Hu Antigen R (HuR) multimerization contributes to glioma disease progression

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SUPPLEMENTAL DATA:

SUPPLEMENTAL FIGURE LEGENDS

Supplemental Figure 1. HuR multimerization in human PDGx cell lines. Examples of HuR protein dimerization/multimerization detected in non-reduced and non-denaturated condition (marked with white bar) versus reduced and denaturated condition (marked with black bar). Note that lanes 1, 2, 3, 4 represent proteins from XD456, JX10, JX6 and JX 12 *PDGx* cell lines. The arrows mark 30, 60, 90 and 120 kDa, respectively.

Supplemental Figure 2. Illustration of the HuR drug discovery approaches and assays used for the selection of compound #5. A) The HuR/cdk AlphaScreen assay was designed to find compounds interfering with HuR hinge region function (the assay was based on the detection of the alteration of HuR phosphorylation at hinge region at residue S202 in the presence of the screening compounds). The assay was not constructed to identify inhibitors of CDK5 dependent phosphorylation but to find inhibitors of Cdk 5 interaction with the HuR hinge. It was specifically set up to find inhibitors of the protein:protein interaction between HuR and Cdk5 by using a high concentration of ATP to reduce false positive hits from kinase inhibitors. Cdk5 was chosen for this assay because it has only one site of interaction with HuR, serine 202 at the hinge region. Briefly, recombinant HuR protein fused to GST binds to Glutathione Donor beads. The Phospho-(Ser) CDK Substrate Antibody, which specifically recognizes the phosphorylated serine in HuR hinge region (pS202) binds to the Acceptor (Protein A) beads. The master AlphaScreen assay was based on the interaction of Donor beads attached to HuR, with protein A beads attached to the phospho specific antibody (a). HuR recognition by N-terminal binding antibody (19F12) served as a control or as a detection of the overall alterations of HuR molecule tertiary structure following interaction with compound. The N-terminal binding antibody (19F12) binds to the Acceptor (Protein A) beads in the secondary AlphaScreen Assay (b). **B)** Examples of positive and negative control values in each plate during AlphaScreen. The averaged Z' factor of 0.73$\pm$ 0.06. The inhibition cut off analysis was based on the Criterion calculated from compound inhibition (>=69.08%). Criterion 69.08% is calculated as average inhibition (13.05%) plus three times standard deviation (56.034%) of compound results. The selected active compounds have been split in two groups: i) active in both master and secondary AlphaScreen assays; ii) active in master AlphaScreen assay but inactive in secondary AlphaScreen assay. Compound #5 belongs to the group (i) of selected compounds. **C)** Illustration of inhibitory dose response of compound #5 in master (left) and secondary (right) AlphaScreen assays.

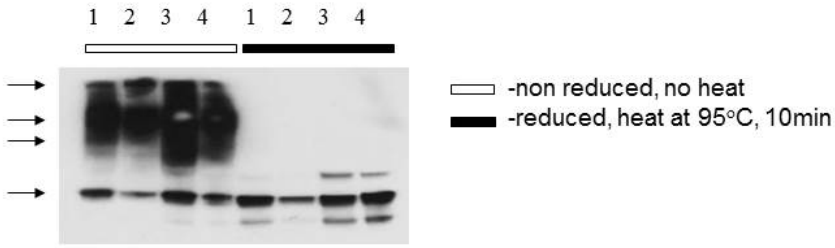
Supplemental Figure 3. MS-444 inhibits HuR multimerization. The known HuR inhibitor, MS-444, is used as a control to demonstrate inhibition of HuR multimer formation in the split Firefly luciferase assay. The red circles represent luminescence signal from U251 cells co-expressing HuR-Nluc and HuR-Cluc constructs and the blue circles represent luminescence signal from U251 cells expressing Fluc construct (control). The MS-444 IC_{50}=87±15 uM (n=4). The compound structure is illustrated in the insert.

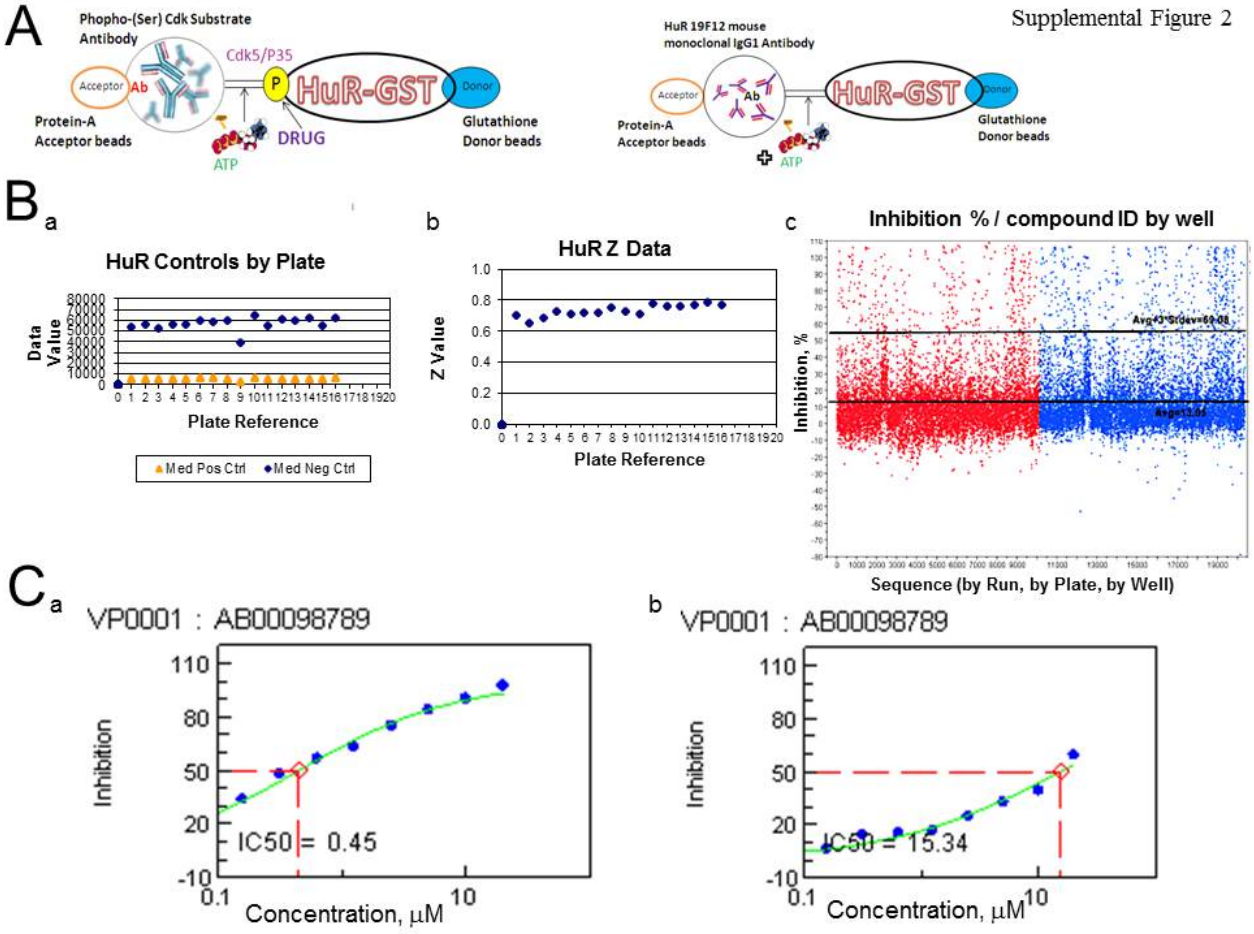
Supplemental Figure 4. Cytotoxicity curves for compounds DHTS and #5 on human PDGx cell lines. The inhibitory dose response curves for DHTS and compound #5 were obtained using PrestoBlue cell viability reagent on XD456 (marked with black circles) and JX10 (marked with black squares) cell lines after 24 hours of treatment. The IC_{50}=1.4±0.2 uM (n=3) and IC_{50}=6±3 uM (n=3) for DHTS and compound #5, respectively, on XD456 cell line. The IC_{50}=3.4±0.5 uM (n=3) and IC_{50}=44±9 uM (n=3) for DHTS and compound #5, respectively, on JX10 cell line. The chemical structure for each compound is illustrated in the insert.

Supplemental Figure 5. Co-expression of endogenous IDH1 and Flag-IDH1-R132H mutant in U251 cells induces the enhancement of HuR /HuR interaction. (Aa) Western blots represent dox-inducible Flag-IDH1-R132H/C/S/wild constructs expression in established U251 cell lines. Flag epitope has been attached to the IDH1-R132H/C/S/wild constructs on N-terminus. **(Ab)** 2D-HG levels after IDH1-R132H/C/S/wild constructs induction. The data has been normalized to the 2D-HG level in cells over-expressing Flag-IDH1-wild construct. **(Ac)** Comparison of HuR/HuR interaction by split luciferase assay in U251 cells following co-expression of endogenous IDH1 plus Flag-IDH1-wild type (as control) or endogenous IDH1 plus Flag-IDH1-R132H mutant. The HuR/HuR interaction increases 1.68±0.07 fold (n=6), the difference is significant, P=0.003). The data has been normalized to the luminescence signal at control condition (endogenous IDH1 plus Flag-IDH1-wild type). **(B**-left**)** HuR immunostaining (with HuR-3A2 antibody, Santa Cruz) in cells co-expressing endogenous IDH1 plus Flag-IDH1-wild type (as control) or endogenous IDH1 plus Flag-IDH1-R132H mutant. Note the granular distribution of HuR protein in the cytoplasmic fraction in cells over-expressing Flag-IDH1-R132H mutant. DAPI has been used to mark cell nuclei. (**B**-right) HuR sub-cellular distribution detected by Western blots from cells co-expressing endogenous IDH1 plus Flag-IDH1-wild type (as control) or endogenous IDH1 plus Flag-IDH1-R132H mutant (Dox-/Dox+ conditions). Tubulin and Lamin A/C have been used to illustrate cytoplasmic and nuclear fractions, respectively.

Supplemental Figure 1

PDGx cell lines, cytoplasmic fraction




A
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Ab
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Donor
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HuR 19F12 mouse monoclonal IgG1 Antibody
Acceptor
Ab
HuR-GST
Donor
Protein-A Acceptor beads
ATP
Glutathione Donor beads
B
a
HuR Controls by Plate
Data Value
Plate Reference
Med Pos Ctrl
Med Neg Ctrl
b
HuR Z Data
Z Value
Plate Reference
c
Inhibition % / compound ID by well
Inhibition, %
Sequence (by Run, by Plate, by Well)
C
a
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Inhibition
IC50 = 0.45
Concentration, µM
b
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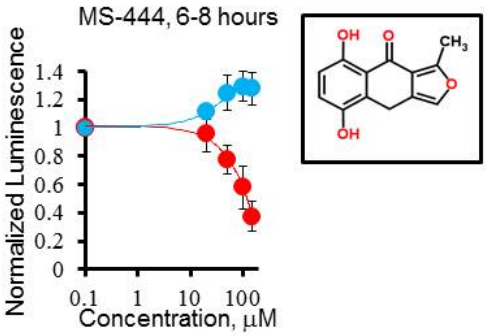
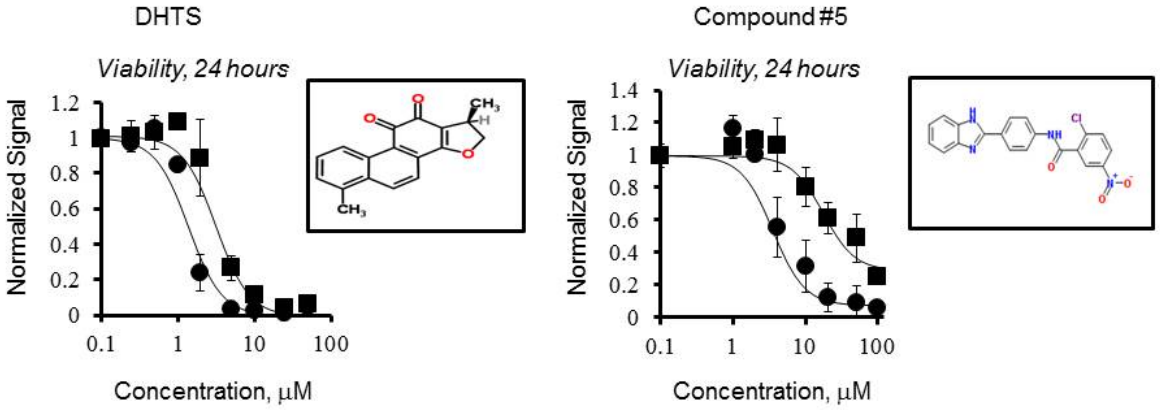
Inhibition
IC50 = 15.34
Concentration, µM

Supplemental Figure 3




DHTS
Viability, 24 hours
Normalized Signal
1.2
1
0.8
0.6
0.4
0.2
0
0.1
1
10
100
Concentration, μM
Compound #5
Viability, 24 hours
Normalized Signal
1.4
1.2
1
0.8
0.6
0.4
0.2
0
0.1
1
10
100
Concentration, μM

Supplemental Figure 5