RNA Regulatory Disruption by 3'-dATP: A Novel Approach to Inhibit Ribosome Biogenesis in Cancer

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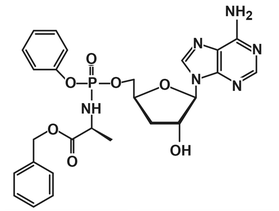
Background

Ribosome Biogenesis & Cancer

- Ribosomes, a complex ensemble of RNA and proteins, play a key role in cell survival, growth, and proliferation
- Ribosome biogenesis (RiBi) is a complex function governed by precise checkpoints and surveillance mechanisms which may become dysregulated in cancer, leading to tumor growth and therapeutic resistance
- Regulation of RNA through polyadenylation is a key post-transcriptional mechanism that influences mRNA metabolism[1]
- Poly(A) tail length of mRNAs, particularly those encoding ribosomal subunits and components of the translational apparatus, is critical for efficient production of proteins
  - These mRNAs are often categorized as 5' terminal oligopyrimidine tract (5'TOP) mRNAs
  - Impairment of 5'TOP mRNA translation can directly impact ribosome biogenesis and function, which in turn can halt cellular proliferation; a hallmark of cancer progression[2,3]
- Targeting polyadenylation machinery to influence the poly(A) tail length of mRNAs encoding ribosomal subunits could be a promising strategy to disrupt the aberrant protein synthesis that supports cancer cell growth
- Ribosome-targeted therapy could provide a promising treatment for cancer

NUC-7738: ProTide transformation of 3'-dA



Phosphoramidate | Nucleoside (3'-dA)

- Generates sustained intracellular levels of anti-cancer metabolite 3'-dATP
- Influences RNA regulatory processes, including poly(A) tail length
  - Leads to alternative polyadenylation (APA) and splicing
  - Resulting in impaired cellular responses

Figure 1: NUC-7738: 3'-deoxyadenosine (3'-dA) phosphorylated and protected with a phosphoramidate moiety attached at the 5'-position

Aim: to investigate the impact of 3'-dATP on RNA regulation and RiBi utilizing a novel bioinformatic pipeline

Methods

Cell Culture

- Melanoma (A375, MeWo, CHL1, SK-MEL28) and renal cell carcinoma (786O & 769P) cell lines were treated with IC_{50} levels of NUC-7738. Cells were harvested 24 hours post-treatment for RNA-sequencing, for protein analysis whole cell lysates were extracted at 24, 48, 72 and 96 hours. All experiments were carried out on 3 biologically independent replicates.

Paired Biopsies

- Patient paired tumor biopsies were collected from 5 cutaneous melanoma patients treated with 1125 mg/m^2 NUC-7738 on days 1, 8 & 15 of a 21-day cycle in combination with 200mg pembrolizumab on day 1
- Biopsies were collected pre-(SCR) and post-(OTB) drug infusion (≤6h post infusion) and preserved in Zymo RNA/DNA Shield (Zymo, Cat#R1100)

RNA Extraction

- RNA extracted from cell lines using RNeasy kits (Qiagen 74004)
- Paired patient biopsies, macroscopically assessed for viable tumor, were homogenized using ceramic beads and Precellys 24 homogenizer. RNA isolated using Quick-RNA-Mini kit.

Long-read RNA-Seq library preparation

- Sequencing libraries for PCR-cDNA sequencing were constructed utilizing SQK-PCS111.24 kit from Oxford Nanopore Technologies

RNA seq data processing and analysis

- Sequencing libraries were processed using R9.4.1 flow cells on PromethION P2 sequencer by Nanopore. The sequenced Fast5 files were basecalled using Guppy v6.0.6, employing the settings --fast5_out and --trim_strategy none. Fast5 files were analyzed with the tailfindr package in R4 to estimate poly A tail lengths for each read.
- For gene expression analysis, basecalled FastQ files were aligned to Gencode v41 transcriptome reference using minimap25 v2.17 aligner. Aligned FastQ files were processed through pychopper to identify and orient full-length reads based on their barcodes. Gene-level expression quantification was conducted using Salmon6 in long-read counting mode (-ont). Differential gene expression was assessed with DeSeq2 package in R.

Poly(A) tail analysis

- Tail lengths extracted from tailfindr output together with unique read_id and transcriptome aligned .bam files. Jupyter Notebook environment with Python programming language was employed to process graphs and execute statistical analysis.

JESS capillary Western blots

- Whole cell protein lysates were probed with RPL5, RPL11, RPS3, RPL6 and YAE1 specific antibodies and analyzed by automated JESS Western blot

Multiplexed IF

- Multiplexed immunofluorescence (IF) was carried out on biopsies using the in-house optimized automated methods and the Leica BOND RX autostainer. Each antibody concentration was optimized using a wide range tissue TMA and single staining and multiplexed staining prior to staining biopsies.

Results

NUC-7738 causes a global reduction in poly(A) tail length in cell lines and biopsies

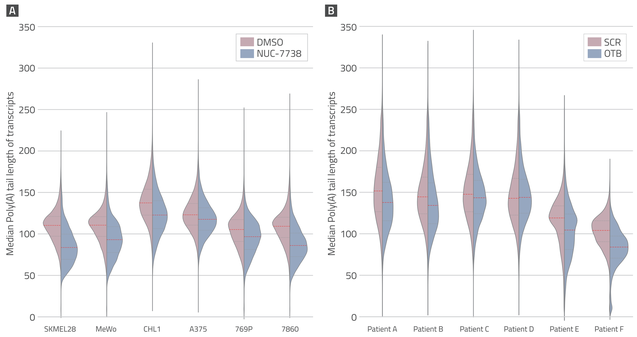


Figure 2: Poly(A) tail changes in cancer cell lines (A) treated with NUC-7738 and paired biopsies from patients (B) treated with NUC-7738 + pembrolizumab. Violin plots depicting the median poly(A) tail length of transcripts from melanoma and renal cell cancer cell lines and paired biopsies. Each plot represents a different cell line or patient paired biopsy, with the median value denoted by a dashed red line. The width of each 'violin' indicates the density of data points at different lengths.

- NUC-7738 caused a shortening of poly(A) tails (blue vs red) in cell lines treated with NUC-7738 and patients' paired biopsies treated with NUC-7738 and pembrolizumab

NUC-7738 shortens poly(A) tails of 5'TOP mRNA and SNHG non-coding RNAs

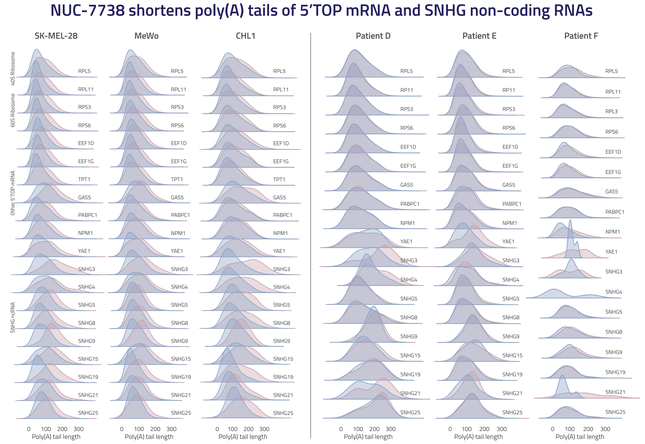


Figure 3A: Ridgeline Plots of Poly(A) Tail Length of genes in cell lines and patients' paired biopsies. The genes are representative of 5'TOP mRNA genes and SNHG non-coding RNA. The x-axis quantifies the poly(A) tail length in nucleotides, while the y-axis represents the kernel density estimation of gene expression.

Results *(cont)*

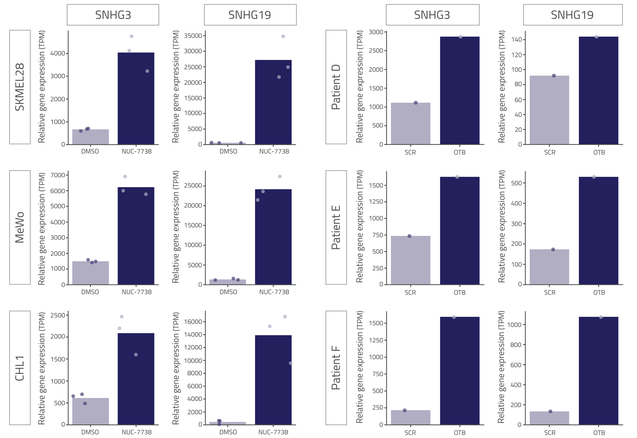


Figure 3B: Transcript levels of SNHG3 and SNHG19 across multiple cell lines and patient paired biopsies. The figure presents bar plots of transcript per million (TPM) measurements for SNHG3 and SNHG19 in a variety of human cell lines treated with NUC-7738 and paired patient biopsies.

- NUC-7738 reduces poly(A) tail length of all 5'TOP genes (median reduction of 15 adenines)
- NUC-7738 reduces poly(A) tail length of SNHG non-coding RNAs (median reduction of 25 adenines)
- NUC-7738 reduces poly(A) tail length of PAXT lncRNAs, SNHG3 and SNHG19, by ~50 adenines, accompanied with an increase in transcript abundance

NUC-7738 reduces the protein levels of ribosomal subunits and YAE1

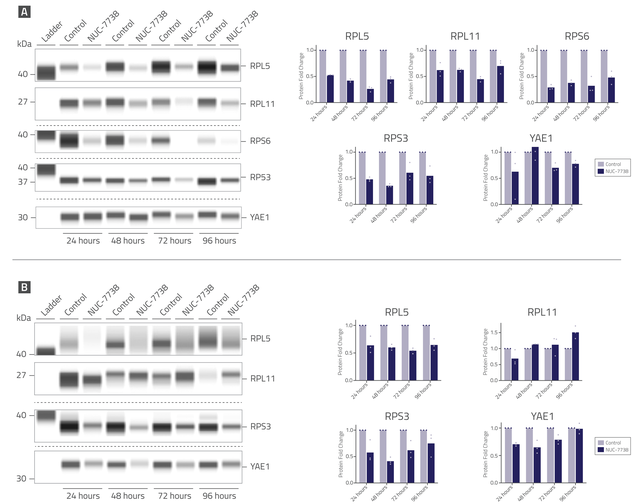


Figure 4: Ribosomal subunit proteins in melanoma cell lines. (A) MeWo and (B) SK-MEL-28 cell lines. Each data point represents an independent biological replicate (n=3).

- NUC-7738 reduces expression of ribosomal subunits proteins by up to 70% across all cell lines
- Expression of YAE1, a regulator of RiBi, also decreased, indicative of impaired RiBi
- These data suggest NUC-7738 targets ribosomal subunits proteins

NUC-7738 + pembrolizumab reduces RPS3 and YAE1 protein

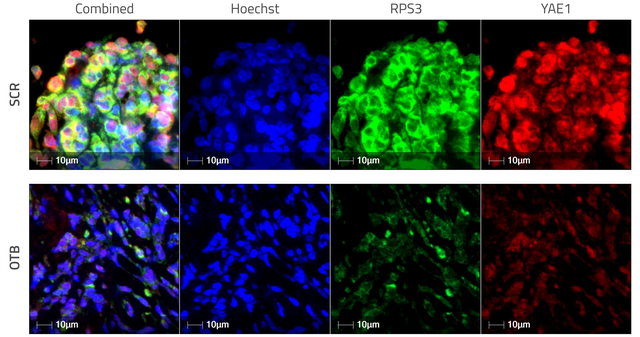


Figure 5: RPS3 and YAE1 protein expression in representative patient paired biopsy treated with NUC-7738 + pembrolizumab. Hoechst staining represents cell nuclei.

- NUC-7738 + pembrolizumab reduces the expression of ribosomal protein RPS3 and YAE1 confirming cell line data

CONCLUSION

A Cancer cell without NUC-7738 | **B Treatment with NUC-7738**

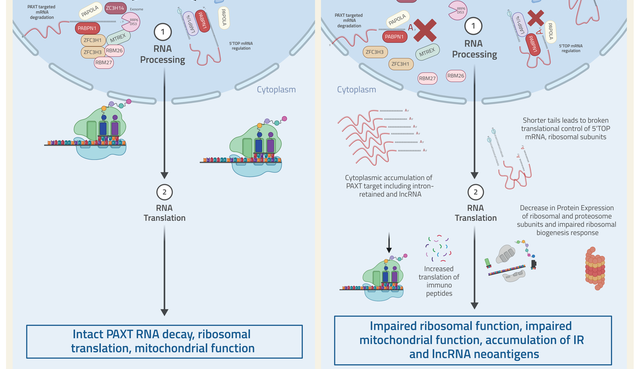


Figure 6: Proposed mechanisms of action of NUC-7738 contributing to impaired ribosome biogenesis.
A) In the absence of NUC-7738, RNA processing and translation proceed regularly, maintaining intact PAXT RNA decay, and ribosomal subunits translation.
B) NUC-7738 generates anticancer metabolite 3'-dATP. Incorporation of 3'-dATP by Poly(A) Polymerase results in the premature termination of poly(A) tail elongation. This inhibition disrupts the poly(A) tail exosome targeting (PAXT), causing an accumulation of SNHG gene transcripts and intron-retained transcripts. Reduction of poly(A) tail length impairs the translational control of protein-coding ribosomal mRNA. Consequently, there is a decrease in protein expression of ribosomal subunits, leading to inhibition of ribosomal biogenesis and protein translation, as well as impaired ribosomal function.

- NUC-7738 significantly modulates RNA stability, particularly affecting 5' TOP genes crucial for translational control
- Global poly(A) tail shortening, observed across cancer cell lines, as well as in paired biopsies, indicates broad and targeted mRNA stability impact
- Specific shortening of poly(A) tails within the 5'TOP gene set suggests interference with mRNA regulation, leading to decreased protein translation
- Preliminary data highlight NUC-7738's potential to influence gene regulation, especially in the translational machinery critical for cancer cell growth and survival

REFERENCES: 1. Xiang, K., Bartel, D.P., 2021. The molecular basis of coupling between poly(A)-tail length and translational efficiency. eLife 10, e66493. https://doi.org/10.7554/eLife.66493 2. Gentilella, A., Morón-Duran, F.D., Fuentes, P., Zweig-Rocha, G., Riaño-Canalias, F., Pelletier, J., Ruiz, M., Turón, G., Castaño, J., Tauler, A., Bueno, C., Menéndez, P., Kozma, S.C., Thomas, G., 2017. Autogenous Control of 5'TOP mRNA Stability by 40S Ribosomes. Molecular Cell 67, 55–70.e4. https://doi.org/10.1016/j.molcel.2017.06.005 3. Pelletier, J., Thomas, G., Volarević, S., 2018. Ribosome biogenesis in cancer: new players and therapeutic avenues. Nat Rev Cancer 18, 51–63. https://doi.org/10.1038/nrc.2017.104 4. (Krause *et al.*, 2019) 5. (Li, 2018) 6. (Patro *et al.*, 2017)

ABBREVIATIONS: 3'-dATP: 3'-deoxyadenosine triphosphate 5'TOP: 5' terminal oligopyrimidine APA: alternative polyadenylation IC_{50} half maximal inhibitory concentration IF: Immunofluorescence mRNA: messenger ribonucleic acid OTB: On Treatment biopsy PAXT:Poly(A) tail exosome targeting RiBi: Ribosome biogenesis RPL3 – Ribosomal Protein L3 RPS6 – Ribosomal Protein S6 SCR: Screen Biopsy SNHG: Small Nucleolar RNA Host Gene TMA: Tissue Microarray