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, immunotherapy and vaccines. It was first tested in 1990, but subsequently found unsuitable because of its innate immunogenicity and low efficiency. More recently, Kariko *et al.* demonstrated that mRNAs containing naturally occurring pseudouridine (Ψ) increased overall protein expression and biological stability of mRNA. Ψ-modified mRNA also reduced Toll-like receptor mediated immune activation, activation of protein kinase R (PKR) and PKR-mediated phosphorylation of eukaryotic translation initiation factor 2 alpha (eIF2α). Activation of innate immune pathways leads to shut down of protein translation. Andries *et al.* found that N1-methylpseudouridine (m1Ψ) substituted mRNA provided higher reporter gene expression than ψ substituted mRNA upon transfection into cell lines or mice.

We prepared seven N1-substituted ψ derivatives: N1-ethyl-Ψ (Et1Ψ), N1-(2-fluoroethyl)-Ψ (FE1Ψ), N1-propyl-Ψ (Pr1Ψ), N1-isopropyl-Ψ (iPr1Ψ), N1-methoxymethyl-Ψ (MOM1Ψ), N1-pivaloxymethyl-Ψ (POM1Ψ), and N1-benzyloxymethyl-Ψ (BOM1Ψ), and their 5′-triphosphates. Uridine depleted polyadenylated Cap1 FLuc mRNAs (1.9 kb) were prepared by *in vitro* transcription with N1-substituted ΨTP in the presence of CleanCap™ (TriLink BioTechnologies) using T7 RNA polymerase and a PCR-amplified template from linearized plasmid DNA. It was found that the yield of mRNA is related to the size and electronic properties of the N1-substitution group of N1-substituted ΨTPs.

The FLuc Ψ-mRNAs with N1-substitutions were tested for expression of luciferase in the THP-1 monocyte cell line, a sensitive model for innate immune activation. Four of seven N1-substituted mRNAs showed activities higher than the activity of N1-unsubstituted Ψ-mRNA and were close to the activity of N1-Me Ψ-mRNA. Importantly, N1-substituted Ψ-mRNAs showed decreased cell toxicity (by MTT assay) compared to WT-mRNA and Ψ-mRNA. Protein translational activity was also measured in an *in vitro* wheat germ extract translation assay but this did not correlate with luciferase activity in THP-1 cultured cells. This suggests that while some N1-modifications may reduce protein translation, this effect is largely offset by a significant reduction of innate immune translational shut down in the cells.

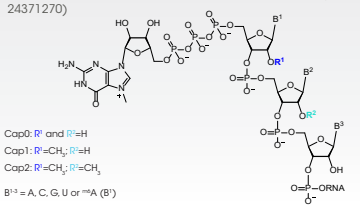
- **Background: Why mRNA therapeutics?**
  - mRNA is a popular new tool for gene expression because it:
    - Does not have a risk of insertional mutagenesis
    - Can transfect difficult cells such as non-dividing cells
    - Is transient
- **Applications**
  - Genome editing (Transposons, Cre, ZFNs, 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 dsRNA

Innate immune sensors recognize mRNA

- **Transfection of cells with unmodified RNAs can lead to cell death due to activation of innate immune pathways**
- **Toll-like receptors 3, 7 & 8 recognize different RNA forms**
  - Found in endosomes where some viruses enter cells
- **Cytosolic sensors**
  - Protein Kinase R (PKR): dsRNA
  - MDA5: long dsRNA
  - IFITs: unmethylated cap structures
  - RIG-I: 5′-triphosphate

Figure 1: Cap0, Cap1 and Cap2 Structures of 5′-Ends of mRNAs

- Eukaryotic 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 PRRs
  - RIG-I is activated by Cap0 RNAs but not Cap1 mRNAs (PMID: 18426922 and 20457754)
  - IFIT1 binds Cap0 RNAs more tightly than Cap1 mRNAs (PMID: 24371270)



- Co-transcriptional capping with CleanCap™ (Cap1) helps evade an immune response

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

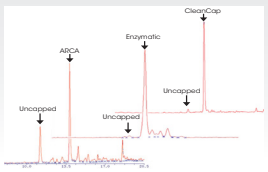
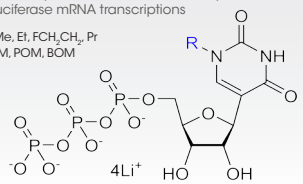


Figure 3: Pseudouridine 5′-Triphosphate Derivatives

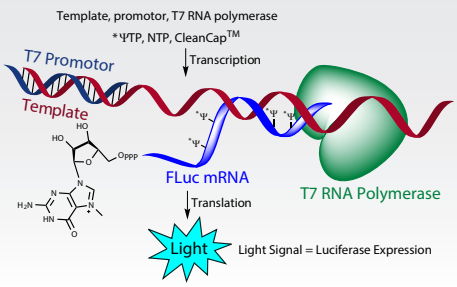
- Incorporation of modified nucleosides in mRNA helps to evade an immune response
- Ψ or m5C/Ψ modifications of mRNA are current industry standard
- Several novel pseudouridine NTPs were synthesized and tested in firefly luciferase mRNA transcriptions

R = H, Me, Et, FCH$_2$CH$_2$, Pr iPr, MOM, POM, BOM



Pseudouridine 5′-triphosphate derivatives; H = pseudouridine, Me = N1-methyl, Et = N1-ethyl, FE = fluoroethyl, Pr = propyl, iPr = isopropyl, MOM = methoxy methyl, POM = pivaloxy methyl, BOM = benzyloxy methyl pseudouridine

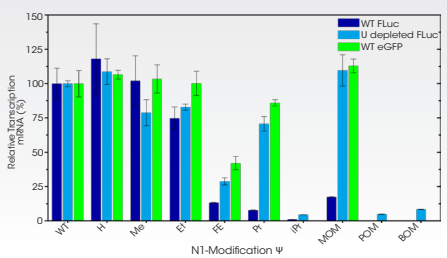
Figure 4: mRNA Synthesis by *In Vitro* Transcription



Schematic representation of transcription and translation with N1-modified pseudouridine 5′-triphosphate derivatives in the presence of CleanCap™. *N1-modified-Ψ

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



Relative efficiency of mRNA synthesis by *in vitro* transcription using T7 RNA polymerase with N1-modified ΨTPs; 7.5 mM triphosphates, 37 °C for 3 h; 2100 Agilent Bioanalyzer with RNA nano chip

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

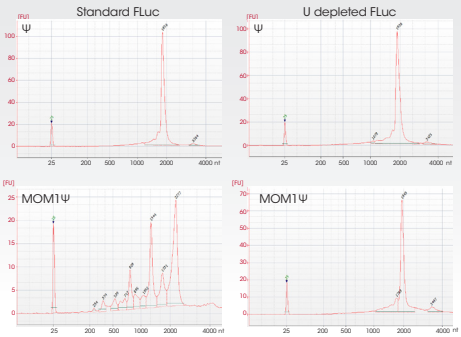
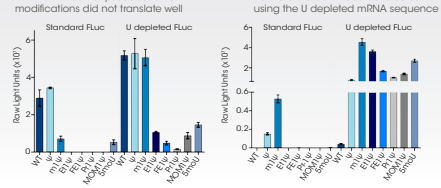


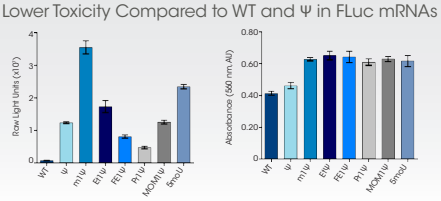
Figure 7: *In Vitro* Translation and Cell Activity of Modified Luciferase mRNAs

- U depleted mRNA sequences translated better in wheat germ extracts
- mRNA with bulkier pseudouridine modifications did not translate well
- U depleted mRNA sequences resulted in higher activity in THP-1 cells
- We therefore continued our studies using the U depleted mRNA sequence



Translation in wheat germ extracts | Activity in THP-1 cells

Figure 8: N1-Substituted Ψ Derivatives Resulted in Lower Toxicity Compared to WT and Ψ in FLuc mRNAs



Activity in THP-1 cells | Toxicity (MTT assay*) in THP-1 cells

*Lower absorbance reflects decreased cellular metabolism indicating toxicity

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 by *in vitro* transcription using WT and U-depleted templates
- Efficiency of transcription using U-depleted templates greatly improved quality and yield
- 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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