Novel N1-Substituted Pseudouridine 5'-Triphosphates for the Synthesis of Modified mRNA and its Effect on mRNA Translation in THP-1 Cells

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Abstract

Messenger RNA (mRNA) therapeutics is an emerging platform technology for the treatment of gene disorders, immunotherapies and vaccines. It was first tested in 1990, but subsequently found unsuitable for human clinical trials due to toxicity and instability. Novel modifications of mRNA are currently being developed and tested in various disease models.

Figure 1: Cap0, Cap1 and Cap2 Structures of 5' Ends of mRNA

- Euukaryotic mRNAs have a Cap1 or Cap2 structure.
- Sensing of proper cap structure is thought to be involved in self/non-self RNA recognition.
- Cap structure influences activation of PBRs.
- RS-1 is activated by Cap1 RNAs, but not Cap1 mRNA (PMID: 18426922 and 20457754).
- RTT binds Cap1 RNAs more tightly than Cap1 mRNA (PMID: 24371270).
- Cap0 cap structure is more stable than Cap1 mRNA.
- Co-transcriptional capping with CleanCap™ (Cap1) helps evade an immune response.

Figure 2: CleanCap™, Enzymatic and ARCA, Capping Comparison

- Background: Why mRNA therapeutics?
  - mRNA is a popular new tool for gene expression because it:
    - Does not have a risk of insertional mutagenesis
    - Can transfer difficult cells such as non-dividing cells
    - Is transient
  - Applications:
    - Genome editing (Transposons, Cre, ZFNs, TALENs and CRISPR/Cas9)
    - Gene replacement
    - Vaccines
  - Limitations:
    - Innate immune response to unmodified mRNA
  - Solutions:
    - Proper capping
    - Chemical modification of mRNA can prevent innate immune stimulation
    - Removal of diSSNA

Figure 3: Pseudouridine 5'-Triphosphate Derivatives

- Incorporation of modified nucleosides in mRNA helps to evade an immune response
- **Ψ** or **Ψ** modifications of mRNA are currently an industry standard
- We synthesized pseudouridine NTPs and tested in vitro luciferase mRNA transcriptions

**Figure 4: In Vitro Translation and Cell Activity of Modified Luciferase mRNAs**

- U depleted mRNA sequences resulted in higher activity in THP-1 cells
- We therefore continued our study using U-depleted mRNA sequences

Figure 5: U Depletion of Primary Luciferase mRNA Sequence Improves Incorporation of N1-Substituted Ψ Derivatives by T7 Polymerase

- Some N1-substituted Ψ derivatives did not incorporate well in WT mRNA
- We decreased the number of uridine residues in the sequence by substituting synonymous codons
- U depletion resulted in good incorporation

Figure 6: Sequence Engineering of FLuc mRNA (Ψ or MOM1Ψ Substitution, Bioanalyzer)

- Standard FLuc, U depleted FLuc
- MOM2FLuc, MOM1FLuc
- U depleted mRNA sequences resulted in higher activity in THP-1 cells

Conclusions

- We have synthesized a number of 5'-triphosphates of N1-modified pseudouridine derivatives
- These nucleoside 5'-triphosphates were used for the synthesis of modified mRNAs in vitro transcription using WT and U-depleted templates
- Efficiency of transcription using U-depleted templates greatly improved yield and quality
- N1-substituted Ψ mRNAs show potential translational and immunological properties
- Translational activity of modified mRNAs in wheat germ extracts did not directly correlate with cell activity, which may indicate differences in immune stimulation by these mRNAs

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