Maximizing the Translation and Activity of mRNA Therapeutics
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Abstract
mRNA-based therapeutics avoid the challenges of viral vector delivery and may be able to successfully target several tissues at once. However, many small-molecule drugs require transient expression of therapeutic proteins with higher levels of translation and activity. Enhancing activity in a range of cell types is crucial for success of mRNA therapies.

Figure 1: Cap0, Cap1 and Cap2 Structures of 5'-Ends of mRNAs
- Eukaryotic mRNAs have a Cap0 or Cap1 structure.
- Sensing of proper cap structure is thought to be involved in self/non-self RNA recognition.
- Cap structure influences activation of PRRs.
- Cap0 is activated by Cap0 RNAs but not Cap1 mRNAs (PMD: 182692 and 20675715).
- 5'-ends Cap0 RNAs more tightly than Cap1 mRNAs (PMD: 24317270).
- Co-transcriptional capping with CleanCap™ (Cap1) helps evade an immune response.

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
  - MD-2: RNA
  - IFI44: 5' triphosphate

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

Figure 3: Pseudouridine 5'-Triphosphate Derivatives
- mRNA body modifications help to evade an immune response.
- Pseudouridine or 5'-methoxypseudouridine/psu are current industry standard.
- Several novel pseudouridine NPs were synthesized and tested in firefly luciferase transcriptions.

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 luciferase 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.

Figure 5: In Vitro Translation and Cell Activity of Modified Luciferase mRNAs
- U-depleted sequences translated better in wheat germ extracts.
- Bulky 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

Figure 7: Slot Blot Demonstrates that HPLC Purification Depletes dsRNA
- An HPLC method depletes mRNAs of contaminating dsRNA.
- This reduces the innate immune response by reducing PRR activation.

Figure 8: 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 mRNA?
- Could this also be due for the Psu2 derivative?

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.
- Interestingly, HPLC purification of 5moU mRNAs did not increase activity.
- One possibility is that 5moU dsRNA is not efficiently recognized by PRRs.
- Translational activity in wheat germ extracts did not directly correlate with cell activity, which may indicate differences in immune stimulation by these mRNAs.

Future Directions
- If 5moU is not recognized by PRRs, then activity of 5moU should be equivalent in PKR−/− and wt +/− mice.
- Measure activity, toxicity and interferon response in THP-1 cells for HPLC purified Psu2 oligonucleotides.

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, CRISPR/Cas9);
  - Vaccines;
  - Gene replacement.
  - Genome editing (Transposons, Cre, ZFNs, TALENs and CRISPR/Cas9).

- Does not have a risk of insertional mutagenesis.
- Transient mRNA expression is also desirable for cellular reprogramming, genome editing and mRNA vaccines.

Limitations
- Innate immune response to unmodified mRNA.
- Solutions
  - Proper capping;
  - Chemical modification of mRNA can prevent innate immune stimulation.
  - Removal of dsRNA.

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