Maximizing Translation of mRNA Therapeutics By Sequence Engineering and Chemical Modification

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Abstract

For maximal expression in cells or target organs, transfected mRNAs must avoid detection by pattern recognition receptors (PRRs) that evolved to sense pathogenic non-self RNAs. These include PRRs that recognize improperly capped RNAs (RIG-I, IFITs) and double stranded RNA (PKR, OAS, RIG-I, TLR3). PRR activation leads to cytokine production, translational arrest and cell toxicity or death. Mammalian mRNAs are modified post-transcriptionally to contain nucleotides with 2'-*O*-methyl residues, pseudouridine (Ψ) and N6-methyladenosine (m6A). Interestingly, these modifications can reduce activation of PRRs and allow maximal translation of the transfected mRNA. For mRNA drugs to achieve their potential, methods to produce multigram batches of properly capped, highly purified and non-immunogenic mRNA will be needed. For vaccines, some immunogenicity may be desirable to serve as an adjuvant.

During RNA capping, Cap0 (m7GpppN) is formed as an intermediate. Methylation of the 2' position of the first and sometimes second nucleotide forms Cap1 and Cap2, respectively. Recombinant enzymes used to generate Cap1 mRNA are expensive, do not always go to completion and the RNA must be purified prior to capping. We developed a novel co-transcriptional capping method called CleanCap™ that yields Cap1 with high efficiency and lower costs in a "one pot" reaction. CleanCap also facilitates modification of the 5' end of mRNAs with diverse functional groups, including m6A. We developed a capping assay that allows direct assessment of mRNA capping. Capping efficiencies as high as 99% can be obtained.

Previously, we identified 5-methoxyuridine (5moU) as a promising modification to avoid innate immune stimulation while supporting efficient translation. To further explore chemically modified bases, we synthesized several Ψ derivatives: N1-ethyl-Ψ (E1Ψ), N1-fluoroethyl-Ψ (FE1Ψ), N1-propyl-Ψ (P1Ψ), N1-isopropyl-Ψ (iP1Ψ) and N1-methoxymethyl-Ψ (MOM1Ψ) 5'-triphosphates. Luciferase mRNAs were fully substituted with these modifications and translational potential was monitored in wheat germ extracts. Activity was also measured in the THP-1 monocyte cell line, which is a sensitive model for innate immune activation. A recent report showed that minimizing uridine content in mRNAs reduced immune stimulation by unmodified mRNAs. Here we show that incorporation of our modified uridine residues or 5moU into uridine depleted luciferase resulted in equivalent activity relative to Ψ in THP-1 cells. We are currently evaluating the use of 5moU in U depleted Renilla, beta-galactosidase, erythropoietin, mCherry and Cas9 mRNAs.

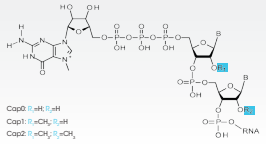
- **Background: Why mRNA therapeutics?**
  - mRNA is a popular new tool for gene expression because it:
    - Does not have a risk of insertional mutagenesis
    - Can transfect difficult cells such as non-dividing cells
    - Is transient
- **Applications**
  - Genome editing (Transposons, Cre, ZFNs, 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: dsRNA
  - IFITs: unmethylated cap structures
  - RIG-I: 5' triphosphate

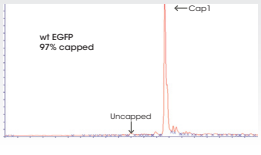
Figure 1: Cap0, Cap1 and Cap2 Structures of 5'-Ends of mRNAs

- 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

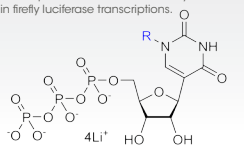
Figure 2: Capping Efficiency Assay Shows CleanCap™ Yields High Levels of Cap1



Capping efficiencies are consistently above 90% depending on reaction conditions. Data collected by liquid chromatography with masses confirmed by mass spectroscopy assay

Figure 3: Pseudouridine 5'-Triphosphate Derivatives

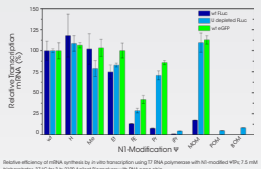
- mRNA body modifications help to evade an immune response
- Pseudouridine or 5-methylcytidine/pseudouridine are current industry standard
- Several novel pseudouridine NTPs were synthesized and tested in firefly luciferase transcriptions.



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: U Depletion of Primary Luciferase Sequence Improves Incorporation of Bulky Pseudouridine Derivatives by T7 Polymerase

- Some pseudouridine derivatives did not incorporate well
- We depleted the Fluc sequence for Us to try and remedy this
- U depletion resulted in good incorporation
- We tested the derivatives that did incorporate for translation and activity



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.

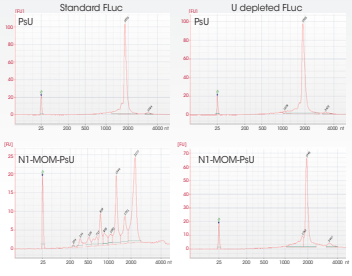


Figure 5: *In Vitro* Translation and Cell Activity of Modified Luciferase mRNAs

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

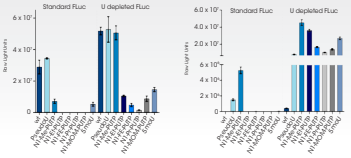
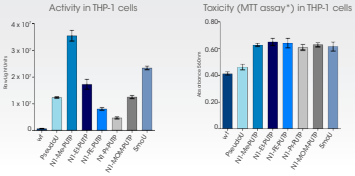


Figure 6: Pseudouridine Derivatives and 5moU Resulted in Lower Toxicity Compared to WT and PseudoU



*Lower absorbance reflects decreased cellular metabolism indicating toxicity

Figure 7: Cell Activity of GFP Modified RNAs in Various Cell Lines

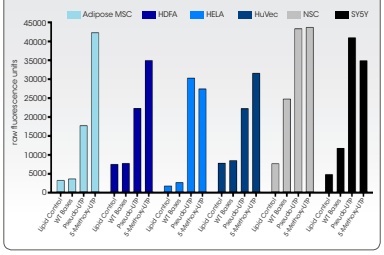


Figure 8: Cell Activity of Standard and U Depleted Firefly Luciferase in THP-1 Dual Cells

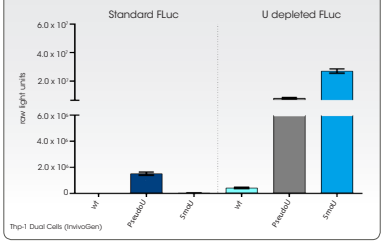


Figure 9: Effect of HPLC Purification on Firefly Luciferase Cell Activity in THP-1 Dual Cells

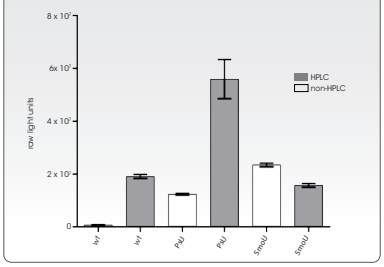


Figure 10: Cell Activity of U Depleted Renilla Luciferase in THP-1 Dual Cells

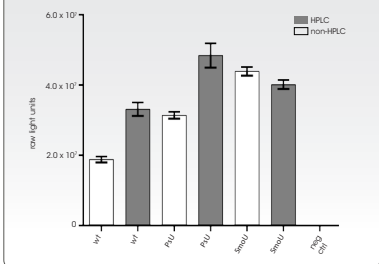


Figure 11: Interferon Reporter Activity of THP-1 Dual Cells in Response to RNA Transfection

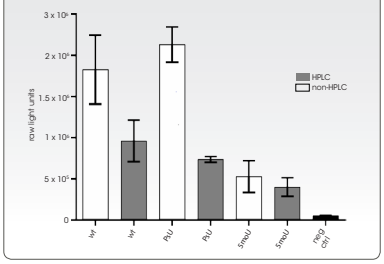
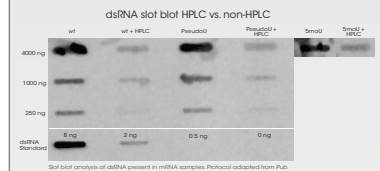


Figure 12: Slot Blot Demonstrates that HPLC Purification Depletes dsRNA

- An RP-HPLC method depletes mRNAs of contaminating dsRNA
- This reduces the innate immune response by reducing PKR activation



Slot blot analysis of dsRNA present in mRNA samples. Protocol adapted from Pub Med ID: 23296926.

- As previously described, HPLC purification depleted dsRNA, reduced toxicity and reduced interferon activation

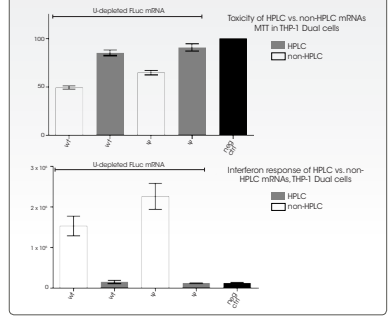
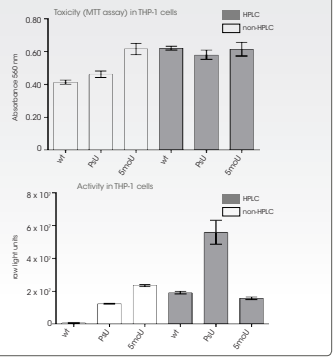


Figure 13: Cell Activity of HPLC vs. non-HPLC Luciferase mRNAs

- HPLC purification dramatically increased the activity of wt mRNA, improved the activity of PsU mRNA but did not alter the activity of 5moU mRNA
- Could this be because PKR does not bind 5moU dsRNA?
- Could this also be true for the PseudoU derivatives?



Conclusions

- We have introduced a number of novel modified bases with interesting translational and immunological properties
- U depletion improved transcription quality, yield and activity
- HPLC purification to remove dsRNA reduced toxicity and interferon response and increased activity
- Translational activity in wheat germ extracts did not directly correlate with cell activity, which may indicate differences in immune stimulation by these mRNAs
- 5moU is a promising modification for reducing innate immune stimulation
- Ability of 5moU to suppress innate immune stimulation is sequence context dependent
- HPLC improves activity of WT and PseudoU modified RNAs but not 5moU modified RNAs. HPLC may not be necessary for 5moU
  - One possibility is that 5moU dsRNA is not efficiently recognized by PRRs

Future Directions

- If 5moU is not recognized by PRRs, then activity of 5moU should be equivalent in PKR -/- and +/+ MEFs
- Measure activity, toxicity and interferon response in THP-1 cells for HPLC purified PseudoU derivative mRNAs

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