Nucleic Acids Research Advance Access published September 2, 2011

Nucleic Acids Research, 2011, 1–10
doi:10.1093/nar/gkr695

Generating the optimal mRNA for therapy: HPLC purification eliminates immune activation and improves translation of nucleoside-modified, protein-encoding mRNA

Katalin Karikó[1], Hiromi Muramatsu[1], János Ludwig[2] and Drew Weissman[3,*]

[1]Department of Neurosurgery, University of Pennsylvania, Philadelphia, PA, USA, [2]Institute of Clinical Chemistry and Pharmacology, University of Bonn, Bonn, Germany and [3]Department of Medicine, University of Pennsylvania, Philadelphia, PA, USA

Received May 26, 2011; Revised August 9, 2011; Accepted August 10, 2011

ABSTRACT

***In vitro*-transcribed mRNA has great therapeutic potential to transiently express the encoded protein without the adverse effects of viral and DNA-based constructs. Mammalian cells, however, contain RNA sensors of the innate immune system that must be considered in the generation of therapeutic RNA. Incorporation of modified nucleosides both reduces innate immune activation and increases translation of mRNA, but residual induction of type I interferons (IFNs) and proinflammatory cytokines remains. We identify that contaminants, including double-stranded RNA, in nucleoside-modified *in vitro*-transcribed RNA are responsible for innate immune activation and their removal by high performance liquid chromatography (HPLC) results in mRNA that does not induce IFNs and inflammatory cytokines and is translated at 10- to 1000-fold greater levels in primary cells. Although unmodified mRNAs were translated significantly better following purification, they still induced high levels of cytokine secretion. HPLC purified nucleoside-modified mRNA is a powerful vector for applications ranging from *ex vivo* stem cell generation to *in vivo* gene therapy.**

INTRODUCTION

Our understanding of the importance of RNA in biological processes and the therapeutic potential has substantially increased with the discovery of non-coding regulatory RNAs. The use of mRNA has also expanded, including the delivery of mRNA to generate induced pluripotent stem (iPS) cells (1–3) and *in vivo* administration to express therapeutic proteins (4). The recognition that the immunogenicity of RNA could be reduced by the incorporation of modified nucleosides with a concomitant increase in translation (5), potentially allows efficient expression of intra and extracellular proteins *in vivo* and *ex vivo* without activation of innate immune pathways. Unfortunately, modified nucleoside-containing RNA transcribed by phage RNA polymerase transcription still retains a low level of activation of such pathways (3,5–7). The remaining activation of RNA sensors by nucleoside modified RNA could be because the modifications do not completely suppress the RNAs ability to activate sensors or due to contaminants with structures that activate in the presence of nucleoside modification. It is well established that RNA transcribed *in vitro* by phage polymerase contains multiple contaminants, including short RNAs produced by abortive initiation events (8) and double-stranded (ds)RNAs generated by self-complementary 3′ extension (9), RNA-primed transcription from RNA templates (10) and RNA-dependent RNA polymerase activity (11).

Large quantities of RNA can be easily prepared by *in vitro* transcription from DNA templates using phage RNA polymerase or solid-phase chemical synthesis. For uses that require further purification, such as NMR (12), crystallography (13) and therapeutic applications (14), a number of techniques have been developed. Preparative denaturing polyacrylamide gel electrophoresis is commonly used to purify *in vitro*-transcribed RNA, however, this method is suitable only for short RNAs [reviewed in (15)]. Long RNAs can be separated on denaturing agarose gels, but they are not translatable due to covalent modifications introduced by the denaturants glyoxal and formaldehyde (16). Chromatography based on size exclusion can efficiently remove unincorporated nucleoside triphosphates, small abortive transcripts and plasmid template from the desired RNA product

*To whom correspondence should be addressed. Tel: +1 215 573 8491; Fax: +215 349 5111; Email: dreww@mail.med.upenn.edu

under native conditions (17,18), but is limited in its ability to remove contaminants with similar sizes and contaminants complementary to the RNA selected to purify. No technique has been reported for purification and preparative isolation of long *in vitro*-transcribed mRNA that removes contaminating complementary strands and preserves its translatability.

The development of mRNA to use as a tool to replace intra- and extracellular proteins *in vivo* and to transdifferentiate, reprogram and differentiate cells *ex vivo* requires the RNA to have high translatability and no RNA sensor activation. In this report, we identify that contaminants from *in vitro*-transcribed RNA are a source of innate immune activation and their removal increases RNA translation and eliminates type I interferon and inflammatory cytokine secretion.

MATERIALS AND METHODS

Cells

Human embryonic kidney 293T cells (American Type Culture Collection) were cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with 2 mM L-glutamine (Life Technologies) and 10% fetal calf serum (FCS) (HyClone) (complete medium). Human and murine dendritic cells (DCs) were generated as described (5). Human keratinocytes were obtained from the Skin Disease Research Core (Penn) and grown in MCDB with bovine pituitary extract (140 μg/ml) (Sigma) and 70 μM Ca^{++} on collagen (0.01 mg/ml) (Invitrogen) coated plates.

mRNA synthesis

mRNAs were transcribed as previously described (5), using linearized plasmids encoding firefly luciferase (pT7TSLuc and pTEVLuc), codon-optimized murine erythropoietin (pTEVmEPO), enhanced green fluorescent protein (pTEVeGFP), Metridia luciferase (pT7TSMetluc) or Renilla luciferase (pT7TSRen and pTEVRen) and T7 RNA polymerase (Megascript, Ambion). All mRNAs were transcribed to contain 30 or 51-nt long poly(A) tails. Additional poly(A) tail was added with yeast poly(A) polymerase (USB) and noted as A_n. Triphosphate-derivatives of pseudouridine (Ψ) and 5-methylcytidine (m5C) (TriLink) were used to generate modified nucleoside containing RNA. All RNAs were capped using the m7G capping kit with or without 2′-*O*-methyltransferase (ScriptCap, CellScript) to obtain cap1 or cap0. We did not observed differences in the immunogenicity of cap0- and cap1-containing nucleoside-modified RNAs. All RNAs were analyzed by denaturing or native agarose gel electrophoresis. Pseudouridine-modified mRNAs encoding KLF4, LIN28, cMYC, NANOG, OCT4 and SOX2 were a kind gift of CellScript, Inc.

HPLC purification of RNA

RNA was purified by High performance liquid chromatography (HPLC) (Akta Purifier, GE Healthcare) using a column matrix of alkylated non-porous polystyrene-divinylbenzene copolymer microspheres (2.1 μm) (21 × 100 mm column). Buffer A contained 0.1 M triethylammonium acetate (TEAA), pH = 7.0 and Buffer B contained 0.1 M TEAA, pH = 7.0 and 25% acetonitrile (Transgenomics). Columns were equilibrated with 38% Buffer B, loaded with RNA and run with a single or 2 linear gradients to 55 or 65% Buffer B over 20–30 min at 5 ml/min. RNA analyses were performed with the same column matrix and buffer system using a 7.8 mm × 50 mm column at 1.0 ml/min.

RNA isolation from column fractions

RNA content from desired fractions was concentrated and desalted using Amicon Ultra-15 centrifugal filter units (30K membrane) (Millipore) by successive centrifugation at 4000*g* for 10 min (4°C) in a Sorvall ST16R centrifuge (Thermo Scientific) and dilution with nuclease free water. The RNA was recovered by overnight precipitation at −20°C in NaOAc (0.3 M, pH 5.5), isopropanol (1 volume) (Fisher) and glycogen (3 μl) (Roche).

Dot blot

RNA (200 ng) was blotted onto super charged Nytran, dried, blocked with 5% non-fat dried milk in TBS-T buffer (50 mM Tris–HCl, 150 mM NaCl, 0.05% Tween-20, pH 7.4), and incubated with dsRNA-specific mAb J2 or K1 (English & Scientific Consulting) for 60 min. Membranes were washed six times with TBS-T and reacted with HRP-conjugated donkey anti-mouse Ig (Jackson Immunology), washed six times and detected with ECL Plus Western blot detection reagent (Amersham). Images were captured on a Fujifilm LAS1000 digital imaging system. dsRNA (25 ng) used as a positive control was derived from sense and antisense strands of T7TS UTR sequence (328 bp). Blots were reprobed with ^{32}P-labeled DNA complementary to the 3′-UTR of the RNA to document the presence of RNA.

Complexing of RNA

Lipofectin (Invitrogen) complexing was performed as described previously (5) using 0.8 μl of Lipofectin and 0.1 μg of RNA per well of a 96-well plate. Complexing of RNA to TransIT mRNA (Mirus Bio) was performed according to the manufacturer combining RNA (0.1 μg) with TransIT mRNA (0.3 μl) and boost (0.2 μl) reagents.

Cell transfections

For Lipofectin complexed RNA, medium was removed and 50 μl of complexed RNA was added to 5 x 10^4 293T or DCs per well. Cells were incubated for 1 h and the Lipofectin-RNA mixture was replaced with 200 μl complete medium. For TransIT complexed RNA, 17 μl of complex was added to cells, 293T, DCs, or 2 × 10^5 keratinocytes cultured in 183 μl complete medium. Cells were lysed in firefly or Renilla specific lysis reagents (Promega) at 24 h post RNA addition. Aliquots were assayed for enzyme activities using firefly and Renilla luciferase assay systems (Promega) and a LUMAT LB 950 luminometer (Berthold/EG&G; Wallac). Expression of

eGFP in DCs was documented using an inverted epifluorescent Nikon microscope mounted with a Nikon D40 digital camera. Murine EPO protein was measured with a specific ELISA assay (R&D Systems).

RNA immunogenicity analyses

DCs (murine or human) (5×10^4 cells/well) in 96-well plates were treated with medium, R-848 (Invivogen), or Lipofectin- or TransIT-complexed RNA or poly(I:C) (Sigma). Supernatant was harvested after 24 h and the levels of IFN-α, IFN-β (PBL InterferonSource), or TNF-α (Biosource International) were measured by ELISA.

Gene array analysis

Human DCs from three donors were generated in 5% FCS. Cells (1×10^6 DCs/well of a 6-well plate) were treated with TransIT-complexed $TEVRenA_{51}$ RNA with or without modification and with or without purification. Six hours later, RNA was isolated using RNeasy (Qiagen). RNA was amplified with the TargetAmp Nano-g Biotin-aRNA labeling kit (Epicentre) and analyzed on an Illumina Human HT12v4 chip in an Illumina BeadStation 500GX. Raw data was processed by the Bead Studio v.3.0 software. Levels in untreated DC were used as the baseline for the calculation of fold increase.

Northern blot

Samples were processed and analyzed as previously described (6). Probes were derived from plasmids and were specific for the coding regions of human IFN-α13, IFN-β (Open Biosystems), TNF-α, or GAPDH (ATCC).

RESULTS

A dot blot assay with J2 and K1 monoclonal antibodies (mAbs) that recognize dsRNA (19) was used to determine whether *in vitro*-transcribed RNA contains dsRNA. These mAbs recognize continuous double stranded structure of at least 40 bp in length (20), which is not found in any of the coding sequences or UTRs in the mRNAs analyzed in this study. Testing mammalian and reporter protein-encoding *in vitro* transcripts containing either no nucleoside modifications, pseudouridine- (Ψ), or 5-methylcytidine- (m5C) and Ψ- (m5C/Ψ) nucleoside modifications, we found that all samples contained dsRNA contamination (Figure 1A and data not shown). Recognition of dsRNA by J2 mAb was not affected by the presence of modified nucleosides, while K1 had reduced binding to dsRNA containing Ψ or m5C/Ψ nucleoside modifications.

Others and we have previously demonstrated that incorporation of modified nucleotides into RNA reduced its ability to activate RNA sensors including Toll-like receptor (TLR)3, TLR7 and TLR8 (21), retinoic acid inducible gene I (RIG-I) (22) and RNA-dependent protein kinase (PKR) (6,23). Monocyte-derived DCs that express these and all other known RNA sensors (24) were used to measure residual immune activation present in modified nucleoside-containing RNA. A series of RNAs with coding sequences for mammalian and reporter proteins flanked by different 5′- and 3′-UTRs were analyzed. The RNAs were cell-delivered following complexing with Lipofectin, a cationic lipid, or TransIT, a membrane active polymer and lipid mixture. RNA complexed with Lipofectin induced high levels of TNF-α and moderate levels of IFN-α, while RNA complexed with

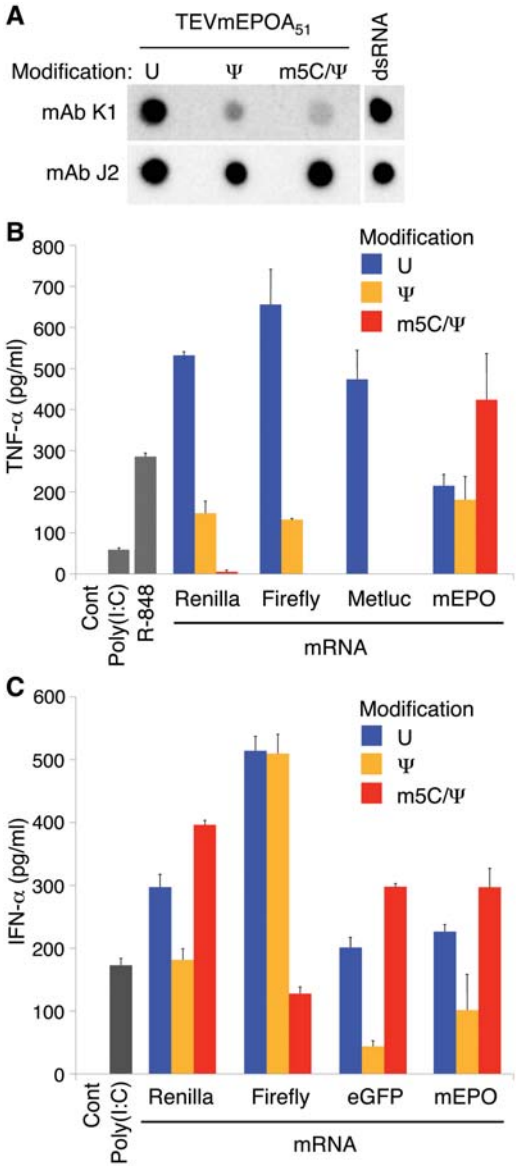

Figure 1. *In vitro*-transcribed RNA is immunogenic and contains dsRNA contaminants. (**A**) 200 ng of *in vitro* transcripts encoding mEPO and containing the indicated modified nucleosides were blotted and analyzed with K1 and J2 dsRNA-specific mAbs. The dsRNA positive control contained a 328 bp long dsRNA (25 ng). (**B**) DCs were treated with Lipofectin-complexed Renilla luciferase ($T7TSRenA_{30}$), firefly and Metridia luciferases ($T7TSLucA_{30}$, $T7TSMetlucA_{30}$), and mEPO ($TEVmEPOA_{51}$) mRNAs. TNF-α levels were measured in the supernatants at 24 h. (**C**) DCs were treated with TransIT-complexed *in vitro* transcripts encoding Renilla and firefly luciferases ($T7TSRenA_{30}$, $T7TSLucA_{30}$), eGFP ($TEVeGFPA_{51}$) and mEPO ($TEVmEPOA_{51}$). IFN-α levels were measured in the supernatants at 24 h. Error bars are standard error of the mean. Data shown is from one experiment that is representative of 3–6.

TransIT induced low levels of TNF-α and high levels of IFN-α (data not shown) with some donor-dependent variation. Typically, Lipofectin-complexed RNA with Ψ or m5C/Ψ modifications induced less TNF-α. (Figure 1B), while TransIT-complexed RNA with or without nucleoside modification induced variable, sequence-dependent effects on IFN-α secretion (Figure 1C). These data suggest that the presence of dsRNA and potentially other contaminants in *in vitro*-transcribed RNA could be responsible for innate immune activation.

Multiple HPLC bead matrix compositions and buffer systems were screened and alkylated non-porous polystyrene-divinylbenzene copolymer matrix and triethylammonium acetate buffer with an acetonitrile gradient was identified as a system capable of removing dsRNA and other contaminants from *in vitro*-transcribed RNA. The HPLC chromatogram of Ψ-modified mRNA encoding enhanced green fluorescent protein (eGFP) demonstrated a major peak (Figure 2), which was collected and identified as the expected RNA product using agarose gel electrophoresis. Additional UV-absorbing products with shorter and longer retention times relative to the main RNA product could also be observed. Reanalysis of the purified RNA by HPLC demonstrated a single peak with the same retention time. RNAs with or without nucleoside modification encoding different sequences yielded similar patterns with varying relative heights for the preceding and succeeding peaks.

HPLC purification of both unmodified and nucleoside-modified RNA reduced staining by dsRNA-specific mAb to baseline levels (Figure 3A). Analysis of Ψ-modified RNA encoding clinically relevant proteins demonstrated that the amounts of dsRNA contamination in the *in vitro* transcripts were dependent on the sequence, but HPLC could successfully remove the contaminants from all of them (Figure 3B). Next, the HPLC-purified RNAs were tested on human DCs. No TNF-α or type-I interferons (IFN-α and β) were induced following transfection of HPLC-purified Ψ- or m5C/Ψ-modified RNAs that were complexed with Lipofectin or TransIT, respectively (Figure 3C and D and data not shown). Similarly, no cytokine induction could be detected when HPLC-purified modified nucleoside-containing RNAs were transfected into murine DCs. HPLC purification similarly ablated IFN-α secretion from DCs transfected with the clinically relevant Ψ-nucleoside modified mRNAs complexed to TransIT used in Figure 3B. However, HPLC-purified RNA without nucleoside modification remained potent inducers of TNF-α and IFN-α (Figure 3C and D).

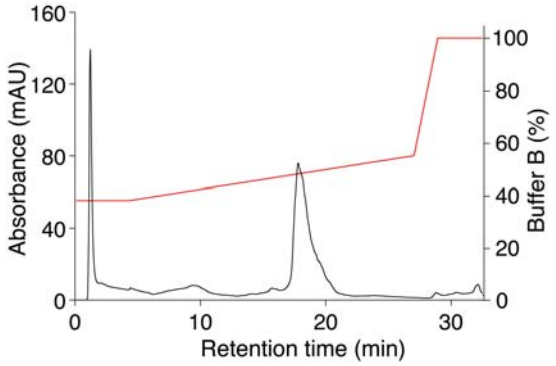

Figure 2. HPLC purification of RNA identifies contaminants eluting before and after the expected product. Chromatogram of Ψ-modified TEVeGFPA$_n$ mRNA. RNA was applied to the HPLC column and eluted using a linear gradient of Buffer B (0.1 M TEAA, pH 7.0, 25% acetonitrile) in Buffer A (0.1 M TEAA, pH 7.0). The gradient spanned 38–55% Buffer B over 22 min (red line). Absorbance at 260 nm was analyzed (black line), which demonstrated the expected sized RNA as well as smaller and larger RNA species. Data shown are from one experiment that is representative of over 200

The impact of HPLC purification of unmodified, Ψ- and m5C/Ψ-modified RNA on gene expression in human DCs was analyzed using gene arrays. Total cellular RNA isolated from DCs from three different donors 6 h after cells were transfected with TransIT-complexed RNA, were analyzed on an Illumina Human HT12v4 chip. RNA modified with Ψ or m5C/Ψ nucleosides induced less expression of type I interferons, interleukins, tumor necrosis factor (TNF) family members, chemokines and markers associated with DC activation, while HPLC purification of Ψ- and m5C/Ψ-modified RNA further reduced induction of these genes to the levels observed in cells treated only with TransIT (Figure 4A). The same sets of total RNA from DCs that were tested on the gene arrays were also analyzed for levels of IFN-α, IFN-β and TNF-α mRNA by northern blot. Lower levels of IFN-α, IFN-β and TNF-α mRNAs were detectable in DCs treated with nucleoside modified as compared to unmodified RNA. More importantly, none of these cytokine mRNAs were detectable when DCs were transfected with HPLC-purified RNAs containing Ψ or m5C/Ψ modification. However, HPLC purified RNA without nucleoside modification remained a potent inducer of IFN-α, IFN-β and TNF-α mRNAs (Figure 4B).

To determine whether HPLC purification affected translatability of *in vitro* transcripts, a series of mRNAs were tested following cell delivery. HPLC-purified Renilla and mouse erythropoietin (mEPO) mRNAs were translated at 2- to 20-fold higher levels compared to unpurified RNA when delivered to 293 T cells by TransIT (Figure 5A). In primary human DCs, the translational enhancement was more robust, resulting in up to a 1000-fold increase when the same sets of unpurified and HPLC-purified mRNAs were transfected with Lipofectin (Figure 5B) or TransIT (Figure 5C). Similar increases in translation were observed for other mRNAs after HPLC purification, including mRNAs encoding firefly luciferase, human EPO, macaque EPO and Metridia luciferase, and other cell types, including mouse embryonic fibroblasts and human primary keratinocytes. Translation levels were much higher with Ψ- and m5C/Ψ-modified eGFP mRNA when the mRNA was HPLC-purified prior to transfection of human DCs (Figure 5D and data not shown).

To characterize the contaminants being removed by HPLC purification, three fractions corresponding to RNAs eluting from the column prior to the major transcription product (fraction I), the full-length transcription product (fraction II) and RNAs eluting after the

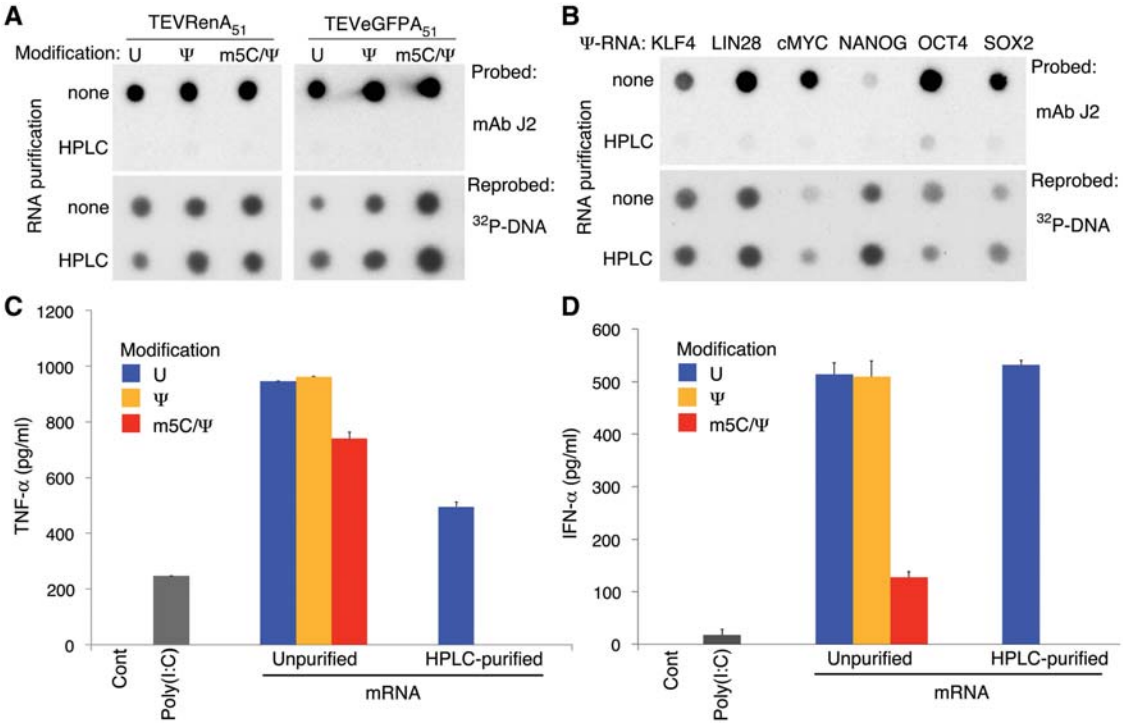

Figure 3. HPLC purification of *in vitro*-transcribed nucleoside modified mRNA removes dsRNA contaminants and eliminates immunogenicity. (**A**) 200 ng of RNA encoding the indicated protein and containing the indicated modified nucleosides with or without HPLC purification were blotted and analyzed with the J2 dsRNA-specific mAb. (**B**) 200 ng of RNA encoding the indicated protein and containing Ψ-modifications with or without HPLC purification were blotted and analyzed with the J2 dsRNA-specific mAb. Blots were reprobed with a ^{32}P-labeled probe for the 3′-UTR of the RNAs to control for amount of RNA analyzed. (**C**) DCs were treated with TEVRenA$_{51}$ RNA containing the indicated nucleoside modifications with or without HPLC purification and complexed to Lipofectin. TNF-α levels were measured in the supernatants at 24 h. Differences in the effect of nucleoside modification on immunogenicity of Renilla-encoding mRNA compared to Figure 1B is likely due to donor variation and differences in UTRs of the RNAs. (**D**) DCs were treated with TEVLucA$_{51}$ RNA containing the indicated nucleoside modifications with or without HPLC purification and complexed to TransIT. IFN-α levels were measured in the supernatants at 24 h. Error bars are standard error of the mean. Data shown is from one experiment that is representative of 3 or more.

expected transcription product (fraction III) were collected (Figure 6A). Nucleoside-modified RNA occasionally demonstrated a second smaller peak overlying the large peak and isolation and purification of both peaks demonstrated similar RNA lengths on denaturing and non-denaturing agarose gel electrophoresis and RNAs with similar levels of translation and immunogenicity. RNA purified from the three fractions was analyzed for immunogenicity. RNA in fractions I and III induced IFN-α secretion from transfected DCs, while the purified full-length RNA (fraction II) was not immunogenic when it contained Ψ- or m5C/Ψ-nucleoside modifications (Figure 6B). Fraction I RNA had low levels of staining with the J2 mAb, while fraction III RNA had high levels of staining similar to the unpurified RNA (Figure 6C).

Primary human keratinocytes and murine fibroblasts treated once with unpurified, unmodified RNA delivered by TransIT complexing demonstrated detachment from the collagen-coated plastic base as evidence of cell death. A second delivery of unmodified RNA 24 h later resulted in the termination of the culture. Substantially less toxicity was observed when the RNA contained Ψ- or m5C/Ψ-nucleoside modifications, but repeated daily delivery of m5C/Ψ-nucleoside modified mRNA to keratinocytes reduced the final cell number by 75% on day 11 (Figure 7). HPLC purification of the RNA greatly reduced toxicity in treated keratinocytes where unmodified mRNA caused minimal cell rounding and death. Daily treatment of keratinocytes for 10 days with HPLC-purified m5C/Ψ-modified mRNA complexed to TransIT showed no signs of cell toxicity and the rate of proliferation was similar to that obtained with TransIT alone treated cells (Figure 7).

DISCUSSION

Modified nucleoside-containing mRNA has previously been used for the induction of iPS cells from fibroblasts with very high efficiency (3). The authors determined that m5C/Ψ-nucleoside modified mRNA yielded the least amount of RNA sensor activation and the highest level of translation, but needed to add the B18R protein, a vaccinia virus decoy receptor for type I interferon (25), for optimal iPS cell generation. We previously reported that modified nucleoside-containing mRNA was efficiently delivered to primary dividing and non-dividing cells and produced high levels of encoded protein in an easily controlled manner (5). In addition, the RNA had reduced innate immune sensor activation. The residual amount of activation of modified nucleoside-containing

DOCKET ALARM

Explore Litigation Insights

Docket Alarm provides insights to develop a more informed litigation strategy and the peace of mind of knowing you're on top of things.

Real-Time Litigation Alerts

Keep your litigation team up-to-date with **real-time alerts** and advanced team management tools built for the enterprise, all while greatly reducing PACER spend.

Our comprehensive service means we can handle Federal, State, and Administrative courts across the country.

Advanced Docket Research

With over 230 million records, Docket Alarm's cloud-native docket research platform finds what other services can't. Coverage includes Federal, State, plus PTAB, TTAB, ITC and NLRB decisions, all in one place.

Identify arguments that have been successful in the past with full text, pinpoint searching. Link to case law cited within any court document via Fastcase.

Analytics At Your Fingertips

Learn what happened the last time a particular judge, opposing counsel or company faced cases similar to yours.

Advanced out-of-the-box PTAB and TTAB analytics are always at your fingertips.

API

Docket Alarm offers a powerful API (application programming interface) to developers that want to integrate case filings into their apps.

LAW FIRMS
Build custom dashboards for your attorneys and clients with live data direct from the court.

Automate many repetitive legal tasks like conflict checks, document management, and marketing.

FINANCIAL INSTITUTIONS
Litigation and bankruptcy checks for companies and debtors.

E-DISCOVERY AND LEGAL VENDORS
Sync your system to PACER to automate legal marketing.