Selective inhibition of ZCCHC11/ZCCHC6 TUTases with genetic and pharmacological tools supports a role in Glioblastoma cell growth

Robinson Triboulet, Khikmet Sadykov, Jessica L. Johnson, Andrew R. Snyder, Sarah K. Knutson, Pavan Kumar, Christopher B. Mayo, Dillon Hawley, Andrew Madanjian, Ross L. Stein, David M. Wilson, Darren M. Harvey, Shomir Ghosh, Robert M. Campbell. Twentyeight-Seven Therapeutics, Watertown, MA, US.

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Introduction

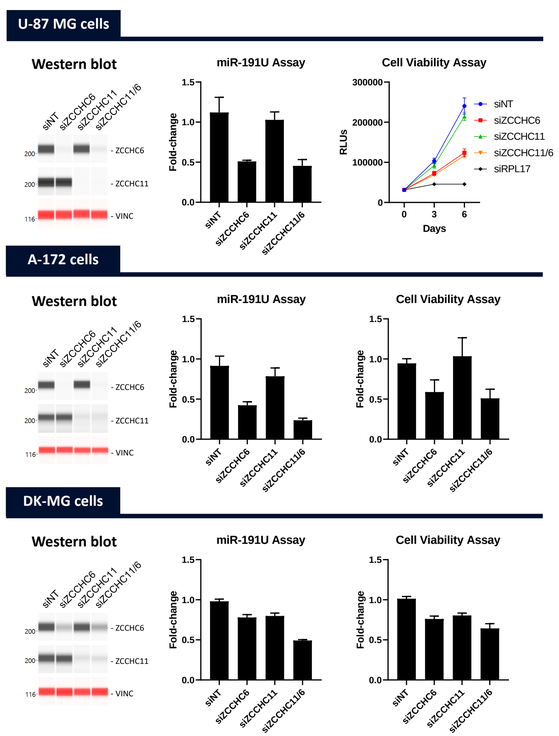
Terminal uridyltransferases (TUTases) ZCCHC11 and ZCCHC6 catalyze 3' uridylation of both protein-coding and noncoding RNA[1]. TUTase-mediated uridylation of RNA 3' ends has been implicated in many biological processes including histone mRNA turnover, RNA decay, RNA surveillance and microRNA biogenesis[1]. Recent evidence indicates that TUTases can play a role in cancer[2]. Kim et al. showed that genetic perturbation of ZCCHC11/6 expression disrupts cell proliferation of both immortalized and patient-derived primary Glioblastoma cell lines[2]. Here we confirm that genetic inhibition of ZCCHC11/6 can decrease viability of U-87 MG and A-172 Glioblastoma cell lines and identify DK-MG as another Glioblastoma cell line with sensitivity to ZCCHC11/6 knockdown. We report the first novel, potent, and selective inhibitor of ZCCHC11/6, TS-1, and its less active enantiomer TS-2, and show that pharmacological inhibition of TUTases with TS-1 reduces cell viability and microRNA uridylation and increases cell cycle arrest and apoptosis in Glioblastoma cell lines.

Material and Methods

siRNA transfection, Western blot, miR-191 assay and phenotypic endpoints: U-87 MG (ATCC), A-172 (ATCC) and DK-MG (JCRB) cells were reverse-transfected using RNAiMAX (Thermo Fisher) with ON-TARGETplus non-targeting siRNA pools (Horizon). CellTiter-Glo assay (Promega) was used to evaluate cell viability. Protein expression was evaluated by Western blot on a Simple Western Jess instrument using 66-440 kDa Jess/Wes Separation Modules (ProteinSimple). miR-191 and miR-191U assays (Somagenics) were used to measure expression of canonical miR-191 and its mono-uridylated form in total RNA. Guava Cell Cycle Reagent and Guava Nexin Reagent were used with a Guava easyCyte HT instrument (Luminex) to evaluate cell cycle and apoptosis.

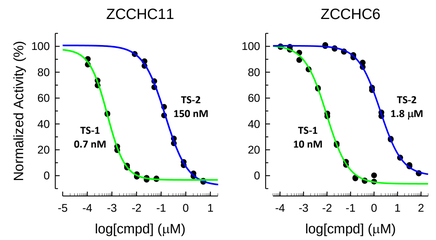
Enzymatic Assays: Dose responses were generated against recombinant ZCCHC11 (residues 212-1420, 100 pM) and ZCCHC6 (full length, 300 pM) using RNA (30 nM) and UTP (40 μM) substrates in an assay with a direct fluorescence readout produced by single nucleotide addition to the RNA substrate. Two replicates are shown for each concentration, and IC_{50} were derived from a four parameter Logistics fit (SigmaPlot), no parameters constrained. Enzyme was expressed in HEK293 cells and purified by tandem affinity chromatography.

1. Genetic Inhibition of ZCCHC11/6 Reduces Viability of Glioblastoma Cell Lines



Knockdown of TUTases caused reduced cell viability in Glioblastoma cell lines U-87 MG (top panels), A-172 (middle panels) and DK-MG (bottom panels). For each cell line, siRNA targeting ZCCHC11 or ZCCHC6 mRNA caused a reduction of the corresponding protein (left). miR-191 mono-uridylation (middle), used here as a surrogate marker of uridylation, and cell viability (right) are driven by ZCCHC6 protein knockdown in U-87 MG and A-172 cells, while these endpoints are driven by both proteins in DK-MG cells. Cell viability (N=6) was evaluated at three and six days after siRNA transfection (U-87 MG) or seven days after siRNA transfection (A-172, DK-MG). Protein expression and mono-uridylation of miR-191 (N=3) were evaluated at six days after siRNA transfection (U-87 MG) or seven days after siRNA transfection (A-172, DK-MG).

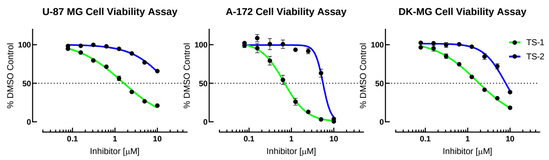
2. Potency of Compounds Against Recombinant ZCCHC11/6



TUTase inhibitor TS-1 represses uridylation activity of recombinant ZCCHC11 (left) and ZCCHC6 (right) proteins in vitro, whereas TS-2, the enantiomer of TS-1, is nearly 200-fold less active against both recombinant enzymes (IC_{50} are indicated on the figure). TS-1 was >1000-fold selective over other terminal nucleotidyltransferases (TENTs) TENT4A, TENT4B, and TENT6.

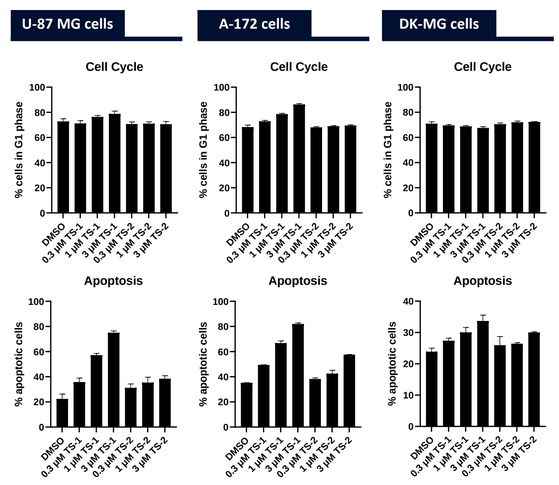
3. Pharmacological Inhibition of ZCCHC11/6 Reduces Viability of Glioblastoma Cell Lines

Compounds	Z11 Enzyme Assay IC_{50} (μM)	Z6 Enzyme Assay IC_{50} (μM)	Cell Viability Assay IC_{50} (μM)			miR-191U Assay IC_{50} (μM)		
			U-87 MG	A-172	DK-MG	U-87 MG	A-172	DK-MG
TS-1	0.0007	0.001	1.57	0.71	1.55	0.023	0.013	0.035
TS-2	0.15	1.8	> 10	5.60	8.21	> 3	> 3	> 3



TUTase inhibitor TS-1 reduces cell viability and miR-191 mono-uridylation in U-87 MG, A-172 and DK-MG cells. In contrast, its less active enantiomer TS-2 is less potent on cell viability and on mono-uridylation of miR-191U. Top table shows cell viability and miR-191 mono-uridylation (miR-191U) IC_{50} for the three Glioblastoma cell lines. miR-191 mono-uridylation was evaluated three days after addition of compounds (N=3). Bottom dose-response curves for TS-1 and TS-2 were generated from 7-day cell viability assays (N=3). RLUs: relative luminescence units.

4. Pharmacological Inhibition of ZCCHC11/6 Induces Cell Cycle Arrest and Apoptosis



TUTase inhibitor TS-1 induces a G1 phase arrest and apoptosis in U-87 MG (left panels) and A-172 cells (middle panels), and apoptosis only in DK-MG cells (right panels). The less active enantiomer TS-2 does not induce cell cycle and apoptosis as potently as TS-1. Phenotypic endpoints were evaluated three days after compound addition (N=3 for U-87 MG, N=2 otherwise).

Discussion and Conclusion

Previously Kim et al reported that U-87 MG and A-172 Glioblastoma cell lines were sensitive to knockdown of ZCCHC11 and ZCCHC6[2]. We confirmed these results as well as the ZCCHC6 genetic dependency reported in DepMap for the DK-MG cell line[3]. By developing a novel inhibitor of ZCCHC11/6, we showed that pharmacological inhibition of uridylation, as demonstrated by the decrease of the mono-uridylated form of miR-191, results in reduced cell viability in these three Glioblastoma cell lines, thereby confirming that U-87 MG, A-172 and DK-MG are vulnerable to inhibition of ZCCHC11/6. We are currently optimizing the pharmacokinetic properties of these compounds for exploration in vivo.

References:
1: "A tale of non-canonical tails: gene regulation by post-transcriptional RNA tailing." Yu S, Kim VN. Nat Rev Mol Cell Biol. 2020 Sep;21(9):542-556.
2: "A Mechanism for microRNA Arm Switching Regulated by Uridylation." Kim H, Kim J, Yu S, Lee YY, Park J, Choi RJ, Yoon SJ, Kang SG, Kim VN. Mol Cell. 2020 Jun 18;78(6):1224-1236.e5.
3: https://depmap.org/portal/