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Genetic and pharmacological inhibition of TUT4/TUT7 sensitizes AML cells to BCL-2 inhibition

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Introduction

Dysfunctional RNA processing has been observed in leukemia and lymphoma[1] implicating potential opportunities as a therapeutic target. The terminal uridyl-transferases (TUTs) are a family of enzymes that modify RNA post-transcriptionally. TUT4/7 (ZCCHC11/6) mono and poly-uridylate the 3'-end of a wide range of RNAs and, as a consequence, are implicated in RNA decay, histone mRNA regulation, viral RNA sensing, and miRNA biogenesis[2]. Our study evaluated the effects of genetic and pharmacological inhibition of TUT4/7 in acute myeloid leukemia (AML) cell line THP-1 and primary AML samples and supports the potential therapeutic benefit of combining TUT4/7[3] and BCL-2 inhibitors.

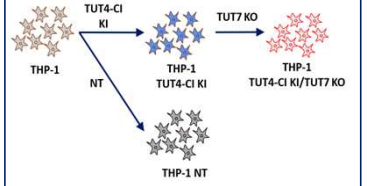
Materials and methods

CRISPR nucleofection, Western blot, miR-191 assay, and phenotypic endpoints: THP-1 cells (ATCC) were nucleofected (Lonza) with CRISPR sgRNAs, Cas9, and donor DNAs (Thermo/Synthego) to generate knock-in (KI) catalytic inactive[4] TUT4 (TUT4-CI) and combination TUT4-CI KI/TUT7 knock-out (KO) cells. 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. miRNA expression was analyzed using Qiagen miRcury LNA primers. Guava Nexin Reagent was used with a Guava easyCyte HT instrument (Luminex) to evaluate apoptosis.

Primary cell assays: Apoptosis was read out at 72 hrs by FACS staining for annexin V and DAPI positive cells. Statistical analysis was performed by Student's *t* test.

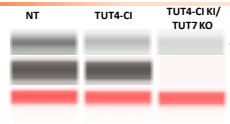
RNA sequencing and gene set enrichment analysis (GSEA): RNA extraction of THP-1 AML cell lines was performed in duplicate and sequenced at Azenta Life Sciences. Reads were aligned against the *Homo sapiens* GRCh38 reference genome (Ensembl 104) using salmon. Differential gene expression was performed using the tximport-DESeq2 workflow (Bioconductor 3.15 / R 4.2). GSEA was performed using fgsea with MSigDB 7.5.1. Similar trends in differential expression and GSEA were observed with independent clones (not shown).

1. Generation of THP-1 cells harboring genetically inhibited TUT4/7

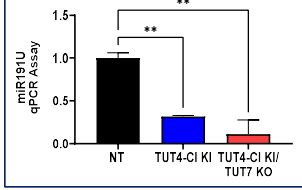


THP-1 cells containing dual genetic inhibition of TUT4/TUT7 were generated by sequential TUT4-CI KI followed by TUT7 KO.

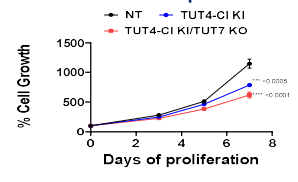
2. Genetic inhibition of TUT4/7 in THP-1 cells reduces growth



Reduction of uridylation

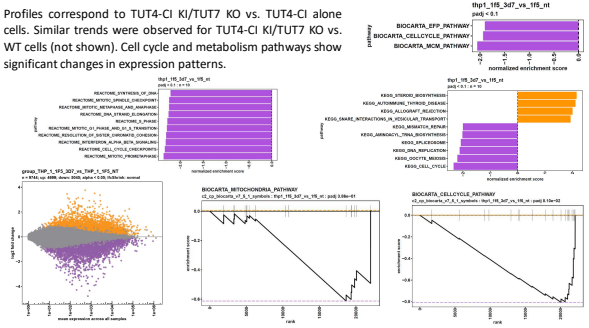


Reduced cell proliferation



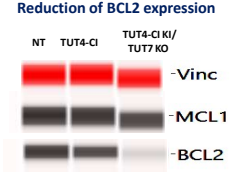
3. GSEA: genetic inhibition of TUT4/7 in THP-1 alters gene expression

Profiles correspond to TUT4-CI KI/TUT7 KO vs. TUT4-CI alone cells. Similar trends were observed for TUT4-CI KI/TUT7 KO vs. WT cells (not shown). Cell cycle and metabolism pathways show significant changes in expression patterns.

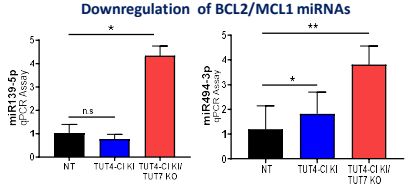


4. Dual genetic inhibition of TUT4/7 sensitizes THP-1 cells to venetoclax

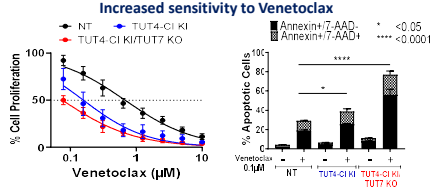
Reduction of BCL2 expression



Downregulation of BCL2/MCL1 miRNAs



Increased sensitivity to Venetoclax

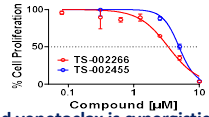


Expression of BCL2 and MCL1 are reduced in TUT4-CI KI and TUT-CI KI/TUT7 KO cells while expression of regulatory miRNAs miR139-5p and miR494-3p are increased. Uridylation deficient cells are more sensitive to venetoclax than control cells.

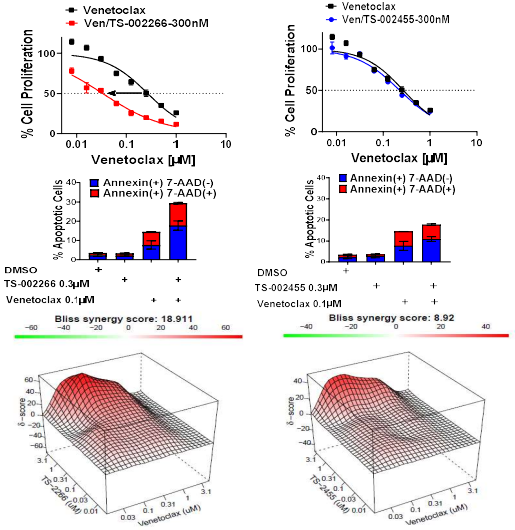
5. TUT4/7 inhibitor shows synergistic combinatorial effect with venetoclax

TS-002266 and weakly active enantiomer TS-002455

Biochemical Assay IC_{50} (μM)	TUT4	TUT7
TS-002266	0.0007	0.0096
TS-002455	0.15	1.8

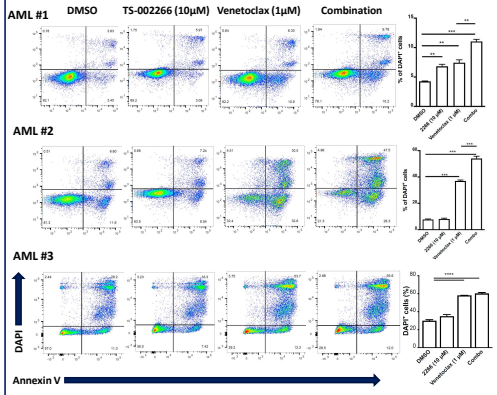


Combination of TS-002266 and venetoclax is synergistic



TUT4/7 inhibitor compound TS-002266, but not its enantiomer TS-002455, potentiates the activity of venetoclax in THP-1 AML cells as read out by cell titer glo and annexin V

6. Treatment of patient derived primary AML samples with TUT4/7 inhibitors



The anti-leukemic effect of TS-002266 was evaluated as single agent and in combination with venetoclax in 8 primary AML samples. Cell death was assessed at 72hrs by annexin V/DAPI staining. Overall results showed a statistically significant synergistic effect of the combination treatment in 6 out of 8 primary samples regardless their sensitivity to each single agent. Representative FACS plots and summary bar graphs are presented for three models.

Discussion and conclusion

Dysregulation of RNA stability, processing, and metabolism have been found to have an important role in leukemogenesis and cancer proliferation and progression[1]. RNA binding proteins play a major role in this regulatory process and have been proposed as potential therapeutic targets for drug development in oncology[1, 5]. TUT4/7 play an important role in post-transcriptional regulation of mRNAs as well as miRNA biogenesis and represent a novel and attractive target with the potential to offer therapeutic benefit in oncology[2, 4]. In these studies we demonstrated that TUT4/7 inhibition can impact metabolism, cell cycle, and mitochondrial stability, thereby sensitizing AML cell lines and primary cells to venetoclax. Our findings indicate a dual TUT4/7 inhibitor is necessary owing to the complementary activity of these enzymes in AML cells. While TS-002266 shows promising *in vitro* activity this compound has weak *in vivo* properties. A better understanding of the *in vitro* to *in vivo* translation will be required to confirm the potential of this type of inhibitors to support therapeutic benefit.

References:
1. Prieto, C., and Kharas, M.G. (2020) RNA regulators in leukemia and lymphoma. *Cold Spring harb Perspect Med* 10(5)
2. Zigackova, D., and Vacacova, S. (2018) The role of 3' end uridylation in RNA metabolism and cellular physiology. *Phil Trans R Soc B* 373
3. Patent WO2021178420, Ericsson, A.M., Ghosh, S., Harvey D.M. Published 2021
4. Labno, A., Warkocki, Z., Kulinski, T., Krawczyk, P.S., Bijata, K., Tomecki, R., Dziembowski, A. (2016) Perlman syndrome nuclease DIS3L2 controls cytoplasmic non-coding RNAs and provides surveillance pathway for maturing snRNAs. *Nuc Ac Res*, 44(21)
5. Kang, D., Lee, Y., Lee, J-S. (2020) RNA-binding proteins in cancer: functional and therapeutic perspectives. *Cancers* 12