (A). 16-nt biotinylated ARE^{Msi1} RNA oligo (Msi1-Biotin) and HuR RRM1/2 protein double titration to determine optimal concentrations. Based on the binding curve and signal intensity, 25 nM Msi1-Biotin RNA and 100 nM RRM1/2 protein were optimal. (B). HuR RRM1/2 protein titration with 25 nM Msi1-biotin or random control RNA (Random-Biotin). (C). False positive test of CMLD−6 and NC1−3. Only NC-1 shows very strong inhibition of the signal at the tested concentration, which is consistent with the unexpected low IC_{50} in the competition assay.

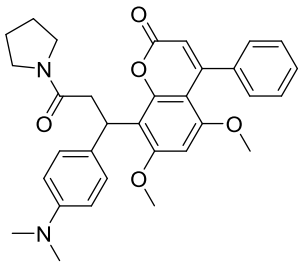
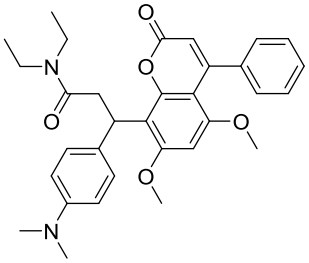
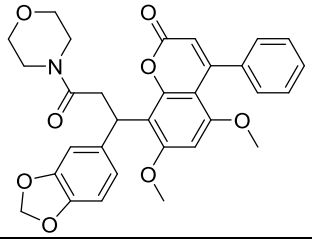
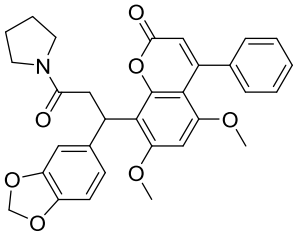
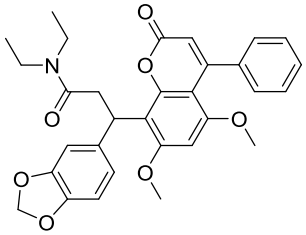
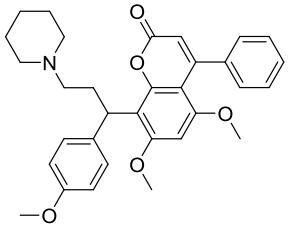
Figure S2. Dose−responses curve of Cluster A compounds and negative controls disrupting HuR−ARE^{Msi1} binding in FP assay using 25 nM HuR RRM1/2 fragment and 2 nM fluorescein-labeled Msi1 RNA. Data are representative of three independent experiments.

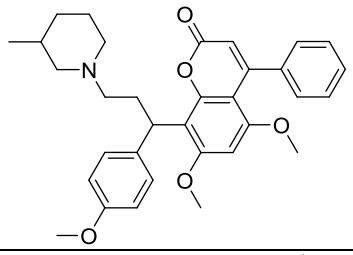
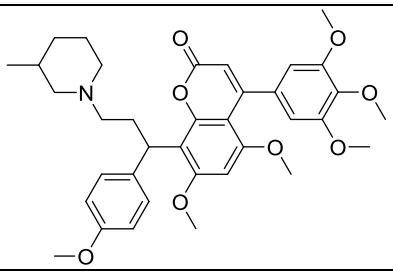
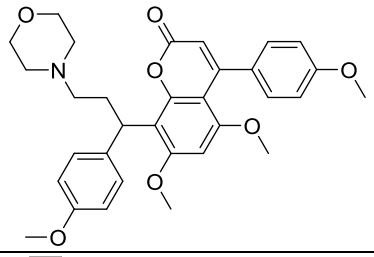
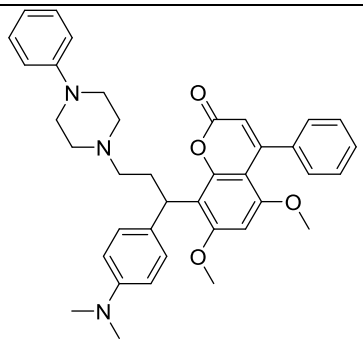
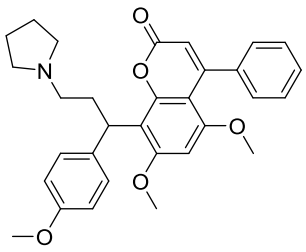
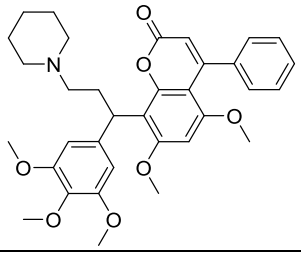
Figure S3. The cytotoxicity of CMLD1−6 and NC1−3 against the human normal colon epithelial cell line CCD 841 CoN. Figures are representative of three independent experiments, IC_{50} Values are mean ± SD from three independent experiments.

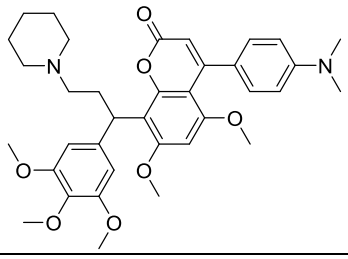
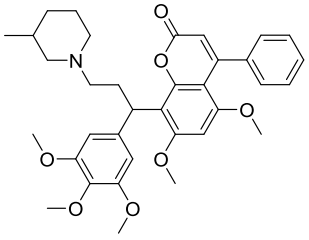
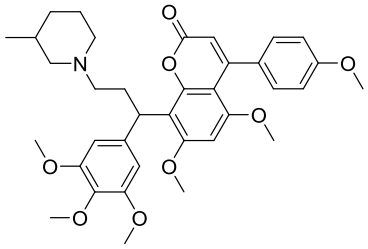
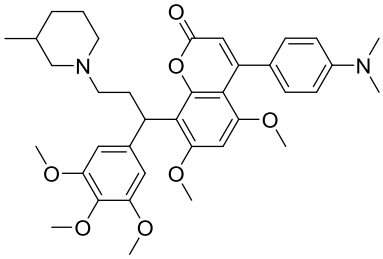
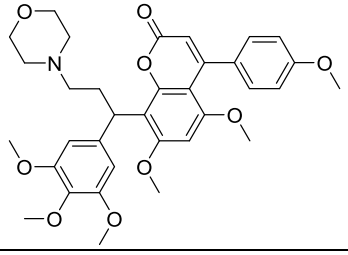
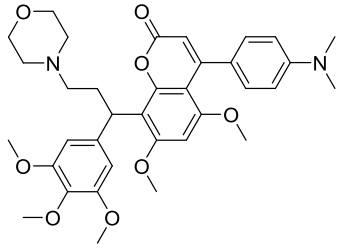
Supporting Information Table 1: cluster A compounds and analogues in the HTS

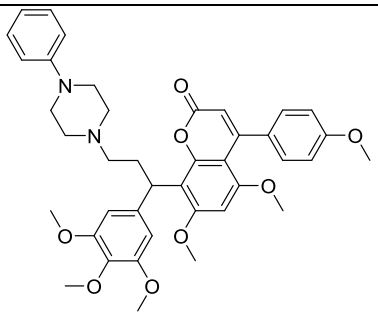
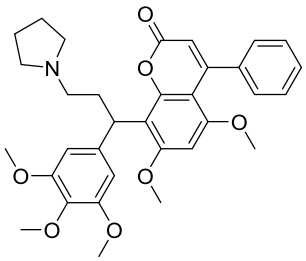
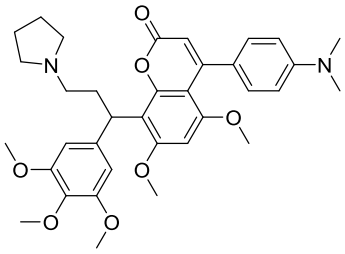
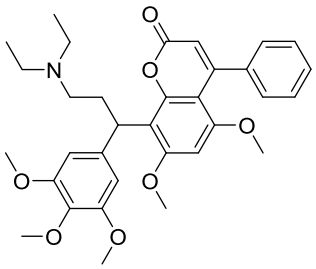
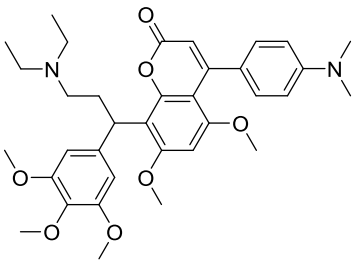
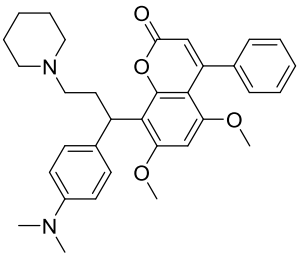
Compound ID	Structure	Percent of inhibition	Active	ID in cluster
KU0104555		2%	No	
KU0104556		2%	No	
KU0104557		-5%	No	
KU0104558		2%	No	
KU0104982		95%	Yes	CMLD-3
KU0104985		84%	Yes	CMLD-2

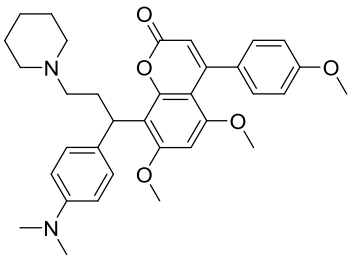
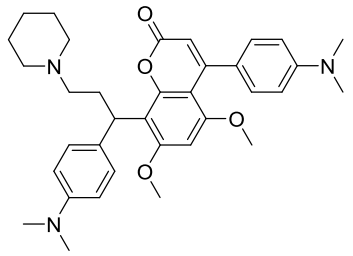
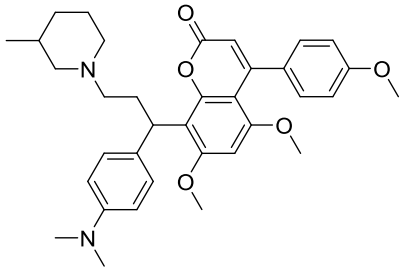
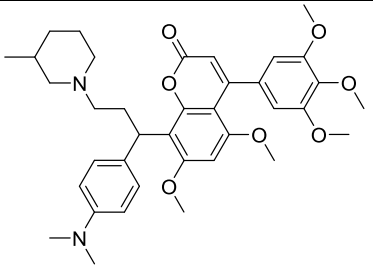
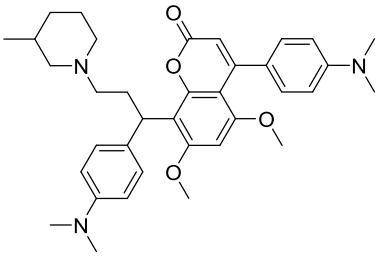
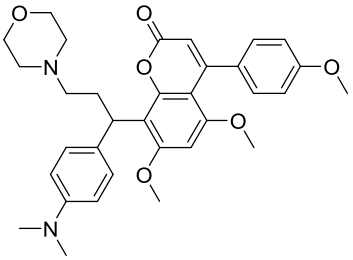
KU0104987		90%	Yes	CMLD-4
KU0104988		-6%	No	NC-3
KU0104991		88%	Yes	CMLD-5
KU0104993		86%	Yes	CMLD-1
KU0104994		2%	No	
KU0104995		-8%	No	

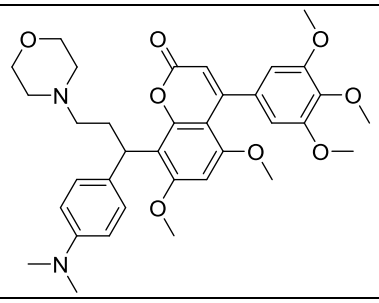
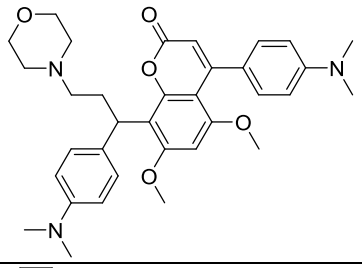
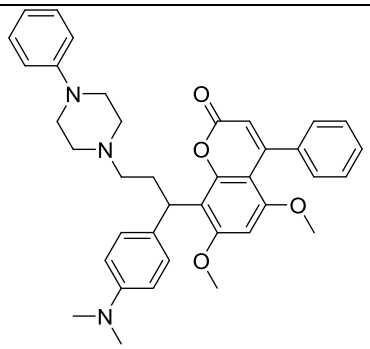
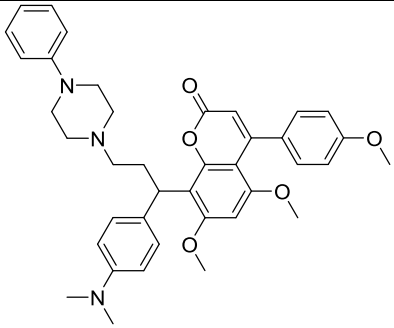
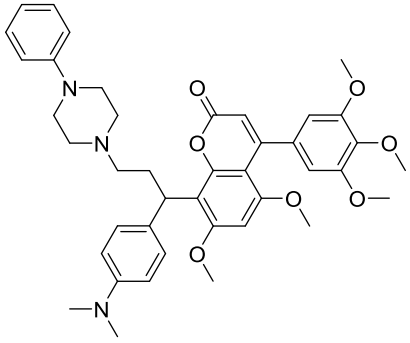
KU0104997		-4%	No	NC-1
KU0104999		6%	No	
KU0105002		-5%	No	
KU0105005		43%	Yes	CMLD-6
KU0105007		-13%	No	
KU0105036		3%	No	

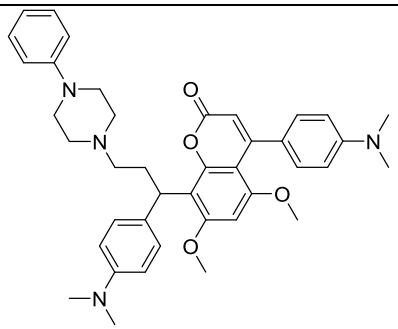
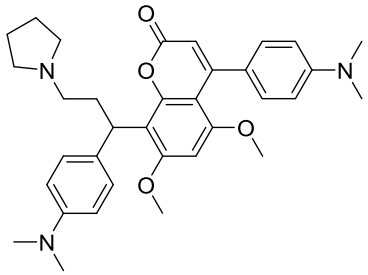
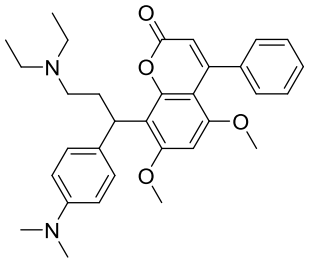
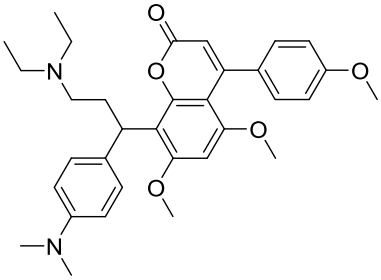
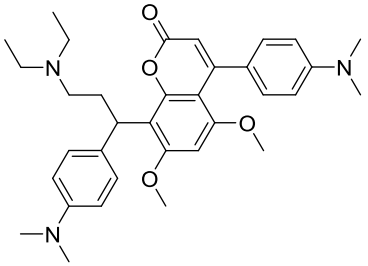
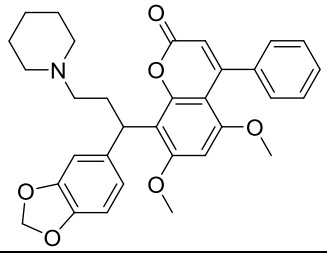
KU0105039		-1%	No	
KU0105041		6%	No	
KU0105043		0%	No	
KU0105045		-10%	No	
KU0105047		-5%	No	NC-2
KU0105051		5%	No	

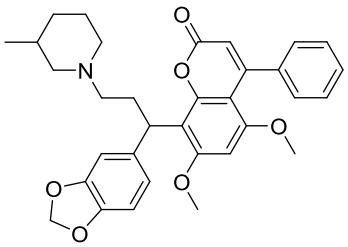
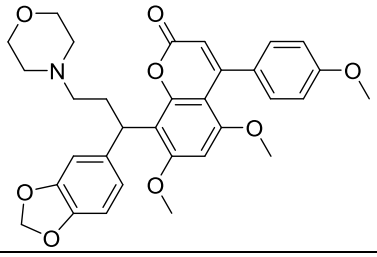
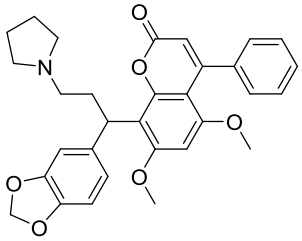
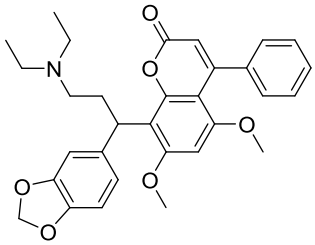
KU0105052		-2%	No	
KU0105054		1%	No	
KU0105056		1%	No	
KU0105057		16%	No	
KU0105060		7%	No	
KU0105061		-1%	No	

KU0105065		4%	No	
KU0105068		0%	No	
KU0105070		0%	No	
KU0105073		3%	No	
KU0105075		0%	No	
KU0105078		-3%	No	

KU0105080		5%	No	
KU0105081		-8%	No	
KU0105084		2%	No	
KU0105085		-2%	No	
KU0105086		3%	No	
KU0105088		2%	No	

KU0105089		3%	No	
KU0105090		2%	No	
KU0105093		-11%	No	
KU0105094		3%	No	
KU0105095		2%	No	

KU0105096		-5%	No	
KU0105099		-10%	No	
KU0105101		-4%	No	
KU0105103		0%	No	
KU0105104		-4%	No	
KU0105106		-2%	No	

KU0105109		-3%	No	
KU0105111		-7%	No	
KU0105116		-5%	No	
KU0105119		-3%	No	

Supporting Information Table 2

Primers	Sequences
Bcl-2 forward	5′-CATGCTGGGGCCGTACAG-3′
Bcl-2 reverse	5′-GAACCGGCACCTGCACAC-3′
Msi1 forward	5′-TTGGCAGACTACGCAGGAAG-3′
Msi1 reverse	5′-TGGTCCATGAAAGTGACGAAGC-3′
XIAP forward	5′-AGTGGTAGTCCTGTTTCAGCATCA-3′
XIAP reverse	5′- CCGCACGGTATCTCCTTCA-3′
GAPDH forward	5′-ATGTTCGTCATGGGTGTGAA-3′
GAPDH reverse	5′-GGTGCTAAGCAGTTGGTGGT-3′

Fig. S1

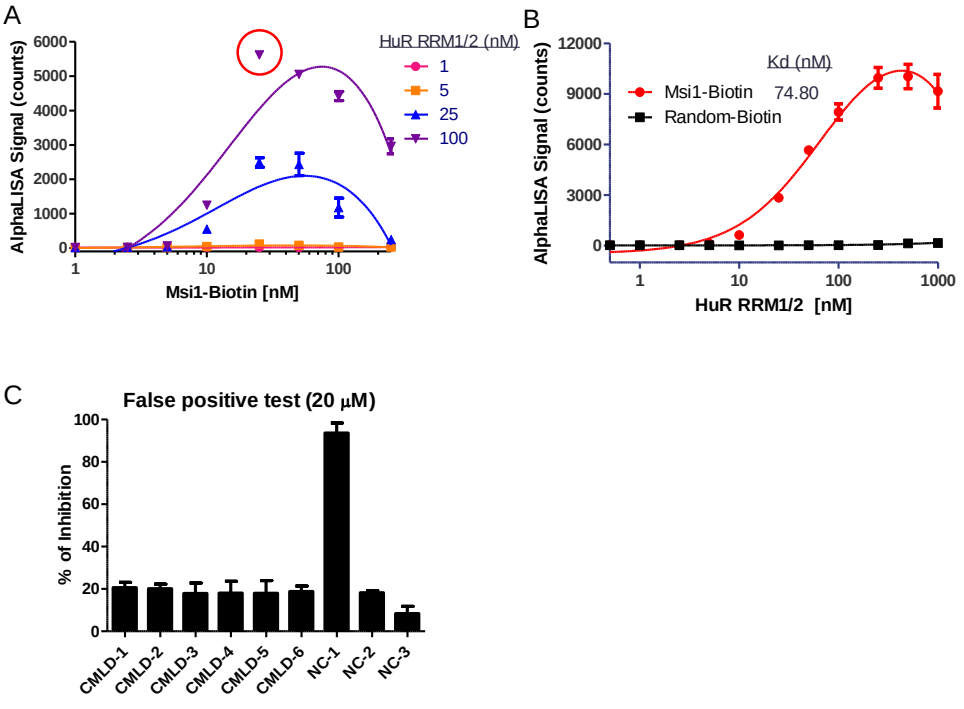

A
AlphaLISA Signal (counts)
6000
5000
4000
3000
2000
1000
0
1
10
100
Msi1-Biotin [nM]
HuR RRM1/2 (nM)
1
5
25
100
B
AlphaLISA Signal (counts)
12000
9000
6000
3000
0
Kd (nM)
Msi1-Biotin 74.80
Random-Biotin
1
10
100
1000
HuR RRM1/2 [nM]
C
False positive test (20 μM)
% of Inhibition
100
80
60
40
20
0
CMLD-1
CMLD-2
CMLD-3
CMLD-4
CMLD-5
CMLD-6
NC-1
NC-2
NC-3

Fig. S2

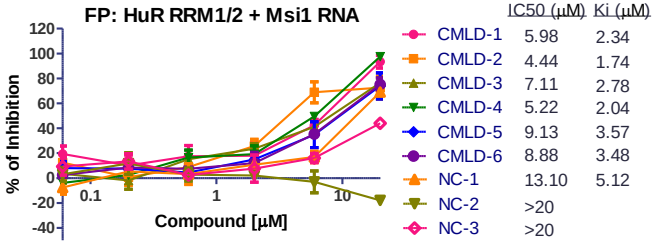
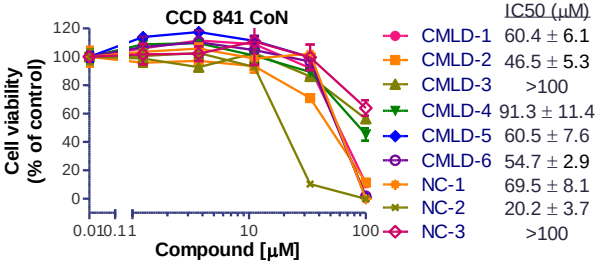

FP: HuR RRM1/2 + Msi1 RNA
% of Inhibition
120
100
80
60
40
20
0
-20
-40
0.1
1
10
Compound [µM]
IC50 (µM)
Ki (µM)
CMLD-1 5.98 2.34
CMLD-2 4.44 1.74
CMLD-3 7.11 2.78
CMLD-4 5.22 2.04
CMLD-5 9.13 3.57
CMLD-6 8.88 3.48
NC-1 13.10 5.12
NC-2 >20
NC-3 >20

Fig. S3


CCD 841 CoN
Cell viability (% of control)
120
100
80
60
40
20
0
0.01
0.1
1
10
100
Compound [µM]
IC50 (µM)
CMLD-1 60.4 ± 6.1
CMLD-2 46.5 ± 5.3
CMLD-3 >100
CMLD-4 91.3 ± 11.4
CMLD-5 60.5 ± 7.6
CMLD-6 54.7 ± 2.9
NC-1 69.5 ± 8.1
NC-2 20.2 ± 3.7
NC-3 >100