Review

Strategic Deactivation of mRNA COVID-19 Vaccines: New Applications for RIBOTACs and siRNA Therapy

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Abstract

The rapid development and authorization of mRNA vaccines by Pfizer-BioNTech (BNT162b2) and Moderna (mRNA-1273) in 2020 marked a significant milestone in human mRNA product application, overcoming previous obstacles such as mRNA instability and immunogenicity. This paper reviews the strategic modifications incorporated into these vaccines to enhance mRNA stability and translation efficiency, such as the inclusion of nucleoside modifications and optimized mRNA design elements including the 5' cap and poly(A) tail. We highlight emerging concerns regarding the wide systemic biodistribution of these mRNA vaccines leading to prolonged inflammatory responses and other safety concerns. The regulatory framework guiding the biodistribution studies is pivotal in assessing the safety profiles of new mRNA formulations in use today. The stability of mRNA vaccines, their pervasive distribution, and the longevity of the encapsulated mRNA along with unlimited production of the damaging and potentially lethal Spike (S) protein call for strategies to mitigate potential adverse effects. Here, we explore the potential of small interfering RNA (siRNA) and ribonuclease targeting chimeras (RIBOTACs) as promising solutions to target, inactivate, and degrade residual and persistent vaccine mRNA, thereby potentially preventing uncontrolled Spike protein production and reducing toxicity. The targeted nature of siRNA and RIBOTACs allows for precise intervention, offering a path to prevent and mitigate adverse events of mRNA-based therapies. This review calls for further research into siRNA and RIBOTAC applications as antidotes and detoxication products for mRNA vaccine technology.

Keywords: adverse event, biodistribution, COVID-19 vaccine, mRNA, RIBOTACs, siRNA, Spike protein

Introduction

Prior to the authorization of the Moderna (mRNA-1273) and Pfizer-BioNTech (BNT162b2) mRNA vaccines in 2020, numerous challenges were addressed to circumvent inherent limitations of mRNA technology. Although researchers previously demonstrated that nucleic acid could be encapsulated and successfully delivered via polymer particles, the advent of mRNA as an effective therapeutic agent required the mitigation of mRNA instability and immunogenicity as well as the ability of mRNA to cross multiple barriers [1, 2]. Even when mRNAs evade nucleases in the extracellular space and target cells, most of the mRNAs become trapped in endosomes and are subsequently degraded [3]. Additionally, endocytosis of exogenous mRNA can induce pattern-recognition receptor-mediated immunogenicity with subsequent inhibition of mRNA translation and reduced mRNA stability [4-8]. Furthermore, efficient mRNA delivery is hampered by the negative charges of the mRNA and the cell membrane [2]. Administered mRNA can also be removed by macrophage phagocytosis or through renal filtration [2].

This paper reviews Pfizer-BioNTech and Moderna's mRNA vaccine development, addressing critical challenges such as mRNA instability, immunogenicity, and delivery barriers, and elucidating how these have been overcome through advanced formulation and encapsulation techniques. We explore the regulatory landscape that shapes the preclinical and clinical pathways for mRNA vaccines, the role of lipid nanoparticles (LNPs) in enhancing mRNA stability and delivery, and the biochemical modifications to mRNA. Additionally, we assess the implications of mRNA vaccine biodistribution, examine mRNA vaccine pharmacokinetics, and discuss the potential impact of these vaccines on human health. Lastly, the possibility of siRNA and ribonuclease targeting chimeras (RIBOTACs) as therapeutic tools to mitigate adverse effects

associated with mRNA vaccines are discussed, highlighting their precision and versatility in gene silencing applications.

FDA Regulations

Preclinical biodistribution studies are conducted to assess target and non-target tissues for the presence, persistence, and clearance of the candidate drug [9-12]. The FDA advises that biodistribution studies for RNA therapeutics should only be performed for new vector classes or when substantial changes have been made to vector backbones, formulations, routes of administration, dose levels and dosing schedules [10]. Furthermore, only when mRNA vaccines use novel adjuvants, formulations, additives, or routes of administration does the US require that biodistribution studies be performed prior to in-human studies [13, 14]. The specific preclinical biodistribution studies requested may be based upon the product's individual components (e.g. RNA therapeutic construct, carrier, etc.) or the composite structure [9, 12, 13]. Of note, no biodistribution preclinical studies were included in the final mRNA-1273 and BNT162b2 COVID-19 vaccine mRNA-lipid nanoparticle (LNP) formulations since these applications relied on previous biodistribution data in which a different nucleoside-modified RNA was contained within LNP [15, 16]. According to the FDA, as a general starting point, preclinical biodistribution studies should examine, minimally, the blood, brain, gonads, heart, injection site(s), kidneys, liver, lungs, and spleen [17]. The selection of tissue panels can be modified depending on the RNA product, the expressed protein, and the route of administration [17]. For example, the FDA requires that the draining lymph node and contralateral site are also included in tissue panel selection when the product is injected intramuscularly [11]. Vervaeke et al. note that no minimal tissue panel is available for mRNA vaccine pharmacokinetic studies [17]. The FDA advises that preclinical

biodistribution rodent studies of RNA therapeutics and mRNA vaccines include at least 5 animals for each sex, experimental group, and sacrifice time point [11, 13].

The FDA granted both BNT162b2 and mRNA-1273 COVID-19 vaccines emergency use authorization in 2020 [18]. These mRNA vaccines are delivered via intramuscular injection (IM) and consist of nucleoside-modified messenger RNA (modRNA) encapsulated in LNP. Multiple techniques were employed to elucidate the biodistribution of the mRNA-1273 and BNT162b2 mRNA COVID-19 vaccines. Specifically, preclinical studies of both vaccines relied on whole-body autoradiography (QWBA) in rats [15, 16]. Additional techniques utilized in preclinical studies of the BNT162b2 and mRNA-1273 vaccines to examine biodistribution included liquid chromatography-mass spectrometry (LC-MS) and a multiplexed branched DNA (bDNA) assay, respectively [15, 16]. Furthermore, Pfizer-BioNTech determined the biodistribution of a LNP-formulated luciferase surrogate reporter through *in vivo* bioluminescence readouts. Peak signals were observed at the injection sites and the liver 6h post-IM injection of LNP-formulated luciferase RNA [15]. Notably, the FDA regards mRNA vaccines against infectious diseases as vaccines and not gene therapeutics [17].

BNT162b2 and mRNA-1273 Vaccine Modifications

The efficient application of mRNA vaccine technology requires successful circumvention of the aforementioned limitations. The BNT162b2 and mRNA-1273 vaccines have incorporated numerous modifications to surmount challenges associated with the therapeutic application of mRNA technology (**Figure 1**) [19]. Both the mRNA-1273 and BNT162b2 mRNA vaccines encode a SARS-Cov-2 prefusion full-length Spike glycoprotein and include stabilizing substitutions at the K986 and V987 positions. Additionally, both mRNA vaccines substituted each uridine with N1-methyl-pseudouridine to enhance mRNA secondary structure stability and decrease inherent

mRNA immunogenicity [20]. Specifically, Mauger et al. demonstrated that the introduction of modified uridines induces global changes in the mRNA secondary structure which may explain the reduced recognition of modified mRNA by RNA-binding proteins involved in innate immunity [20, 21]. Furthermore, the introduction of N1-methyl-pseudouridine (ψ) ostensibly increases the melting point of mRNA and, therefore, could enhance mRNA stability of the mRNA-1273 and BNT162b2 vaccines before administration. [21, 22].

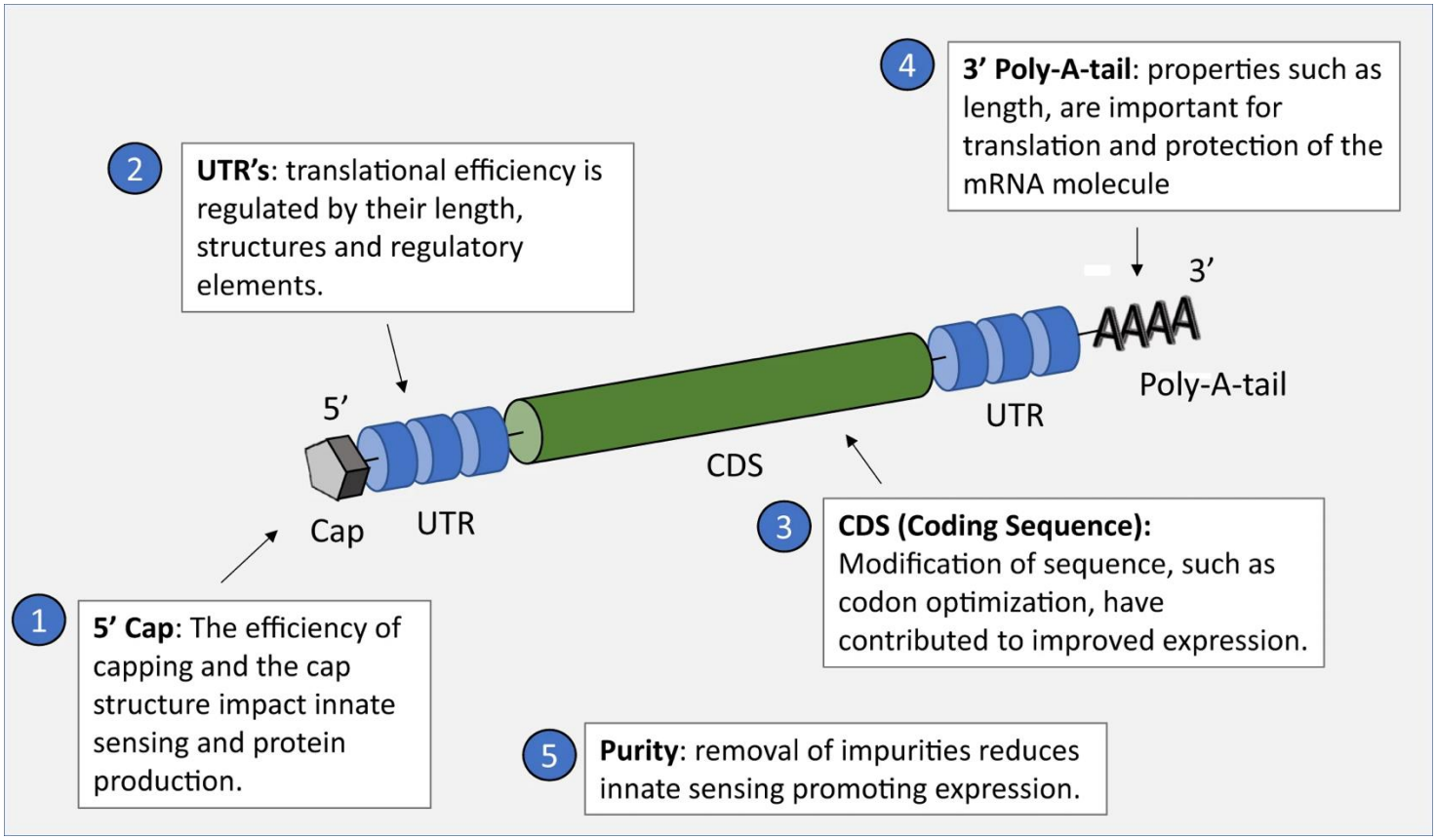

Figure 1: mRNA Construct Design for Therapeutic Applications. Five primary modifications include 5′ capping efficiency and structure; UTR structure, length, and regulatory elements; modification of coding sequence; poly-A-tail properties; mRNA purity.

**Figure reprinted from Jackson et al [19]. The figure legend title has been adapted. Permission to use this figure has been granted in accordance with the open access Creative Common CC BY 4.0 license.*

The 5’ cap, 5’ untranslated region (UTR), open reading frame (ORF), 3’ UTR and poly (A) tail constitute the five primary elements of mature eukaryotic mRNA [2]. These elements were incorporated into the BNT162b2 and mRNA-1273 vaccines to optimize mRNA design and to enhance mRNA translation efficiency. Mechanistically, the natural eukaryotic 5’ cap is a 7-methylguanosine (m7G) bound at the 5’ end of mRNA. It sterically inhibits nuclease-mediated degradation of mRNAs and binds to factor 4E to promote translation initiation [2]. The inclusion of the 5’ cap in the mRNA vaccines stabilizes mRNA molecules and promotes translation while the use of the poly(A) tail protects mRNA from enzymatic degradation and confers efficient mRNA translation initiation. The UTRs are modified in mRNA vaccine design to enhance protein expression and immune response. BNT162b2 incorporates the 5’-UTR of the highly expressed human α-globin gene with a minor Kozak consensus sequence modification [23]. The 5’-UTR in Moderna’s mRNA-1273 consists of element SEQ ID N01 followed by a GC-rich second element whose resultant secondary structure may increase mRNA stability and translation accuracy [24]. These 5’-UTR elements reside upstream of the Kozak consensus ACCAUG. Moderna and Pfizer-BioNTech utilized distinct approaches in their mRNA vaccines to optimize their respective 3’-UTR elements. Specifically, Moderna inserted the 110-nt 3’-UTR of human α-globin gene between the terminal stop codon and a poly (A) tail while the SELEX optimization protocol was utilized in the design of the Pfizer-BioNTech mRNA vaccine to identify two candidate RNA segments, one from the human mitochondrial 12S rRNA and the other region from human AES/TLES gene [23, 25]. A slightly modified 136-nt AES segment was inserted downstream from the second stop codon with the 139-nt human mitochondrial 12S rRNA inserted directly after [23]. The incorporation of pseudouridine in the ORF can enhance the stability and accuracy of mRNA translation. However, the inclusion of N1-methyl-pseudouridine introduces greater base pair wobble as it can pair with

A,G,C and U [26]. This substitution can result in misreads by near-cognate tRNA. Moreover, since all U nucleotides were substituted in the BNT162b2 and mRNA-1273 mRNA vaccines to avoid mRNA degradation, the stop codons use the more promiscuous N1-methyl-pseudouridine [15, 16, 27]. Such substitutions increase the chance of readthrough and the generation of longer proteins of unknown fate [24]. Consequently, both mRNA vaccines were designed with consecutive stop codons to prohibit readthrough. The selection of stop codons for BNT162b2 (ψGAψGA) and mRNA-1273 (ψGAψAAψAG), however, are potentially problematic as the stop codon context UGAU promotes a +1 frameshifting which would presumably be observed in the presence of the stop codon context ψGAψ, as well [28]. It should be noted that mRNA-1273 and BNT162b2 were designed with an increase in CGN codons and a decrease in AGR codons to encode arginine residues [23]. The elevated CpG content in vaccine mRNA confers greater stability than AU-rich regions [29]. Because host zinc finger antiviral proteins (ZAP), which target CpG dinucleotides and prompt viral RNA genome degradation, are virtually absent in skeletal muscle, intramuscular injection is a sensible route of vaccine administration [23]. The high CpG content in the mRNA vaccines provides the additional benefit that, in the improbable event vaccine mRNAs recombine into a SARS-CoV-2 virus, the abundant CpG presence would trigger ZAP-mediated degradation. Xia proposes that CGC and not CGG is the optimal arginine codon for mRNA vaccines as it is preferred by ribosomal protein genes and highly expressed muscle genes alike [23]. Regarding codon families in mRNA-1273, Moderna has applied the fundamentalist strategy of selecting the major codon while Pfizer-BioNTech also considered the demand on cognate tRNAs in their BNT162b2 codon optimization strategy [23]. Interestingly, Krawczyk et al. describe that the Moderna mRNA-1273 has been engineered with a ψCψAG sequence following the long poly (A) tail and that this ψCψAG sequence is removed after intramuscular injection. Although removal of

the ψCψAG sequence promotes mRNA-1273 de-adenylation and subsequent degradation *in vitro*, the injected mRNA-1273 becomes re-adenylated *in vivo* [30]. Furthermore, synthesized mRNA must be purified from reaction components in accordance with clinical quality standards. Impurities such as double-stranded RNA and DNA-RNA hybrid molecules have been shown to elicit an innate immune response [31]. Additionally, purification of mRNA has been shown to reduce the expression of type 1 interferon and increase protein translation while substantially impacting mRNA vaccine safety [32]. Pfizer-BioNTech and Moderna employed magnetic purification and oligo-dT, respectively, to purify the generated mRNA utilized in their COVID-19 vaccines (27, 33-36).

Since mRNA is readily degraded by RNases in the serum and plasma, and because exogenous RNA can induce an immune response, mRNA encapsulation within a LNP is an effective strategy to mitigate such outcomes [37, 38]. LNPs typically consist of an ionizable lipid portion, a stabilizing agent such as cholesterol or sphingolipid, a phospholipid, and polyethylene glycol (PEG) which increases half-life and circulation time by assisting metabolic and reticuloendothelial system (RES) evasion. In contrast to nucleic acids which are negatively charged, LNPs are essentially neutrally charged at physiological pH and, consequently, they are readily ingested by negatively charged biological membranes. Upon reaching the cell surface, these encapsulated mRNAs are ingested via endocytosis. The acidic environment of the endosomal compartment induces lipid ionization which consequently promotes endosomal escape and cytoplasmic transport of the mRNA cargo. Once docked at the ribosome, the mRNA is translated into the encoded antigen protein. The antigen epitopes are presented to the B cells to induce an immune response. The application of polyethylene glycol (PEG) to the surface of LNPs can enhance biocompatibility and reduce toxicity. The mRNA-1273 LNP composition is SM-102, polyethylene

glycol-2000-dimyristoyl glycerol (PEG2000-DMG), cholesterol and 1,2-distearoyl-sn-glycero-3-phosphocholine (DSPC) [39]. The LNP composition of the BNT162b2 mRNA vaccine consists of an ionizable amino lipid, phospholipid, cholesterol, and a PEGylated lipid [20, 40, 41]. The observation that increasing temperature reduces mean half-life of encapsulated mRNA is reflected in the requirements of BNT162b2 and mRNA-1273 vaccines that they be stored at -90ºC to -60ºC and at -20ºCC, respectively [15,16].

Detection of Vaccine mRNA *In Vivo*

The fate of nucleoside-modified synthetic mRNA (*nms*-mRNA) *in vivo* was not interrogated prior to emergency use authorization of mRNA vaccines for human use [42], and conflicting reports regarding the longevity of such constructs have subsequently emerged. The Infectious Diseases Society of America (IDSA) states that vaccine mRNA is quickly degraded intracellularly and that long-term detection of mRNA vaccines by RNA-seq is not evident [43]. Research by Pardi et al. using a murine model demonstrates that LNP-encapsulated mRNAs injected subcutaneously, intramuscularly, or intradermally translate for up to 10 days [44]. Surprisingly, full length or fragments of vaccine SARS-CoV-2 Spike mRNA were observed in 9.3% of patients' blood up to 28 days following administration of the mRNA-1273 or BNT162b2 mRNA vaccine [45]. The authors surmise that the mRNA detected in plasma is encased within LNPs since naked mRNA would rapidly degrade. Similarly, vaccine mRNA was observed in the germinal centers of lymph nodes 60 days after the second dose of mRNA-1273 or BNT162b2 was administered [46]. Furthermore, a study by Hanna et al. substantiates earlier findings that vaccine mRNA leaves the injection site to distribute systemically. The breast milk of 13 lactating women who had received either the BNT162b2 or mRNA-1273 COVID-19 vaccine was assessed for vaccine mRNA. Trace

amounts of vaccine mRNA which displayed reduced integrity were detected in some breast milk samples up to 45h post-vaccination [47].

Biodistribution and Pharmacokinetics of RNA Therapeutics and Endogenous mRNA

Endogenous mRNA exits the nucleus to localize in the cytosol where the level of gene expression is mediated by rates of mRNA synthesis and degradation. The mRNA decay pathway is initiated via Pan2-Pan3 and Ccr4-Not complex-mediated de-adenylation [48]. Subsequently, the mRNA can be processed through Xrn1- or exosome-mediated degradation. That is, 5'-3' degradation occurs when the Lsm1-7/Pat 1 complex binds to the 3' end and recruits the Dcp1-Dcp2 decapping complex, thus exposing the 5' end to Xrn1 enzymatic activity [49-53]. Alternatively, 3'-5' degradation by the cytoplasmic exosome occurs without decapping. Based upon thousands of transcript decay rates, Yang et al. estimate that the median mRNA half-life in human cells is 10h. Interestingly, both gene function and sequence motifs are correlated with human mRNA decay rates [54].

The advent of RNA therapeutics relied on novel formulations to protect nucleic acid from RNases and to facilitate cellular uptake. LNPs mitigate many challenges associated with RNA therapeutics and, hence, have emerged as a key mRNA delivery technology. LNP biodistribution studies utilizing encapsulated DiR molecules or deuterated lipids demonstrated that, following IM injection, the LNPs distributed throughout the body, with elevated levels detected in the liver [55]. Additionally, researchers discovered that particle size and delivery route impact LNP biodistribution and gene expression levels [55]. These results are consistent with previous findings regarding the biodistribution of vaccine mRNA. Lindsay et al. report using positron emission tomography-computed tomography (PET-CT) to track delivery of an mRNA vaccine against yellow fever in IM-injected Cynomolgus macaques [56]. The labeled mRNA was apparent at the

site of injection as well as in the inguinal, iliac, and para-aortic lymph node regions at the selected 4h and 28h post-injection time points. *In vivo* imaging indicates that the fluorescent-labeled mRNA continues to accumulate in the lymph nodes for at least 28h post-injection [56].

Biodistribution and Pharmacokinetics of BNT162b2 and mRNA-1273

A comprehensive understanding of synthetic mRNA LNP pharmacokinetics is lacking. Consistent with FDA regulations, BNT162b2 and mRNA-1273 COVID-19 vaccine applications relied on the biodistribution data obtained from a different nucleoside-modified RNA sequence encapsulated within LNP formulations [15, 16].

Pfizer-BioNTech incorporated the ionizable lipid ALC-0315 (4-hydroxybutyl)azanediyl) bis(hexane-6,1-diyl)bis(2-hexyldecanoate) into its BNT162b2 formulation because the lipid's neutral charge at physiological pH facilitates internalization while its positive charge in the endosome promotes RNA release into the cytosol [57, 58]. A second novel lipid component of the BNT162b2 formulation, ALC-0159 (2-[(polyethylene glycol)-2000]-N,N-ditetradecylacetamide), was introduced to provide a steric barrier. Studies were conducted to evaluate the biodistribution of formulations with these novel components as LNP composition may affect distribution. To assess the pharmacokinetics of the novel lipids, Wistar Han rats were IV-bolus injected with a LNP formulation containing luciferase-encoding RNA, ALC-0315 and ALC-0159 [15]. LC-MS/MS was used to detect the presence of ALC-0315 and ALC-0159 in plasma, liver, urine, and feces for up to 2 weeks. Both ALC-0315 and ALC-0159 concentrations were reduced to less than 1% of the maximum plasma concentrations 24h after the bolus injection. Evidence suggests that the liver is the major organ for ALC-0315 and ALC-0159 distribution following plasma clearance [15]. The maximum concentrations of ALC-0315 and ALC-0159 detected in the liver were at 3h and 0.5h, respectively, post-IV injection. After 2 weeks, liver concentrations of ALC-0315 and ALC-0159

were determined to be at approximately 25% and 0.04%, respectively, of the maximum detected concentrations [15]. Pfizer also examined the biodistribution of a LNP having an identical lipid composition to that used in BNT162b2 and bearing a surrogate luciferase modRNA [15]. Using a murine model, *in vivo* bioluminescence measurements were taken at various time points post IM-injection. Injection site signal peaked at 6h post-injection with levels approximately 10,000 times that of the control and diminished to about 7 times that of the control level by day 9. The liver demonstrated peak signal 6h post-injection which reduced to background levels 48h after injection [15]. A rat study using radiolabeled LNP and luciferase modRNA was also conducted to determine biodistribution as radiolabeling is considered a more sensitive approach. At the earliest time point chosen (0.25h post-IM injection) most of the selected tissues demonstrated radioactivity with notable distribution to the injection site and liver. Highest plasma levels were observed 1-4 h following dose with distribution mainly detected in the liver, adrenal glands, spleen, and ovaries over a 48h interval post-injection. The percentages of the injected dose of radiolabeled LNP + modRNA recovered from the liver, spleen, adrenal glands, and ovaries were approximately 21.5%, $\leq$1.1%, $\leq$0.1% and $\leq$0.1%, respectively [15]. Additionally, metabolic studies were performed which suggest that ALC-0315 is metabolized via sequential ester hydrolysis reactions to produce a monoester metabolite and a doubly de-esterified metabolite. The monoester was detected *in vivo* in rat plasma and liver while the doubly de-esterified metabolite was detected *in vivo* in rat plasma, urine, feces, and liver. ALC-0159 is ostensibly metabolized via amide bond hydrolysis to yield N,N-ditetradecylamine. No discernible elimination of ALC-0315 and ALC-0159 was detectable in the urine. Regarding ALC-0315 and ALC-0159, approximately 1% and 50%, respectively, of the dose was excreted unchanged in the feces [15]. It should be noted that different RNAs likely

display distinct kinetics such that the luciferase expression profile may differ somewhat from the full-length Spike protein expression profile.

The biodistribution of Moderna's mRNA-1273 is predicted to reflect the biodistribution profile observed for mRNA-1647, an mRNA vaccine composed of 6 mRNAs encoding cytomegalovirus (CMV) antigens encapsulated in a proprietary LNP. Although the same lipid nanoparticle composition was used for each construct, the size of the LNPs differed slightly between mRNA-1273 and mRNA-1647. An *in vivo* rat study was conducted whereby a qualified multiplex branched DNA (bDNA) assay was performed to quantitate the 6 mRNA constructs in blood or specified organs/tissues pre-dose or at various time points following IM injection of mRNA-1647 [16]. Peak concentrations of mRNA were observed for tissues with exposures above plasma levels at between 2h and 24h following IM injection. In addition to the injection site, vaccine mRNA localized to the lymph nodes, liver, spleen, blood, heart, lung, testis, eye, and brain, demonstrating movement of the mRNA construct across the blood-brain barrier [16]. Half-life values of mRNA-1647 at the site of injection, in proximal popliteal and axillary distal lymph nodes, and in spleen were estimated as 14.9h, 34.8h, 31.1h and 63.0 h, respectively [16]. The mRNA construct was cleared from plasma within the first 24 hours and was not measurable in tissue beyond 3 days with the exceptions of muscle, lymph nodes and spleen [16]. **Figure 2** illustrates the putative biodistribution of mRNA after BNT162b2 and mRNA-1273 COVID-19 vaccine administration.

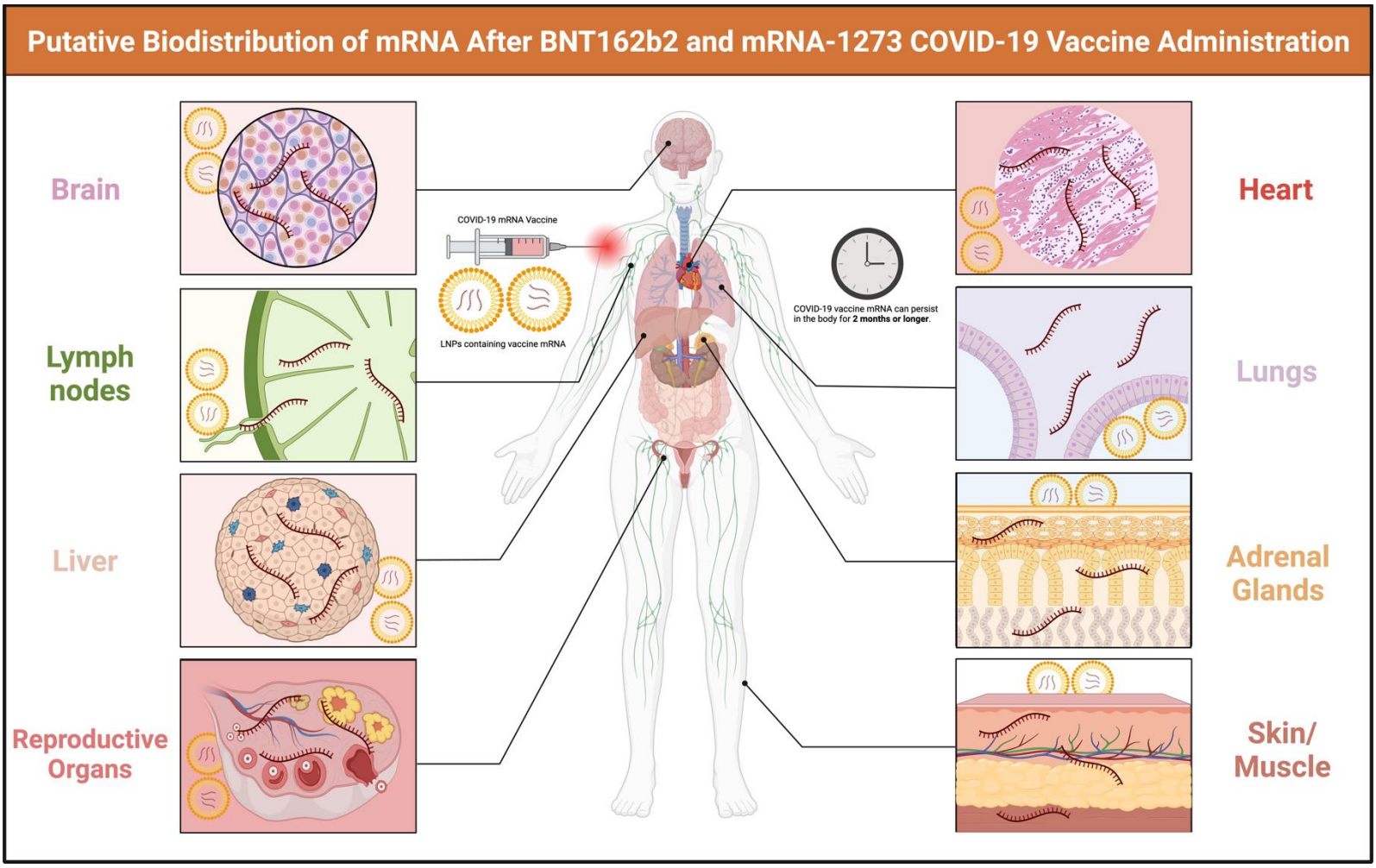

Figure 2: Putative Biodistribution of mRNA after BNT162b2 and mRNA-1273 COVID-19 Vaccine Administration. COVID-19 vaccines inject lipid nanoparticles (LNPs) containing mRNA that encodes for the Spike protein. Proxy construct studies demonstrate that LNPs and mRNA are widely distributed and persist throughout the body.

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Rationale for COVID-19 Vaccine mRNA Degradation

The regulation of cytoplasmic mRNA expression is tightly controlled as evidenced by the operation of parallel, partially redundant mRNA degradation pathways [59]. Such mRNA decay mechanisms include the enzymatic activities of exo- and endoribonucleases which themselves require proper activation [59]. The stability of mRNAs can vary substantially; the half-life of the

c-*fos* proto-oncogene transcript is approximately 10'-15' whereas globin mRNA exhibits a half-life of several hours [60, 61]. As detailed, the BNT162b2 and mRNA-1273 vaccines have been designed with enhanced stability. Clinical studies which demonstrate the presence of mRNA vaccines in blood and lymph nodes weeks and months after vaccine administration, respectively, attest to the stability of these constructs [45, 46]. The physiological effects in humans of the enhanced BNT162b2 and mRNA-1273 mRNA longevity is not fully understood. However, several potential concerns regarding the mRNA vaccine design have been identified. Both mRNA vaccines substituted all uridines with the N1-methyl-pseudouridine (ψ) residue which exhibits increased wobble [26, 62]. Consequently, near-cognate tRNAs could incorporate incorrect amino acids into the growing peptide chain. Additionally, the replacement of uridine with N1-methyl-pseudouridine in the stop codons increases the likelihood of readthrough and the production of longer protein with potentially harmful effects. Rubio-Casillas et al. found that the addition of 100% of N1-methyl-pseudouridine to an mRNA vaccine stimulated cancer growth and metastasis in a melanoma model [63]. Furthermore, *in vitro* studies conducted in the human liver cell line suggest the possibility that the mRNA in the BNT162b2 COVID-19 vaccine may integrate into the host genome using a LINE1-mediated retro-position mechanism [64]. The observed wide biodistribution of mRNA-nanolipid vaccine compounds coupled with the stability of synthetic modified mRNA suggests that mRNA vaccines could induce a prolonged inflammatory response in various tissues through production of Spike protein, which has been shown to possibly be toxic to all organ systems and results in adverse events such as myocarditis, thrombosis, and death (65-67). Thus, suppressing uncontrolled Spike protein production via targeted COVID-19 vaccine mRNA neutralization may be a promising strategy to attenuate or prevent adverse events.

siRNA and RIBOTAC Applications

COVID-19 vaccine adverse events may be mitigated with small interfering RNA (siRNA) and ribonuclease targeting chimeras (RIBOTACs) (**Figure 3**). RNA interference (RNAi) is a eukaryotic defense mechanism whereby invading genetic material is degraded via a microRNA (miRNA)- or siRNA-mediated pathway [68]. The latter RNAi pathway is initiated upon the introduction of double-stranded RNA (dsRNA) which consequently induces Dicer to cleave the dsRNA into short fragments (~20 nt long). These fragments are then incorporated into the RNA-induced Silencing Complex (RISC). Subsequently, the guide strand binds to the complementary sequence of the target mRNA to activate Argonaute 2 endonuclease (Ago 2) within RISC. Ago 2 cleaves the target mRNA, thus compromising protein expression [69]. The therapeutic potential of siRNAs arises from their high specificity, restriction to the cytoplasm which precludes genome integration, remarkable efficiency, and target versatility [69]. However, despite their numerous advantages, substantial obstacles must be surmounted to effectively harness the power of siRNAs. Barriers to the successful implementation of siRNAs as a therapeutic intervention include their susceptibility to degradation by endogenous nucleases in serum [70], rapid renal clearance [71], activation of the innate immune system [72, 73], plasma protein sequestration and entrapment by the reticuloendothelial system (RES) [74-76], membrane impermeability [77], endosomal entrapment [78, 79] and off-target effects [80]. Chemical changes, such as ribose [81, 82] and terminal [83] modifications, have been introduced to overcome inherent challenges. Additionally, lipid-based delivery systems [84, 85], viral vectors [86-88], and other delivery strategies have been developed to mitigate obstacles to siRNA therapy. Inclisiran (trade name Leqvio®) is an example of one such siRNA therapy which has received FDA approval for the treatment of hypercholesterolemia [89]. Proprotein convertase subtilisin/kexin type 9 (PCSK9) promotes liver cell LDL receptor degradation, thereby reducing clearance of low-density lipoprotein cholesterol

(LDL-C) from the blood [90]. Inclisiran was engineered with an affinity and specificity for the asialoglycoprotein receptor (ASGPR) on liver cell membranes [90]. Once in the hepatocytes, the siRNA binds RISC to direct the degradation of PCSK9 mRNA and, consequently, promotes LDL-C clearance. The main advantage of inclisiran over anti-PCSK9 monoclonal antibodies is its favorable dosing regimen which should improve compliance. While inclisiran shows promise for patients at high cardiovascular risk, considerations such as long-term safety and efficacy as well as interactions with other compounds warrant further investigation. However, seven clinical trials have shown this siRNA therapy to be safe and well-tolerated for long-term administration [91]. Similarly, patisiran (Onpattro®), an LNP-based siRNA therapeutic, received FDA approval in 2018 for the treatment of polyneuropathy caused by hereditary transthyretin-mediated (hATTR) amyloidosis. The intravenously administered, encapsulated siRNA accumulates in the liver. Upon hepatic uptake of the LNPs, siRNAs degrade transthyretin (TTR) mRNA to reduce TTR protein production [92]. Therefore, siRNA therapy represents a promising approach to selectively deactivate and degrade undesirable and persistent vaccine mRNA, thus shutting off further systemic exposure to the toxic and potentially lethal Spike protein.

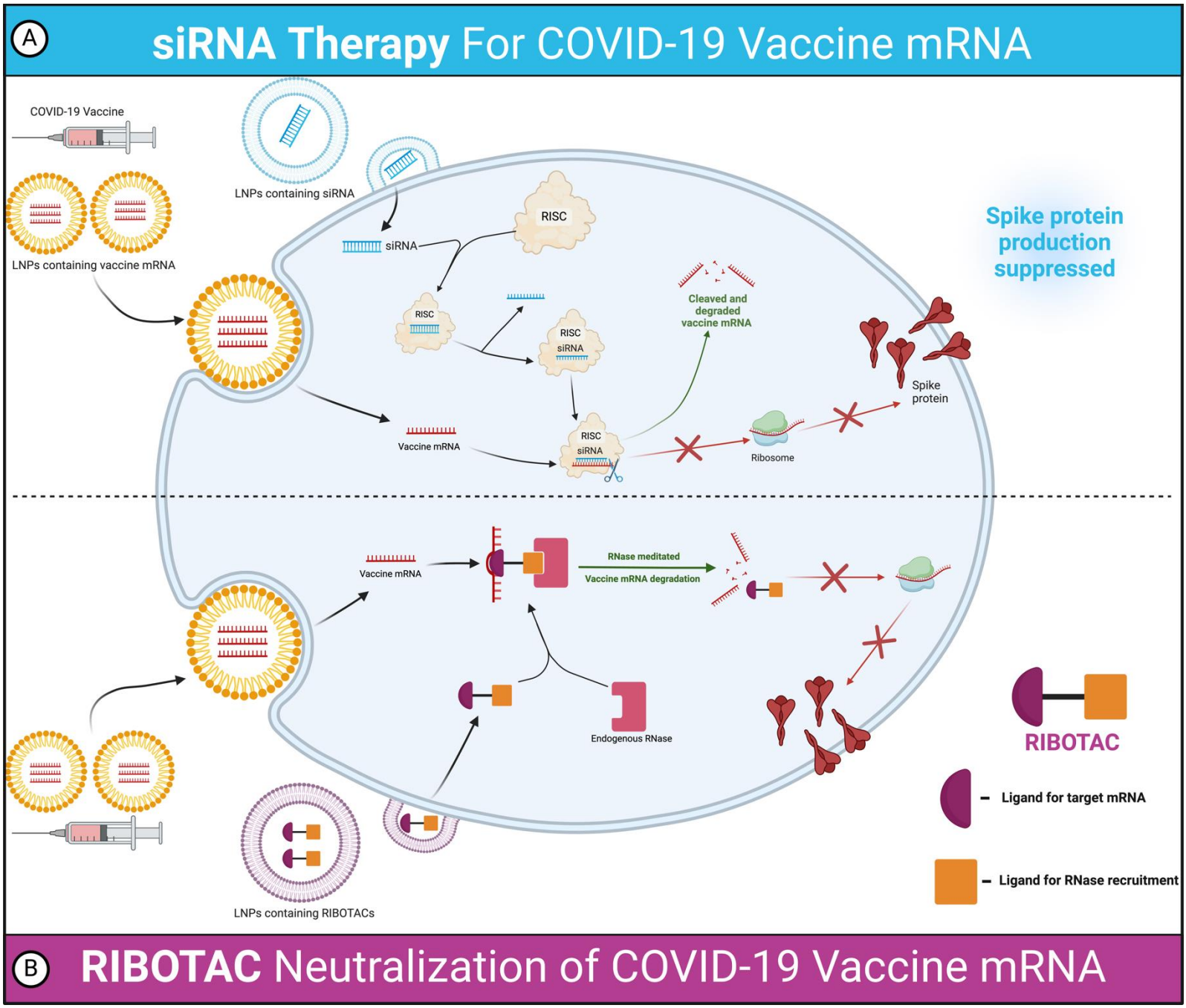

Figure 3: Methods to Target and Degrade Residual and Persistent COVID-19 Vaccine mRNA. A: siRNA targeted against COVID-19 vaccine mRNA enters the vaccinated cell via LNPs, where it incorporates into the RISC. The siRNA in RISC binds to the complementary sequence of the target vaccine mRNA and cleaves it, thus suppressing Spike protein production. **B:** RIBOTACs targeted against COVID-19 vaccine mRNA enter the vaccinated cell via LNPs, where they bind to both the target vaccine mRNA and endogenous RNase. This results in RNase-mediated vaccine mRNA degradation and the suppression of Spike protein production.

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Abbreviations: LNPs: Lipid Nanoparticles, mRNA: Messenger Ribonucleic Acid, RIBOTACs: Ribonuclease Targeting Chimeras, RISC: RNA-induced Silencing Complex, RNase: Ribonuclease, siRNA: Small Interfering Ribonucleic Acid.

In addition to the siRNA approach, the novel emerging technology, ribonuclease targeting chimeras (RIBOTACs), consists of bivalent molecules containing an RNA-binding module and a ribonuclease (RNase) recruitment module. Once they bind to the target RNA, RIBOTACs recruit an endogenous RNase in close proximity to the target, thereby facilitating its degradation [93]. RIBOTACs were created by Costales et al. where they targeted the microRNA-210 hairpin precursor (pre-miR-210), which is overexpressed in hypoxic cancers. They successfully cleaved pre-miR-210 substoichiometrically and induced apoptosis in breast cancer cells [94]. The primary limitations of RIBOTACs are that they exhibit low bioavailability and poor cellular uptake due to having a high molecular weight [95]. This problem may be countered by incorporating even smaller RNase-binding ligands in the molecular structure. As RIBOTACs are novel therapeutics, it will take some time to bypass limitations and establish safety and efficacy. Nonetheless, RIBOTACs offer another promising platform to degrade rogue vaccine mRNA and limit production of the Spike protein.

Conclusions

The Pfizer-BioNTech and Moderna biodistribution studies refute the assertion that nanolipid-bound *nms*-mRNA remains in the deltoid muscle or axillary lymph nodes. Detectable vaccine mRNA levels remaining in various tissues raises potential safety concerns. The possibility of vaccine mRNA integration into the host genome and the prospect of unintended protein production due to readthrough advocate for a mechanism to eliminate lingering synthetic mRNA and halt

damaging Spike protein production. The use of siRNA and RIBOTACs to target and degrade vaccine mRNA are promising approaches to mitigate deleterious health effects. The ability to readily tailor the siRNA and RIBOTACs to target an mRNA of interest makes these techniques particularly appealing, although further investigation is warranted to address challenges which include possible off-target effects and immune system activation.

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