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Functional Comparison of Novel Bioengineered RNA let-7-5p Isoforms to Modulate Target Gene Expression and ADME Function in Liver Cancer Toward Development of Improved Therapies

By

JOSEPH MATTHEW CRONIN
DISSERTATION

Submitted in partial satisfaction of the requirements for the degree of

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in

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in the

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Dedications

This research and dissertation are dedicated to my incredible family, friends, and girlfriend, who were so supportive and understanding through many long days in the lab and long nights writing. This work is a testament to the outstanding faculty at UC Davis, the exceptional mentorship of my major professor Dr. Ai-Ming Yu, and the amazing lab colleagues I have had the pleasure of working alongside.

Dissertation Abstract

Hepatocellular carcinoma (HCC) is a highly lethal liver malignancy often characterized by multidrug resistance, leading to significant challenges in advanced systemic treatment. In HCC and other cancers, aberrant expression of the genes governing the processes of drug metabolism and pharmacokinetics (DMPK) can alter therapeutic drug exposure, efficacy, and safety. Elucidating the mechanisms underlying variable drug disposition in cancer is critically important to overcoming multidrug resistance and developing therapies that are safe and effective. Novel approaches in cancer therapy and DMPK research are needed to address these shortcomings and expand the scope of druggable targets. In pursuit of this goal, research efforts have uncovered posttranscriptional gene regulation by endogenous microRNAs (miRNAs) as important regulatory factor in countless cellular processes, including natural DMPK function, multidrug resistance, and cancer progression.

Historically, recombinant DNA technologies have been foundational for the heterologous expression, purification, and structural characterization of absorption, distribution, metabolism, and excretion (ADME) proteins, such as the cytochrome P450 enzymes. Building upon this legacy, our lab has recently optimized a novel *in vivo* fermentation-based bioengineering platform that produces recombinant RNA (BioRNA) from small volumes of bacterial culture. Utilizing a stable htRNA/pre-miR34a carrier system, this platform achieves high-yield production of fully humanized BioRNAs that accurately recapitulate the structures and modifications of endogenous microRNAs (miRNAs), overcoming the limitations of inconsistently modified synthetic miRNA mimics and providing superior tools for studying ADME gene regulation.

As RNA research has advanced, the clinical landscape has rapidly expanded to include various FDA-approved small RNA and oligonucleotide therapeutics. This emergent therapeutic class encompasses aptamers that selectively bind target proteins, antisense oligonucleotides (ASOs) that modulate protein expression through mRNA degradation, alternative splicing, or steric hindrance, and small interfering RNAs (siRNAs) that leverage the intrinsic RNA interference (RNAi) pathway via the RNA-induced silencing complex (RISC) to regulate protein expression of target mRNA transcripts. To overcome inherent biological instability and delivery barriers, these drugs heavily rely on structural innovations, including phosphorothioate backbone linkages, ribose sugar modifications, and targeted delivery strategies like N-acetylgalactosamine (GalNAc) conjugation for hepatocyte specific uptake. Oligonucleotides' unique structural features and ADME properties are distinct from traditional small-molecule or protein based drugs, requiring specialized strategies to characterize their PK profiles and drug-drug interaction (DDI) risks. Exogenous RNA therapeutics also present novel challenges for conventional DDI risk assessment, introducing the risk of competitively saturating shared intrinsic RNAi machinery, such as Argonaute (AGO) proteins and the nuclear export protein exportin-5 (XPO5). This competition threatens to disrupt the natural PTGR homeostasis of endogenous ADME and pharmacodynamic genes, potentially leading to pharmacodynamic-mediated DDIs that are not predicted by traditional small-molecule assessment frameworks.

To interrogate miRNA-mediated PTGR of relevant oncogenes and ADME genes, the aforementioned BioRNA platform was utilized to systematically compare the effectiveness of 7 major human let-7-5p isoforms in modulating target protein expression and inhibiting HCC cell

viability. The let-7 miRNA family is frequently downregulated in HCC, contributing to the overexpression of multidrug resistance transporters. The comparative evaluation revealed that the regulatory efficacy of these biologic let-7-5p agents is highly target-dependent, sequence-specific, and variable across differing cellular contexts. Specifically, let-7c-5p and let-7d-5p demonstrated the broadest and most effective suppression of the multidrug resistance transporter ABCC5/MRP5 across three heterogeneous HCC cell lines. Additionally, let-7d-5p emerged as the most potent repressor of the oncogenic RNA-binding protein LIN28B, correlating with its high degree of target complementarity outside of the conserved seed sequence. Furthermore, these bioengineered isoforms exhibited variable, cell line-specific efficacy in inhibiting the growth and viability of different HCC cells.

Collectively, this body of work demonstrates the potential of let-7 miRNA replacement therapy in HCC and highlights the profound utility of bioengineered RNA agents in elucidating the fundamental PTGR mechanisms underlying variable DMPK function and therapeutic disposition in cancer, harnessing unparalleled BioRNA research tools to identify specific let-7-5p miRNA isoforms capable of modulating critical ADME targets to combat therapeutic resistance and disease progression in HCC.

Complete List of Abbreviations

ABC, ATP-binding cassette; ABR, Annualized bleeding rate; ADME, Absorption, distribution, metabolism, and excretion; AGO, Argonaute; AHP, Acute hepatic porphyria; ALAS1, Aminolevulinate synthase 1; ALS, Amyotrophic lateral sclerosis; ALT, Alanine aminotransferase; AMD, Age-related macular degeneration; Apo B-100, Apolipoprotein B-100; ASCVD, Atherosclerotic cardiovascular disease; ASGPRS, Asialoglycoprotein receptors; ASOs, Antisense oligonucleotides; AST, Aspartate aminotransferase; AT, Antithrombin; BCA, Bicinchoninic acid; BEBM, Bronchial epithelial cell basal medium; BioRNA, Bioengineered RNA; BSA, Bovine serum albumin; cDNA, Complementary DNA; CLIP, Cross-linking immunoprecipitation; CMV, Cytomegalovirus; CRISPR, Clustered regularly interspaced short palindromic repeats; CYP / P450, Cytochrome P450; DDIs, Drug-drug interactions; DMD, Duchenne muscular dystrophy; DMEM, Dulbecco's modified eagle medium; DMPK, Drug metabolism and pharmacokinetics; EMEM, Eagle's modified essential medium; FCS, Familial chylomicronemia syndrome; FDA, Food and Drug Administration; FISH, Fluorescence in situ hybridization; GA, Geographic atrophy; GalNAc, N-acetylgalactosamine; GalNAc3, Tri-antennary GalNAc ligand; HAE, Hereditary angioedema; HAO1, Hydroxyacid oxidase 1; hATTR, Hereditary transthyretin-mediated amyloidosis; HCC, Hepatocellular carcinoma; HeFH, Heterozygous familial hypercholesterolemia; HoFH, Homozygous familial hypercholesterolemia; IE2, Immediate early region 2; ISS-N1, Intronic splicing silencer N1; IV, Intravenous; IVT, Intravitreal; LDHA, Lactate dehydrogenase A; LDL-C, Low-density lipoprotein cholesterol; LNP, Lipid nanoparticle; lpp, Lipoprotein; MDR, Multidrug resistance; MDS, Myelodysplastic syndromes; miRNAs, MicroRNAs; MREs, miRNA response elements; mRNA, Messenger RNA; MRP, Multidrug resistance-associated proteins; ncRNA, Noncoding RNAs; PCSK9, Proprotein convertase subtilisin/kexin type 9; PD, Pharmacodynamic; Pdx, Putidaredoxin; PEG, Polyethylene glycol; PH1, Primary hyperoxaluria type 1; piRNAs, PIWI-interacting RNAs; PK, Pharmacokinetic; PK/PD, Pharmacokinetic/pharmacodynamic; PMOs, Phosphorodiamidate morpholino oligonucleotides; pre-miRNAs, Precursor miRNAs; pri-miRNAs, Primary miRNAs; PS, Phosphorothioate; PTGR, Post-transcriptional gene regulation; PVDF, Polyvinylidene difluoride; QWBA, Quantitative whole body radiography; rDNA, Recombinant DNA; RISC, RNA induced silencing complex; RNAi, RNA interference; rop, Repressor of primer; rrrnC, Ribosomal RNA operon termination sequence; RT-qPCR, Quantitative real-time PCR; saRNA, Small activating RNA; SD, Standard deviation; SELEX, Systematic evolution of ligands by exponential enrichment; shRNAs, Short hairpin RNAs; siRNAs, Small interfering RNAs; SMA, Spinal muscular atrophy; SMN, Survival motor neuron; SOD1, Superoxide dismutase 1; SQ, Subcutaneous; SRP, Signal recognition particle; TDMD, Target directed miRNA degradation; TEV, Tobacco etch virus; TTR, Transthyretin; US, United States; UTR, Untranslated region; VDR, Vitamin D receptor; VEGF, Vascular endothelial growth factor; XPO5, Exportin-5; 2'-MOE, 2'-O-(2-methoxyethyl); 3'UTR, 3'-untranslated region;

Table of Contents

Chapter 1: Recombinant Technologies Facilitate Drug Metabolism, Pharmacokinetics,

<u>and General Biomedical Research</u>	1
Abstract	2
Significance Statement.....	3
Introduction.....	3
Recombinant DNA Technology	6
Recombinant ADME Proteins for Functional and Structural Studies	17
Recombinant RNAs for Studying ADME Gene Regulation	27
Conclusions and Perspectives	33
Tables	36
Figures.....	39

Chapter 2: Small RNA or Oligonucleotide Drugs and Challenges in Evaluating

<u>Drug-Drug Interactions (DDIs)</u>	42
Abstract	43
Introduction.....	44
Common Mechanistic Actions of RNA Therapeutics	47
Aptamer Drugs.....	49
Antisense Oligonucleotide Drugs	52
RNA Interference Drugs	58
Challenges in the Evaluation of DDI of RNA Drugs	63
Conclusions and Perspectives	72
Tables	74

Figures.....	79
<u>Chapter 3: Comparative Effectiveness of 7 Major Human let-7-5p Isoforms to Modulate</u>	
<u>Target Gene Expression in Liver Cells</u>	82
Abstract.....	83
Significance Statement.....	84
Graphical Abstract	85
Introduction.....	86
Materials and Methods.....	90
Results.....	96
Discussion.....	104
Tables.....	111
Figures.....	112
<u>References</u>	119

Chapter 1: Recombinant Technologies Facilitate Drug Metabolism, Pharmacokinetics, and General Biomedical Research

Abstract

The development of safe and effective medications requires a profound understanding of their pharmacokinetic (PK) and pharmacodynamic (PD) properties. PK studies have been built through investigation of enzymes and transporters that drive drug absorption, distribution, metabolism, and excretion (ADME). Like many other disciplines, the study of ADME gene products and their functions has been revolutionized through the invention and widespread adoption of recombinant DNA (rDNA) technologies. Recombinant DNA technologies utilize expression vectors such as plasmids to achieve heterologous expression of a desired transgene in a specified host organism. This has enabled the purification of recombinant ADME gene products for functional and structural characterization, allowing investigators to elucidate their roles in drug metabolism and disposition. This strategy has also been utilized to offer recombinant or bioengineered RNA (BioRNA) agents to investigate the posttranscriptional regulation of ADME genes. Conventional research with small noncoding RNAs (ncRNA) such as microRNAs (miRNAs) and small interfering RNAs (siRNAs) has been dependent on synthetic RNA analogs that are known to carry a range of chemical modifications expected to improve stability and PK properties. Indeed, a novel tRNA fused pre-miRNA carrier-based bioengineering platform technology has been established to offer consistent and high-yield production of unparalleled BioRNA molecules from *E. coli* fermentation. These BioRNAs are produced and processed inside living cells to better recapitulate the properties of natural RNAs, representing superior research tools to investigate regulatory mechanisms behind ADME.

Significance Statement

In this review article, we summarize recombinant DNA technologies that have been an incredible boon in the study of drug metabolism and pharmacokinetics, providing investigators with powerful tools to express nearly any ADME gene products for functional and structural studies. We further overview novel recombinant RNA technologies and discuss the utilities of bioengineered RNA agents for the investigation of ADME gene regulation and general biomedical research.

Introduction

The understanding of drug metabolism and pharmacokinetic (DMPK) processes represents a critical and necessary step in the development of new drugs. Elucidating these processes and understanding how they differ between species is key in translating basic scientific research into an efficacious and safe clinical treatment. The importance of DMPK in drug development and safety was first made widely evident by the tragedy of thalidomide, a morning sickness drug developed with rat models that caused severe birth defects in humans^{1,2}. While the cytochrome P450 (CYP or P450) enzymes were identified and characterized spectroscopically in 1962, widespread appreciations of P450s in xenobiotic metabolism and inter-species differences in drug disposition had not yet been established³. Further research with thalidomide determined that its teratogenic effect could be attributed to toxic metabolites discovered in the urine of treated animals⁴. Notably, this toxic bioactivation of thalidomide was absent in rat liver microsomes and only observed in rabbits, primates, and humans⁴. This tragedy and subsequent discovery illustrated

the importance of inter-species differences in drug metabolism and resulting toxicity, driving forward DMPK research and influencing key regulatory law still in effect today.

Soon afterward, key advancements in molecular biology and the subsequent birth of recombinant DNA (rDNA) technologies (**Figure 1**) would equip DMPK researchers with the tools to investigate unprecedented research questions in drug metabolism and disposition⁵. This was not limited to CYP enzymes, but also included other genes and proteins involved in absorption, distribution, metabolism, and excretion (ADME) processes. Nucleotide sequencing technologies enabled the generation of complementary DNA (cDNA) libraries for CYP enzymes^{6,7} (**Figure 1**) and other ADME gene products for use in molecular cloning^{5,8-10}. Generated cDNA sequences were used to produce recombinant P450 enzymes through heterologous expression in model systems such as yeast (*Saccharomyces cerevisiae*) and *E. coli* (*Escherichia coli*)¹¹⁻²⁰. With previous research limited to the use of liver microsomes or P450s purified from the tissues²¹⁻²³, the development of rDNA strategies propelled the field forward by enabling the characterization of individual P450 enzymes *in vitro* (**Figure 1**)^{5,11,18-20,24-27}. This approach proved to be a significant boon to the fields of DMPK, empowering researchers to interrogate key differences between human P450 reactions and those relevant to pre-clinical animal models⁵. Recombinant DNA technologies have proven invaluable in the screening and hazard identification of countless drugs, enabling characterization of ADME gene products and arming medicinal chemists and pharmacologists with the knowledge of common moieties likely to undergo biotransformation.

While innovation in molecular biology has given researchers the tools to answer many questions regarding ADME genes and drug metabolism, it has also illuminated a complex system of drug

disposition that varies widely across species, sex, and tissue type⁵. Not only does the expression of individual CYP enzymes vary by organism and tissue, but their catalytic activity can show significant interspecies differences²⁸. Even within the same species some CYP enzymes are highly polymorphic, exhibiting interindividual variations as well as broader racial differences such as those in observed in poor CYP2D6 and CYP2C19 metabolizers^{19,29}. Further complicating things, CYP enzyme expression can be induced through exposure to a wide variety of drugs and environmental chemicals³⁰. Although a variety of induction mechanisms have been elucidated, this largely occurs through xenobiotic interaction with nuclear receptors to drive an increase in CYP gene transcription³¹. Natural products have also been identified to inhibit critical drug-metabolizing CYP enzymes rather than induce them, with the most widely known example being grapefruit juice's inhibition of CYP3A4³². Similar to expression level and catalytic activity, induction of CYP genes in response to environmental chemicals or drugs can vary widely between species as well as between individuals^{30,33}. Understanding these differences in ADME gene expression, protein activity, and gene regulation in response to chemicals is critical to designing safe drugs that account for species and individual differences in drug metabolism and toxicity.

Although significant advancements have been made, continuing to broaden our understanding of ADME gene regulation and function in DMPK is necessary to inform the development of safe and efficacious medications. When analyzed by exome sequencing, ADME genes were found to have significantly more variability and possible variants compared to non-ADME genes³⁴. ADME gene variations between individuals, populations, and species can have drastic effects on DMPK, therefore affecting therapeutic safety and efficacy. These variations between species can have a dramatic impact on the success of drug development, decreasing the face validity of pre-clinical

animal models and negatively affecting whether scientific research can be translated into an effective clinical therapy. Approximately 54% of novel therapeutics failed in clinical development from 1998 to 2015, with the large majority of failures being attributed to inadequate efficacy or safety³⁵. In addition to this, therapies that were successfully approved took an average time of 17 years for research discoveries to reach the clinic³⁶. Recent updates to US FDA guidelines have also removed the requirement that all drugs be tested in animals, instead welcoming more representative models of human disease such as organoid or organ-on-a-chip approaches³⁷. Keeping these shortcomings in mind, it is clear that novel technologies and approaches are needed to further elucidate ADME gene regulation and function in DMPK. Advancement of these fields is critical to educate researchers and improve the success rate of developing drugs, as well as shorten the lag time observed bringing a drug from “bench to bedside”. This minireview will examine rDNA technologies in the context of DMPK research, highlighting historical successes expressing recombinant proteins for structural and functional studies as well as discussing new recombinant RNA approaches that have offered unique RNA molecules to investigate ADME gene regulation and novel research questions.

Recombinant DNA Technology

Recombinant DNA technology is characterized by altering genetic material outside an organism with the goal of transforming desired characteristics into a living organism or their products. Recombinant technologies would not be imaginable without those that laid the scientific foundation of how we understand heredity, genetics, and molecular biology. In this way, all modern successes of rDNA technologies stand on the shoulders of giants like Gregor Mendel that first

began to elucidate these key genetic processes³⁸. These researchers broadened the scientific understanding of genetics and DNA, enabling the development of critical technologies that set the stage for genomic sequencing, molecular cloning, and modern rDNA technologies.

rDNA Technology: A Brief History

In 1952, Joshua Lederberg proposed the term “plasmid” for an extrachromosomal genetic material that had been recently observed (**Figure 1**)³⁹. At the time, nobody could predict that the plasmid would one day become the workhorse at the center of countless recombinant technologies across a diverse range of industries. We know now that plasmids are circular, double-stranded DNA molecules naturally present in the cytoplasm of bacteria and other prokaryotes that are capable of replicating independently from the host cell^{10,40}. Plasmids encode the information needed for their own replication, and can confer specific characteristics to the host organism such as antibiotic resistance¹⁰. However, research at the time of their discovery was hampered by a lack of viable strategies to introduce these circular double-stranded DNA molecules into cells⁹. This research problem would lead to a monumental breakthrough for rDNA technologies by driving collaboration between experts in plasmid technology and targeted DNA manipulation⁹. This collaboration and the intersection of these critical techniques resulted in the construction of the first biologically functional recombinant plasmid *in vitro*⁴¹ (**Figure 1**). Restriction endonucleases EcoRI and EcoRII were utilized to cut the double stranded plasmid, leaving single stranded 3' and 5' sticky ends that could be joined together by DNA ligase to generate a circular double stranded DNA molecule and functional plasmid⁴¹. This key experiment combined separate *E. coli* plasmid species pSC101 and pSC102, which contain genes encoding for resistance to tetracycline and

kanamycin respectively. The resulting biologically functional plasmid conferred resistance to both antibiotics, which was utilized to identify positive transformants via antibiotic treatment⁴¹. This represents the first use of what is commonly known now as a selection marker gene; a critical characteristic of plasmids and other cloning vectors that allows for selection of positively transformed cells. This approach utilizing selective advantage to identify positive transformants has proven invaluable to rDNA technologies and is still widely used today.

The following year in 1974, this innovative strategy was successfully used to achieve the replication and transcription of eukaryotic *Xenopus laevis* DNA in *E. coli*⁴². Detection of *X. laevis* rDNA in *E. coli* transformants from this experiment demonstrated that *E. coli* could be used to clone genes with eukaryotic species of origin⁴². The next logical step was to test whether this approach could be employed to not only transcribe a eukaryotic gene, but also to express and produce human proteins. Shortly after, the human hormone somatostatin was successfully cloned in *E. coli* using a pBR322 plasmid vector, demonstrating that recombinant DNA technologies could be used to produce functional human proteins⁴³ (**Figure 1**). Only a few years later, Genentech's recombinant human insulin named Humulin gained United States (US) Food and Drug Administration (FDA) approval and entered the US market⁴⁴. This landmark advancement in recombinant DNA technology would serve as the inspiration for the development of countless biotechnologies, showcasing vector-driven transformation in microorganisms like *E. coli* as a robust strategy to achieve the expression of a human gene product. With 621 biologic drugs currently FDA-approved in the US, it is clear these initial advances in rDNA technologies have inspired decades of innovative scientific research⁴⁵.

Expression Vectors: Characteristics and Examples

Since a bacterial plasmid was first used to express a eukaryotic gene in *E. coli*, our understanding of plasmids and other cloning vectors has greatly advanced. The bacterial plasmid was the first vector identified and optimized for the purpose of expressing recombinant gene products. However, there are also components present in the genomes of bacteriophages, yeasts, and viruses that can be utilized to accomplish effective transfer, cloning, and expression of a transgene in a heterologous system^{9,46}. Specialized vectors that are optimized for transgene expression in a desired host organism are referred to as expression vectors, and often contain additional noncoding sequences such as promoters, enhancers, and exons from the parent sequence that improve the transcription or expression of the gene product⁹. Although vectors vary in their source, size, structure, capacity, and replication efficiency, all of them must retain several key attributes. To facilitate the discussion of key vector principles and their impact on successful transformation and expression of a gene insert, we will refer to the widely used and iterated-upon plasmid pBR322 as an example (**Figure 2**). This was one of the first vectors constructed with the intent of generating and selecting for recombinant clones, with pBR322 and its derivatives such as the pUC plasmids being commercially available and still widely used in rDNA technologies today⁴⁷.

Notable sequences used to construct the pBR322 plasmid include the ampicillin resistance gene (*amp^R*), a promoter from the ColE1-related plasmid named pRSF214, and the tetracycline resistance gene (*tet^R*) from the pSC101 plasmid discussed earlier^{41,48,49} (**Figure 2**). As established, cloning and expression vectors must contain a selection marker gene that confers a selective advantage to vector bearing host cells^{9,41}. Selection markers commonly encode antibacterial

resistance or metabolic advantages, providing a survival advantage to positive transformants so they can be selected for through antibiotic treatment or nutrient deprivation^{9,10,49} **(Figure 2)**. Strategies to select for recombinant clones are not only limited to those providing a survival advantage to positive transformants, with other successful selection approaches utilizing targeted DNA insertion to functionally disrupt a gene⁴⁹. This can confer traits to positive transformants that allow for their negative selection, such as tetracycline sensitivity in *tet^R* disruption and the formation of colorless colonies when the gene encoding the alpha protein of beta-galactosidase is disrupted^{46,49}.

In addition to containing a selection marker gene, a suitable expression vector must also be able to replicate in the host organism of choice. In plasmids, phagemids, and bacteriophage-based vectors, this is ensured through the inclusion of an origin of replication (*ori*) site⁹. The *ori* site is recognized by host replication machinery, serving as a point of assembly for the components involved in DNA replication^{9,40}. Some *ori* sites are specific to a certain host bacteria, while others are able to be recognized by the replication machinery of multiple species⁹. The *ori* site in the pBR322 plasmid is derived from the ColE1 plasmid native to *E. coli*, making it a suitable choice for cloning and expression in this host organism⁴⁹ **(Figure 2)**. The inclusion of more than one *ori* site in a cloning vector can allow replication across multiple different host species, expanding the flexibility of vectors for use in other model systems. *ori* sites within expression vectors are often optimized for more efficient recruitment of replication components, with these sequences having a dramatic effect on the copy number of the vector^{9,50}. Another determinant of pBR322's copy number is encoded by the repressor of primer (*rop*) gene shown in red^{51,52} **(Figure 2)**. The protein encoded by this gene regulates plasmid copy number by promoting interaction of inhibitory RNA

components that prevent primer maturation to modulate plasmid replication⁵²⁻⁵⁴. This limits the copy number of pBR322, a disadvantage in cloning that would be addressed by future derivatives of this vector.

Expression vectors must also contain multiple cloning sites that serve as unique locations for restriction endonucleases to recognize, enabling the specific digestion of the plasmid at these sites to generate a linearized double stranded DNA molecule. The transgene of interest is digested using the same restriction enzymes to enable gene insertion into the plasmid. The compatible ends generated by restriction digest are joined together by DNA ligase, creating a recombinant vector that can undergo replication using host machinery^{9,10} (**Figure 2**). The pBR322 plasmid has been optimized to include a variety of multiple cloning sites for gene insertion, with many of them falling within critical segments of the vector including both antibiotic resistance genes, the *amp^R* promoter, and the origin of replication⁴⁹ (**Figure 2**). The functional disruption of the *tet^R* gene via DNA insert that was discussed earlier utilizes the unique multiple cloning sites present within this gene. This allows for identification of host cells bearing the plasmid that contains the transgene DNA insert, with positive pBR322 transformants that successfully inserted and ligated the DNA fragment via HindIII, Sall, or BamHI endonucleases no longer having a functional *tet^R* gene^{9,49}. This creative utilization of multiple cloning sites to generate specialized selection markers highlights the vast utility of restriction endonucleases, not only proving invaluable to the targeted insertion of a transgene but also in the customization of expression vectors and their features. While there are many context-specific applications of these enzymes and the cloning sites they recognize, this general strategy of enzyme-mediated transgene insertion, ligation, and replication has been widely applied in the cloning and expression of countless gene products as therapeutics

and for functional study, including those integral to ADME processes such as P450 enzymes^{11,12,19} **(Figure 2)**.

In addition to the discussed components, an effective expression vector must also promote the efficient expression of the inserted transgene in the host system. Inclusion of a strong promoter that is compatible with the host species is critical to achieve adequate expression of a recombinant gene product¹⁰. The promoter sequence must also be compatible with the modality of the gene product, with different promoters being utilized depending on whether the recombinant gene product is a protein, mRNA, or small interfering RNA⁹. Some specialized expression vectors can also be designed with dual-promoter systems that aim to further increase the strength of transgene expression⁵⁵. These dual-promoter systems can consist of two twin promoters activated under the same conditions, as well as two non-identical promoters activated during separate steps to aid production processes in biotechnology⁵⁵. Similarly, the inclusion of a strong termination sequence following the promoter and transgene can enhance expression and represents an important component of an efficient vector^{9,56,57}. As recombinant technologies have continued to advance, expression vectors have become more readily adaptable tools that can be customized to efficiently express a wide variety of transgene products across many host systems. Recently developed expression vectors may also aid the purification of recombinant proteins through the installation of a polyhistidine affinity tag. Once purified, the affinity tag can be cleaved by tobacco etch virus (TEV) protease to return the recombinant protein to its native conformation⁵⁸. This strategy has been utilized to generate several novel expression vectors from the pET series capable of expressing and more easily purifying recombinant proteins⁵⁸.

Like the choice of expression vector, choosing an appropriate host organism for transgene expression is a critically important step when utilizing rDNA technologies. Different model organisms possess distinct characteristics that can make them best suited to a particular application or gene product¹⁰. Model organisms for recombinant expression can vary in their yield capacity, post synthetic modification capability (glycosylation, acetylation), ability to secrete active proteins, and ability to express specific gene products or modalities. Common model species for transgene expression include bacteria (*Escherichia coli*, *Lactococcus lactis*, *Pseudomonas*), yeast (*Saccharomyces cerevisiae*), mammalian cells (CHO, HEK, COS), and insect cells (Sf9)^{11,59-61}. Research efforts continue to improve upon these expression systems and remedy their shortcomings. One of the most relevant examples of this is in *E. coli*, where advancements have enabled glycosylation of protein products and the use of antibiotic-free selection plasmids⁶⁰.

Applications of rDNA Technology in DMPK Research

Since the inception of recombinant DNA technology, innovative researchers have been developing creative ways to adapt the rDNA platform to address their research questions with cDNA-expressed P450s (**Figure 1**). One critical example of this was the development of site-specific mutagenesis methods utilizing M13 bacteriophage cloning vectors^{62,63}. This method is used to elicit specific and targeted changes to a gene of interest for transformation into *E. coli* and other model organisms^{62,63}. With the ability to introduce targeted single-nucleotide changes to any DNA fragment, researchers were able generate novel mutants for functional study. Often this approach is used to investigate the role of individual amino acid residues in contributing to the function or interactions of a specific protein. In DMPK research, this method has been used to generate novel

P450 mutants to elucidate the role that specific residues play in protein behavior and enzyme function⁶⁴. Site-directed mutagenesis has also been widely applied in the characterization of other ADME related proteins such as ABC transporters and multidrug efflux pumps^{65,66}. Distinct from site-directed mutagenesis, random mutagenesis has also been utilized in DMPK research to generate novel CYP enzymes capable of withstanding harsh environmental conditions and performing biotransformations previously unseen in nature⁶⁷⁻⁷⁰. Through many cycles of random mutagenesis and screening, beneficial mutations are accumulated that alter metabolic function and often promote an intrinsic promiscuity or side-reactivity of the natural enzyme⁶⁷. This strategy developed by Frances Arnold is termed directed evolution, and it was awarded the 2018 Nobel Prize in chemistry. This innovative approach has been utilized to develop novel CYP enzymes capable of catalyzing many new and non-natural reactions such as those valuable to medicinal chemistry or biotechnology processes^{67,68}.

Another valuable application of rDNA technologies in DMPK research has been improving the field's access to robust research tools. A prominent example of this is the production of recombinant antibodies and nanobodies utilizing phage or yeast display libraries^{71,72}. Specific and sensitive antibodies are critical reagents for any biomedical research, serving as important probes to detect and quantify target antigens. Recombinant antibodies may also serve as inhibitors to block specific protein-protein interactions, proving useful in elucidating the role of specific protein epitopes and interactions in determining protein function⁷³.

Previous methods of antibody production relied on hybridoma technology, requiring mature B cells be harvested from an animal immunized with the antigen⁷³. Because this relies on host

immunization, the antibody generated is in response to host presentation of the antigen via the major histocompatibility complex. The antigen must undergo proteolysis to a smaller peptide for host presentation to occur, and because of this the antibodies generated by hybridoma methodology do not always successfully recognize the native conformation of the antigen they target^{73,74}. However, when generating recombinant antibodies by phage display, the antigen of interest is present in its native conformation and is less likely to undergo alteration compared to hybridoma approaches⁷³. This represents a distinct advantage of recombinant antibody production when compared to previous hybridoma methods, especially when considering the importance of conformation in ADME related protein function. Additionally, recombinant antibody production is significantly cheaper, does not require the use of animals, and allows for the inclusion of additional features such as an amino acid tag to aid the purification process⁷³.

This success utilizing rDNA technology to develop superior research tools has not only occurred with antibodies, but has also extended to other modalities such as microRNAs (miRNAs), small interfering RNAs (siRNAs), and aptamers⁷⁵. MicroRNAs are understood as epigenetic regulators with a vast array of targets and functions, with one miRNA able to regulate several mRNA transcripts at the posttranscriptional level^{76,77}. Understanding miRNA's role in ADME gene regulation and function is critical to addressing a comprehensive picture of DMPK. The field of miRNA research has been somewhat limited by the widespread usage of chemically synthesized miRNA mimics as research tools. These commercially available miRNA mimics carry extensive chemical modifications that are needed to improve their stability and PK properties^{78,79}. The way in which these synthetic mimics are modified is not always disclosed and modifications between manufacturers can vary in identity, location, and number⁷⁸. Although the nucleotide sequence is

unaffected, these inconsistent modification practices generate miRNAs that are constitutively different from natural miRNAs and may lead to conflicting results between investigators^{78,80,81}.

To address these shortcomings, the Yu lab has developed an innovative bioengineering platform to express and purify recombinant miRNA molecules in *E. coli*^{82,83}. Echoing the success of biologic proteins, these miRNAs are synthesized and folded inside living cells and better recapitulate the structures, modifications, and properties of endogenous miRNAs and thus represent superior tools for DMPK research and as potential therapeutics⁷⁸. Later in this review, we will discuss the optimization of this recombinant strategy as well as its utilization in elucidating the impact of endogenous miRNA on ADME gene regulation and DMPK.

In addition to developing improved tools and reagents for research, advancements in rDNA technologies have also enabled novel approaches to generate improved cellular and animal models for the study of drug metabolism and human disease⁸⁴⁻⁸⁷. One prominent advancement is the 2020 Nobel Prize winner in chemistry, an adaptable gene editing platform that utilizes clustered regularly interspaced short palindromic repeats (CRISPR) associated with a Cas9 endonuclease⁸⁸. This promising technology commonly known as CRISPR-Cas9 has gained widespread popularity over the past decade, with many *ex vivo* gene therapies that utilize the platform currently undergoing clinical trials across a variety of disease states⁸⁹. In the context of DMPK research, this targeted gene editing technology has been utilized to generate valuable *in vivo* and *in vitro* knockouts to characterize individual ADME gene and allele function⁹⁰. Examples of ADME gene products knocked out or modified for functional study using CRISPR-Cas9 include CYP enzymes, ABC transporters, nuclear receptors, and solute carrier proteins^{84,91-95}. This approach can

accomplish specific knockout of individual or multiple alleles of a target gene, allowing researchers to interrogate their role in xenobiotic metabolism, drug-drug interactions (DDIs), and pharmacokinetics^{84,86}. While the CRISPR/Cas9 platform holds great promise to enable research and treat genetic disorders, future iterations of the technology must continue to improve upon its accuracy and precision as well as address concerns surrounding its unintended epigenetic impact⁹⁶.

Recombinant ADME Proteins for Functional and Structural Studies

Before rDNA technologies gained widespread usage in the development of therapeutics and research tools, these recombinant advancements were utilized in the functional and structural study of ADME gene products such as CYP enzymes (**Figure 1**). Indeed, early research of P450 enzymes before rDNA technology was limited to approaches such as preparing microsomes and purifying individual CYPs from animal tissues²¹. This strategy proved effective in reconstituting soluble cytochrome P450 enzymes with intact catalytic activity, allowing researchers to study P450-mediated biotransformation of endogenous compounds and xenobiotics alike^{21,97,98}. Additionally, these early works from Lu & Coon identified the necessary components that constitute catalytic activity in liver microsomes: P450 hemoprotein, cytochrome c reductase, and a heat-stable factor²¹. These research efforts broadened the field's understanding of *in vivo* drug metabolism by identifying numerous unique forms of soluble P450 enzymes, elucidating key components critical to catalytic activity, and illuminating characteristic differences between species^{21,98-100}.

Microbial P450_{CAM}: A Window Into P450 Structure and Function

Despite some success with liver microsomes, the DMPK field was in need of a more suitable model system for the structural and functional characterization of individual P450 enzymes¹⁰¹. This role would be filled by the microbial cytochrome P450_{CAM}, a camphor-metabolizing monooxygenase that was identified in *Pseudomonas putida*¹⁰¹⁻¹⁰³ (**Table 1**). This enzyme was purified for study prior to the advent of recombinant DNA technologies, and instead was isolated from bacterial culture according to a procedure optimized by Gunsalus and Wagner¹⁰³. P450_{CAM} was the first CYP enzyme that had been purified in both soluble and crystalline forms, making it an ideal biological model to study P450 structure and function^{101,103,104}. High-resolution crystal structures were generated of P450_{CAM} both bound to camphor and in the absence of its substrate, providing valuable insight into the substrate binding site and the structure-function relationship of the enzyme^{102,105}. This crystallography data also elucidated the secondary structures and overall conformation of the polypeptide, illustrating important structural features of the enzyme such as the oxygen/heme binding pocket as well as a metal cation interaction that may stabilize active site formation¹⁰².

These advancements in P450_{CAM} visualization were also paired with amino acid sequencing techniques, further improving the detail of structural and functional characterization and enabling the comparison of P450_{CAM} to eukaryotic P450 sequences¹⁰². Compared P450 sequences were found to be fairly homologous, with residues that form the oxygen binding area being highly conserved between species¹⁰². Amino acid sequencing also revealed that eukaryotic P450s uniquely possess a hydrophobic NH2 terminus, a domain that was speculated at the time to contribute to the membrane solubility observed in mammalian P450s¹⁰². While bacterial cytochrome enzymes like P450_{CAM} are easily solubilized in the cytoplasm, eukaryotic P450

enzymes reside in the membranes of the endoplasmic reticulum and to a lesser extent the mitochondria¹⁰⁶. The functional role that this hydrophobic NH2 terminus plays in mammalian P450 membrane association and its effect on catalytic activity would later be elucidated using rDNA technologies^{11,107}. P450_{CAM} has also served as an effective model system to assess the role of accessory proteins, with crystal structures of the enzyme complexed to its redox partner putidaredoxin (Pdx) providing insight into the structural changes induced by Pdx and how they may facilitate electron transfer^{108,109}. Even today, P450_{CAM} is utilized as a model system to further investigate the allosteric roles of accessory proteins and how they may impact conformational dynamics¹¹⁰.

Although P450_{CAM} was purified from a gram-negative bacterium, its extensive study aided our understanding of mammalian P450 enzymes by enabling the thorough characterization of well conserved domains and functions^{7,17-20,102,111}. With isolation of pure P450 enzymes from human tissue being an arduous and time-consuming process, this P450_{CAM} model system was invaluable in establishing initial structure-function relationships and in elucidating the basic catalytic mechanism conserved across nearly all P450s^{5,110}. P450_{CAM} was also one of the first P450 enzymes cloned for recombinant expression, utilizing a plasmid-derived vector under strict promoter control to produce catalytically active P450 monooxygenase in *P. putida* and *E. coli* host systems^{12,59}. This was not the only microbial P450 enzyme investigated utilizing rDNA technologies, as the crystal structure of the prokaryotic form of 14 α -demethylase (CYP51) present in *Mycobacterium tuberculosis* was also elucidated this way¹³ (**Table 1**). While CYP51 exists in most species as a membrane-bound enzyme that is localized to microsomes, the *M. tuberculosis* genome possesses a uniquely soluble form of the enzyme that was expressed in *E. coli* for easier determination of the

crystal structure^{13,112}. This newfound structural information would inform rational drug design of improved antifungal agents that target CYP51.

cDNA Directed Expression of Mammalian P450 Proteins

Not long after the crystal structure and amino acid sequence of P450_{CAM} were elucidated, rDNA strategies would gain widespread usage in the study of mammalian CYP enzyme structure and function in drug metabolism^{11,14-20,26,113-117} (**Figure 1 and Table 1**). Although some P450 amino acid sequences had been elucidated through methods discussed earlier, the development of cloning, nucleotide sequencing, and DNA manipulation technologies would equip researchers with powerful tools to generate libraries of P450 cDNA sequences^{7,102,110}. Complementary DNA sequences for human P450 enzymes were generated using mature mRNA from liver and lung tissue lysates⁷. Purified mRNA was then used to generate complementary DNA strands via reverse transcriptase, which then underwent amplification by polymerase chain reaction to generate double stranded DNA fragments⁹. These gene segments were then ready be digested with complementary endonucleases and ligated into a vector for transformation of the amplified cDNA into a suitable host organism^{7,9,12}. Due to observed sequence homology between species, human P450 sequences could be identified from these libraries through the use of cDNA probes and antibodies against rodent forms of P450^{7,19}. Once cDNA sequences of interest were identified and cloned using this approach, researchers set out to optimize the expression of these recombinant gene products in model organisms so their catalytic activities, substrate specificities, and structure-function relationships could be characterized^{5,7,11,16-19}.

One important example that utilized cDNA-directed expression to illuminate P450 structure was the investigation of rabbit microsomal P450 and its co-translational insertion into rough microsomes from dog pancreas^{107,113} (**Table 1**). This would serve as an effective model of mammalian P450 integration into membranes of the endoplasmic reticulum, allowing researchers to interrogate the role of key structural features responsible for initiating co-translational insertion and for conferring membrane solubility. Initial research demonstrated that co-translational insertion of P450 could not occur without the presence of a ribonucleoprotein complex referred to as the signal recognition particle (SRP)¹¹³. However, further investigation was required to identify the way in which microsomal P450 recognizes the SRP to influence P450 function and membrane topology. Hybrid cDNAs were generated to express chimeric forms of the P450 protein for study, with each mutant possessing unique modifications impacting the length and identity of the N-terminal amino acid chain¹⁰⁷. Of the several chimeric P450 proteins generated, only those possessing an NH2 terminus at least 29 AAs in length were able to co-translationally insert into the membrane¹⁰⁷. This demonstrated that the insertion signal that enables SRP-mediated co-translation to the membrane was contained within this 29 AA segment of the enzyme's NH2 terminus¹⁰⁷. This research broadened the understanding of the NH2 terminus present in eukaryotic P450 forms, demonstrating the impact that NH2 truncation has on their topogenic function and membrane solubility¹⁰⁷.

Interestingly, recombinant research efforts expressing rat P450_{MC} in *Saccharomyces cerevisiae* also generated a truncated form of the enzyme that lacked an extended NH2 terminus¹¹. Spectral and catalytic analysis revealed almost identical properties between the truncated and non-truncated forms of the enzyme, demonstrating that this NH2 terminus is not required for P450 catalytic

activity¹¹ (**Table 1**). This knowledge would be widely applied to solubilize mammalian P450 through deletion of the NH2 terminus, allowing researchers to more easily purify and crystallize mammalian P450 enzymes for structural and functional characterization^{13,15,16,26,114-116,118-124}. This cDNA-directed approach would soon be utilized to express truncated rabbit CYP2E1 in *E. coli* using a pKKHC vector, an optimized derivative of the pBR322 plasmid discussed earlier^{26,125} (**Table 1**). These research efforts further supported the notion that the NH2 terminus is not necessary to retain P450 monooxygenase function, with recombinant truncated CYP2E1 possessing identical spectral and catalytic properties compared to full length CYP2E1 isolated from rabbit liver microsomes^{26,125}.

Similar success was also achieved in the recombinant expression of rabbit CYP2C3 as a soluble dimer in *E. coli*. The CYP2C3 gene insert with a modified NH2 terminus was digested with endonucleases NdeI and EcoRI and transformed into an optimized pCWOri+ vector that had been linearized with the same enzymes¹²² (**Figure 1**). This general procedure is highlighted in (**Figure 1**), serving as an example of heterologous P450 expression in *E. coli*. Additionally, the key components of this vector that allow for strong transgene expression and inducible control are summarized in (**Figure 1**). This pCWOri+ derived expression vector would also later prove useful in the recombinant study of CYP2C5^{115,118,122}.

Not long afterwards, two mammalian P450 enzyme crystal structures (CYP2B4, CYP2C5) were identified through recombinant expression in *E. coli*^{114,115,118} (**Table 1**). Both enzymes were expressed in soluble form through truncation of the NH2 terminus, allowing for their crystallization and structural characterization. Crystallization in the presence and in the absence of

substrate allowed investigators to examine conformational differences that can determine substrate recognition and catalytic function¹¹⁸. These experiments were also one of the first to add a histidine tag to the C terminus of the recombinant protein to aid the purification process¹²². This strategy would also be employed in the study of human CYP2C8, which was uniquely expressed in *E. coli* as stable homodimer complexed by two palmitic acid molecules¹⁶. These peripheral fatty acid binding sites can alter characteristics of the active site and have potential to modulate substrate binding as well as contribute to DDIs¹⁶ (**Table 1**). This observed dimerization of CYP2C8 in addition to its significantly larger active site cavity suggest a distinct metabolic function of this enzyme when compared to previously elucidated P450s. This makes sense when considering that the primary substrates of CYP2C8 are comparatively larger molecules such as taxol¹⁶. Further research crystallized CYP2C8 with a variety of different substrates to examine the role of protein flexibility and ligand-induced conformational changes in accomplishing substrate oxidation¹²⁶.

The crystal structure of human CYP2C9 was also determined through heterologous expression of a NH2 truncated mutant, which revealed a similarly large active site capable of accommodating multiple warfarin molecules or an additional substrate of similar size¹⁵ (**Table 1**). Given that CYP2C9 can be activated by some exogenous compounds including warfarin, it was hypothesized that the binding of some ligands may serve an allosteric function that promotes enzyme activity¹⁵. Compared to CYP2C9, the crystal structure of recombinantly expressed human CYP3A4 revealed a slightly smaller active site as well as a characteristic cluster of phenylalanine residues above it, some of which are known to cooperate in enzyme selectivity and function^{116,117}. X-ray crystallography data was generated for CYP3A4 in the absence of ligand, in the presence of an inhibitor metyrapone, and with its substrate progesterone¹¹⁶ (**Table 1**).

To determine how the properties and structures of CYP3A4 and CYP3A5 differ, both enzymes were recombinantly expressed in *E. coli* and complexed with a high-affinity substrate ritonavir¹²⁴. Crystallography data for these enzymes revealed differences in the shape and plasticity of their active sites upon substrate binding ¹²⁴ (**Table 1**). This structural data may reflect notable differences in the substrate dynamics and catalytic accessibility of these enzymes, potentially leading to divergent metabolic pathways or varying enzymatic efficiencies for shared metabolic pathways¹²⁴. Additionally, enzymes of the CYP3A family can be differentially expressed across ethnic populations, with allelic variations having a significant effect on enzyme abundance and metabolic capability^{127,128}. Given that the CYP3A family is the largest single contributor to drug metabolism, illuminating the structural features and processes that govern their catalytic activity and specificity is critically important in designing safe therapies, predicting DDIs, and understanding individual differences in drug metabolism¹²⁹.

One recent advancement in the structural understanding of this enzyme family is the solved crystal structure of neonatal CYP3A7, a critical enzyme in hormone homeostasis and pediatric drug metabolism^{130,131}. Structural characterization of CYP3A7 was previously lacking, as the enzyme proved difficult to crystallize in its wild-type form. Using site-directed mutagenesis, a recombinant CYP3A7 variant capable of crystallization was generated to allow for structural characterization and comparison to other CYP3A enzymes ¹³⁰ (**Table 1**). In many ways rDNA technologies have provided unprecedented insight into the catalytic structure, conformational dynamics, and substrate specificities of the most integral drug metabolizing enzymes. Even today we continue to build upon our understanding of P450 monooxygenases, with recent investigations

providing additional context regarding the nature of active site plasticity and redox partner selectivity¹¹⁰.

Another Cytochrome P450 enzyme that performs a significant share of xenobiotic biotransformations is human CYP2D6, as it is estimated to metabolize up to 20-30% of commonly prescribed drugs. The crystal structure was solved once again through recombinant expression of a soluble recombinant CYP2D6 in *E. coli* utilizing a pCWOri+ derived vector¹²⁰ (**Table 1**). This experiment revealed a structure most closely resembling that of CYP2C9, and the crystallography data would enable further research efforts building a more detailed understanding of the structural basis of ligand binding and specificity¹²⁰. This information would prove especially critical in understanding the many biotransformations mediated by CYP2D6 and CYP2C9, as their allelic variants between ethnic groups and polymorphisms between individuals can have a dramatic effect on metabolic activity, drug disposition, and therapeutic outcomes^{14,132-135}. The rDNA platform has been adapted to interrogate the impact of specific CYP polymorphisms present in the population, uncovering mechanistic rationale for unique phenotypes of drug metabolism. The ability to recombinantly express specific P450 polymorphisms has allowed researchers to quantify enzymatic differences in the presence of any substrate, providing critical knowledge in our understanding of pharmacogenomic variability between individuals and populations. This knowledge has proven invaluable in drug design and clinical practice, as CYP polymorphisms and genetic variants can result in altered ADME processes, DDIs, and unexpected adverse effects. One key example of this is observed in warfarin-treated individuals with CYP2C9 polymorphisms, who are at increased risk of major bleeding events and excessive anticoagulation associated with increased drug exposure¹³⁵. CYP2C9 polymorphisms have also been shown to promote genotype

dependent DDIs when warfarin or other major substrates are co-administered with an inducer or inhibitor of CYP2C9¹³⁵⁻¹³⁷. Research utilizing rDNA technologies has revealed many genotype-specific differences in PK function and individual drug disposition, providing understanding that is critical to rational drug design, clinical dose management, and DDI prediction. Elucidating these pharmacogenomic differences has been key in developing and maintaining safe therapies, and this knowledge will prove increasingly useful as pharmacogenomic testing of patients becomes more commonplace and cost effective^{138,139}.

In addition to providing valuable insight into the most predominant drug metabolizing P450s, rDNA technologies have also been used to characterize P450 enzymes that biosynthesize critical endogenous compounds. One predominant example of this is human CYP17A1, which is known to catalyze the synthesis of androgens and other important steroids¹⁴⁰. Because of this, CYP17A1 inhibition represents an attractive strategy to prevent androgen synthesis and treat castration resistant prostate cancer. The crystal structures of CYP17A1 bound to two inhibitors were determined by recombinant expression of a truncated in version in *E. coli*¹⁴¹ (**Table 1**). These initial findings would elucidate the structural features integral to CYP17A1's catalytic activity, being further enhanced soon afterwards by crystal structures of CYP17A1 with all four of its major endogenous substrates and clinically relevant inhibitors^{142,143}. Structural characterization of CYP17A1 enabled by recombinant expression would provide critical information in the rational drug design of CYP17A1 inhibitors. Additionally, rDNA technologies would allow investigators to develop more selective CYP17A1 inhibitors through assessment of off-target interactions with other steroidogenic P450 enzymes such as CYP21A2¹⁴⁴.

Another enzyme that mediates critical biosynthetic reactions is cytochrome P450 aromatase (CYP19A1), which is known to synthesize estrogens from androgens. While the crystal structure of CYP19A1 was first determined using aromatase purified from human placenta, subsequent research efforts recombinantly expressed truncated CYP19A1 to further its structural characterization and interrogate the functional roles of specific amino acid residues¹⁴⁵⁻¹⁴⁷ (**Table 1**). This insight into the structure of CYP19A1 and its androgen-specific active site aided the intelligent design of aromatase inhibitor drugs used to treat hormone receptor-positive breast cancers¹⁴⁸. Indeed, this same approach has proven similarly useful in the characterization of many P450 enzymes that catalyze the biosynthesis of important endogenous compounds¹⁴⁹⁻¹⁵¹. These investigations have supported the rational design of selective inhibitor drugs, with recent examples being the characterizations of P450 11B1, P450 11B2, and P450 8B1, which catalyze the production of cortisol, aldosterone, and bile-acid respectively¹⁴⁹⁻¹⁵¹ (**Table 1**).

Recombinant RNAs for Studying ADME Gene Regulation

New advancements in epigenetics in DMPK as well as ADME gene regulation and function are critical in designing the next generation of safe and effective therapeutics. One of the most important factors in the posttranscriptional regulation of ADME genes are the aforementioned miRNAs, whose wide range of regulatory targets and functions allow them to drive therapy resistance or sensitivity depending on the nature of miRNA, disease, and context¹⁵²⁻¹⁵⁵. As discussed earlier, successes in recombinant technologies have also provided scientists with the tools to produce biologic RNA molecules as research tools and potential therapeutics. To this end, the Yu lab has established a novel approach to generate recombinant or bioengineered RNA

(BioRNA) molecules in high yield utilizing *E. coli* fermentation^{82,83} (**Figure 3**). These BioRNAs synthesized and processed inside living cells more accurately represent the properties and chemical modifications of endogenous miRNAs compared to commercially available chemical mimics^{75,78}. This approach has been utilized to generate a collection of humanized miRNAs more representative of their natural state, allowing for more accurate interrogation of their epigenetic regulation of ADME genes to influence drug disposition and therapeutic outcomes^{78,82}.

To bioengineer these RNA molecules in *E. coli*, the pBSTNAV vector is utilized to accomplish cloning and expression. This vector contains an ampicillin resistance gene, compatible ColE1 and pBR322 ori sites, a strong lipoprotein (lpp) promoter, and a ribosomal RNA operon termination sequence (rrnC) (**Figure 3**)^{82,156}. These features allow for antibiotic-based selection of positive transformants and enable cloning and expression of a noncoding RNA (ncRNA) segment within the plasmid¹⁵⁷. This is accomplished using a gene insert encoding a fused tRNA/pre-miRNA carrier molecule that can be stably expressed in *E. coli*⁸³ (**Figure 3**). The identity of the tRNA and pre-miRNA sequences influence expression efficiency, and research efforts investigating different tRNA scaffolds and pre-miRNA species have resulted in an optimized stable carrier system capable of consistently expressing BioRNAs at levels surpassing 30% of total bacterial RNA^{82,158}. This stable hybrid carrier has been refined to achieve production at high yield, with human tRNA fused hsa-pre-miR-34a (htRNA/hsa-pre-miR-34a) demonstrating consistently high expression with many different ncRNA cargos⁸³. Within a stable htRNA/hsa-pre-miR34a carrier, the miR-34a duplex can be replaced with payload miRNA sequences of interest to generate recombinant miRNA agents (**Figure 3**)⁸². This duplex can also be replaced with other small RNAs such as siRNA or even multiple payload RNA sequences, highlighting the flexibility of this approach¹⁵⁹.

Through these research efforts, the Yu lab has developed a robust bioengineering platform capable of producing humanized BioRNAs in high yield to investigate their role in the posttranscriptional regulation of ADME genes as well as their impact on therapeutic susceptibility and treatment outcomes. Websites such as miRBase, miRDB, and Targetscan are valuable resources in identifying ncRNA segments of interest as well as investigating gene transcripts that are predicted regulatory targets of specific miRNA sequences. Sequences encoding target BioRNAs are modified with appropriate cloning sites for vector insertion and amplified by polymerase chain reaction utilizing forward and reverse cloning primers with 15 nucleotide overlaps^{82,83}. Amplified BioRNA segments are then ligated into the pBSTNAV plasmid following linearization with the restriction enzymes EcoRI and PstI, with strategies utilizing EagI and SacII being used to some success in the past^{82,83} (**Figure 3**). Plasmids containing target BioRNA inserts are confirmed by sequencing analyses and used to transform HST08 competent *E. coli* cells to achieve overexpression of recombinant RNAs^{82,83}. Total RNA is then isolated from bacterial culture, and successful expression of target BioRNA is verified through the appearance of a strong band of expected size on an acrylamide-urea gel⁸². Anion exchange fast protein liquid chromatography is utilized to separate the RNAs, allowing the collection of fractions containing the target BioRNA⁸². These fractions can be run on a gel to determine their purity in a semi-quantitative manner, with the purest of these being pooled together to undergo desalting and concentration procedures. These isolated BioRNAs then have their purity quantitatively assessed by high performance liquid chromatography and the level of bacterial endotoxin within each is determined⁸².

The streamlined procedure outlined here in brief has been utilized to express and purify a collection of humanized BioRNA agents for use in research. These BioRNAs serve as prodrugs for the warhead miRNAs they contain, being processed by endogenous machinery to generate high levels of target miRNAs as detectable by stem-loop RT-qPCR or RNA sequencing⁸³. Selective processing of bioengineered miRNA prodrugs to their target miRNAs has been demonstrated in several *in vitro* and mouse models, as well as their ability to regulate gene expression to impact cellular processes and chemosensitivities^{83,158,160} (**Table 2**). This innovative platform has been leveraged to investigate the therapeutic potential and epigenetic role of many small RNAs across several disease states and target protein types. In the remainder of this review, we will highlight several recent studies utilizing these strategies to interrogate miRNA's role in the posttranscriptional regulation of gene products integral drug metabolism and disposition (**Table 2**).

The first investigation utilizing recombinant miRNAs to modulate ADME gene expression was published in 2014, and this study only used a tRNA scaffold to achieve expression of hsa-pre-miR-27b¹⁶¹. Previous research had shown miR-27b to be a negative regulator of CYP3A4 and vitamin D receptor (VDR) through targeting the 3'-untranslated regions of these transcripts¹⁶², making miR-27b an ideal choice to test the effectiveness of recombinant miRNA molecules in accomplishing target gene regulation. The VDR is a nuclear receptor which is known to play a critical role in the transcriptional control of CYP3A4¹⁶³. The tRNA fused to hsa-pre-miR-27b was successfully expressed in *E. coli* but demonstrated low overall abundance with recombinant RNA making up only 2% of total bacterial RNA¹⁶¹. Despite this challenge, tRNA/hsa-pre-miR-27b was successfully purified from bacteria and transfected into human cells for further study. The recombinant RNA was successfully processed to mature miR-27b in LS180 cells, and further

analysis demonstrated the suppression of CYP3A4 and VDR protein expression as well as mRNA transcript abundance¹⁶¹. This reduced protein expression also corresponded with a decrease in CYP3A4 enzymatic activity, demonstrated by a reduced capacity to perform the 1'-hydroxylation of probe drug midazolam¹⁶¹. In 2015, this same strategy was used to produce recombinant tRNA/hsa-pre-miR-1291 for functional study in human carcinoma cells¹⁶⁴. This chimeric RNA was processed to mature miR-1291 in MCF-7 and PANC-1 cell lines, where it was able to effectively downregulate the protein expression of target ABCC1¹⁶⁴ (**Table 2**). Overexpression of ABCC1 and similar transporters is a common occurrence in cancers, which is believed to contribute to therapeutic evasion and multi-drug resistance. Introduction of recombinant tRNA/hsa-pre-miR-1291 into PANC-1 cells also enhanced their sensitivity to doxorubicin, a known substrate of the ABCC1 transporter¹⁶⁴. These results highlighted the early potential of recombinant miRNAs in modulating ADME gene expression to alter drug exposure, overcome resistance, and improve therapeutic outcomes.

The identification and use of stable tRNA/pre-miRNA carrier to produce functional small RNA agents was first reported in 2015 as most target RNAs cannot be heterogeneously expressed with tRNA scaffold¹⁶⁵. A few years later in 2018, another research paper was published that optimized the use of the discussed hybrid tRNA/pre-miRNA carrier to achieve substantially higher yields of target ncRNA sequences from bacterial fermentation, with a greater success rate¹⁵⁸. Utilizing this approach, recombinant miR-124 was generated and used to transfect human cells. The administered agent was then selectively processed to miR-124-3p within the cells, effectively downregulating its established targets ABCC4, as well as other cancer-related genes, and inhibiting proliferation of carcinoma cells¹⁵⁸ (**Table 2**). Further research in 2019 utilized these innovations

in the tRNA/pre-miR-34a carrier to generate BioRNA/miR-27b-3p¹⁵³ (**Table 2**), which was much more highly expressed in *E. coli* compared to when it was produced with only a tRNA carrier¹⁶¹. Once administered to LS-180 cells, BioRNA/miR-27b-3p was processed to mature miR-27b-3p and again successfully suppressed expression of its targets CYP3A4 and VDR. This once again corresponded with a reduction in enzyme activity as demonstrated by a decrease in midazolam 1'-hydroxylase metabolism¹⁵³. This study also produced BioRNA/miR-328-3p for functional study, and it was selectively processed to mature miR-328-3p in human lung carcinoma cells. MiR-328-3p is a known regulatory miRNA for ABCG2, which is a critical drug transporter implicated in chemotherapeutic resistance that is also commonly known as breast cancer resistance protein¹⁶⁶. Transfection with BioRNA/miR-328-3p resulted in knockdown of ABCG2 protein levels and mRNA transcripts, sensitizing chemo-resistant MCF7/MX100 cells to mitoxantrone treatment via promoting the drug's intracellular accumulation¹⁵³ (**Table 2**). Mitoxantrone is an established substrate of ABCG2, providing an explanation as to why downregulation of this transporter could sensitize cancer cells to treatment. Intracellular accumulation of the drug was assessed by examining mitoxantrone fluorescence intensity, which was higher across BioRNA/miR-328-3p treated cells¹⁵³.

Another study published in 2020 investigated the role of epigenetic role of BioRNA/let-7c-5p, an agent with demonstrated promise to inhibit hepatocellular carcinoma growth^{160,167}. This BioRNA was processed to mature let-7c-5p within living cells, effectively suppressing the protein levels of ABCC4 and ABCC5 transporters¹⁶⁷ (**Table 2**). Downregulation of these transporters by BioRNA/let-7c-5p promoted intracellular accumulation of cancer drug 5-FU, explaining the synergistic effects observed with these agents in the inhibition of cancer cell viability¹⁶⁷. Although

most ADME research utilizing this platform so far has been centered on miRNAs, it has also been used to elucidate the epigenetic role of NRF2 through a corresponding siRNA. Nuclear factor erythroid 2-related factor 2 (NRF2) is a transcription factor understood to play a critical role in the induction of ADME gene products such as ABC transporters, with NRF2 overexpression being a commonly observed phenomenon in multidrug resistant cancers¹⁶⁸⁻¹⁷⁰. Administration of BioRNA/NRF2-siRNA resulted in significant downregulation of the transporters ABCC3, ABCC4, and ABCC5, sensitizing human cancer cells to commonly employed chemotherapeutics doxorubicin, cisplatin, and sorafenib¹⁷⁰.

Conclusions and Perspectives

Recombinant DNA technologies have revolutionized the field of drug metabolism and pharmacokinetics, equipping investigators with the tools to produce pure ADME gene products for research and development. Advancements in our understanding and utilization of expression vectors and host organisms has provided researchers with a versatile and adaptable toolbox to express and purify almost any gene of interest. This strategy has been widely employed in the study of P450 monooxygenases, where heterologous expression of these drug metabolizing enzymes has allowed investigators to examine their conformational interactions with xenobiotics and endogenous compounds. In this way, recombinant technologies have provided novel insights into CYP enzymes and other ADME gene products, catapulting forward the fields of DMPK. These research efforts have armed investigators with critical understanding of the molecular mechanisms underlying xenobiotic biotransformation and disposition, providing information that is invaluable to the development of safe and effective therapeutics. Recombinant protein expression continues

to hold incredible potential for future DMPK research, improving availability of robust research tools in addition to aiding the design of new antibodies and specific inhibitor drugs by providing facile means to express and purify specific transgenes of interest.

As recombinant technologies have granted us further insight into the gene products behind DMPK processes, they have also revealed an increasingly complex picture of drug metabolism that can vary widely between populations and individuals. Keeping this in mind, it is essential that investigators continue to employ rDNA technologies in new and innovative ways to interrogate the function and regulation of ADME gene products. Recently, miRNAs have emerged as important factors in this equation, with their roles in posttranscriptional regulation of ADME genes having a significant impact on drug metabolism and transport as well as PK and efficacy^{171,172}. To build upon our understanding of miRNA-directed regulation and address the widespread usage of synthetic miRNA mimics in research, an innovative bioengineering platform has been developed, capable of generating tens milligram quantities of pure recombinant RNA agents from only a liter of bacterial culture. These humanized BioRNA agents are produced and processed inside living cells, serving as a more accurate representation of natural endogenous miRNAs. These BioRNAs do not require added chemical modifications to maintain their intracellular stability. It has proven these biologic RNA agents are selectively processed to the target miRNAs or siRNAs in human cells to selectively regulate target gene expression. Additionally, the hybrid tRNA/pre-miRNA carrier utilized in this technology can achieve high levels of expression with a wide variety of small RNAs^{81,173}. Using this novel platform, the Yu lab has amassed a collection of humanized BioRNA agents so that their utilities in modulating ADME gene expression may be interrogated. This novel bioengineering strategy has been successfully utilized in the study of several ADME

genes, effectively regulating expression of targets such as P450 enzymes and ABC transporters to alter drug metabolism, transport, exposure, and therapeutic outcome. The BioRNA platform has greatly increased the quality, adaptability, and availability of miRNA research tools, making it a unique and valuable addition to DMPK research as well as rDNA technologies as a whole.

Table 1. Expression of Some Recombinant P450 Monooxygenases for Structural or Functional Studies. Abbreviations: NDMA, *N*-Nitrosodimethylamine; SRP, signal-recognition particle; *T. ni*, *Trichoplusia ni*

P450 Enzyme	Species of Origin	Investigative Area	Host Organism	References
P450 _{CAM}	<i>P. putida</i>	Crystal structure of three named orientations of the enzyme	<i>P. putida</i>	101
P450 _{C21}	Bovine	Genetics of adrenal P450 steroid 21-hydroxylation	<i>E. coli</i>	17
Microsomal P450	Rabbit	Role of NH2 terminus (SRP) in membrane solubility	SP-6 transcription vector (<i>in vitro</i>)	107,113
P450 _{MC}	Rat	Role of NH2 terminus (SRP) in catalytic activity	<i>Saccharomyces Cerevisiae</i>	11
P450PCN1	Human	Drug metabolism activity	<i>COS cells</i>	18
CYP2D6	Human	Enzyme function and genetic polymorphism	<i>COS cells</i>	19
CYP2E1	Human	Function in drug metabolism	<i>COS cells</i>	20
CYP2E1	Rabbit	Role of NH2 terminus (SRP) in catalytic activity (NDMA deethylation)	<i>E. coli</i>	26
CYP51	<i>Mycobacterium tuberculosis</i>	Crystal structure, expression of soluble MT form, substrate requirements	<i>E. coli</i>	13
CYP2B4	Mammalian	Crystal structure, conformational flexibility at active site	<i>E. coli</i>	114
CYP2C5	Mammalian	Crystal structure, expression of NH2 truncated form	<i>E. coli</i>	115
CYP2C9	Human	Crystal structure, binding pocket in the presence of warfarin	<i>E. coli</i>	15
CYP2C8	Human	Crystal structure, ligand-induced conformational changes	<i>E. coli</i>	16
CYP2D6	Human	Crystal structure, specificity of ligand binding	<i>E. coli</i>	120
CYP2D6.1, 2, 10, & 17	Human	Functions of allelic isoforms and genetic polymorphism	<i>T. ni</i> (insect)	14

CYP3A4	Human	Crystal structure in multiple conformational states, residues involved in midazolam oxidation	<i>E. coli</i>	116,117
CYP3A4/5	Human	Differences in active site plasticity and substrate binding complexed with Ritonavir	<i>E. coli</i>	124
CYP3A7	Human	Crystal structure, comparative analysis of ligand binding	<i>E. coli</i>	130
CYP17A1	Human	Crystal structure, complexed with major substrates and inhibitors	<i>E. coli</i>	141
CYP19A1	Human	Crystal structure, functional roles of key residues	<i>E. coli</i>	145
CYP11B1	Human	Crystal structure, cortisol synthesis	<i>E. coli</i>	150
CYP11B2	Human	Crystal structure, aldosterone synthesis	<i>E. coli</i>	149
CYP8B1	Human	Crystal structure, bile acid synthesis	<i>E. coli</i>	151

Table 2. Modulation of ADME gene expression as well as drug metabolism and transport by recombinant RNA agents. Abbreviations: BCRP, Breast cancer resistance protein; 5-FU, Fluorouracil.

BioRNA	ADME Genes	Effects	References
miR-27b-3p	CYP3A4	Downregulates CYP3A4 mRNA transcript abundance, protein expression, and 1'-hydroxylase activity in LS180 cells	153,161
miR-1291-5p	ABCC1	Downregulates ABCC1 protein expression in MCF-7 and PANC-1 cells and sensitizes them to doxorubicin treatment	164
miR-124-3p	ABCC4	Downregulates ABCC4 and STAT3 protein expression in A549	158
miR-328-3p	ABCG2	Downregulates ABCG2/BCRP mRNA transcript abundance and protein expression in MCF7/MX100 cells to increase intracellular accumulation of mitoxantrone and sensitize cells to treatment	153
let-7c-5p	ABCC5 ABCC4	Downregulates ABCC5 and ABCC4 protein expression in Huh7 cells, promotes intracellular accumulation of 5-FU, and exhibits synergistic effects inhibiting Huh7 cell viability	167

Figure Legends

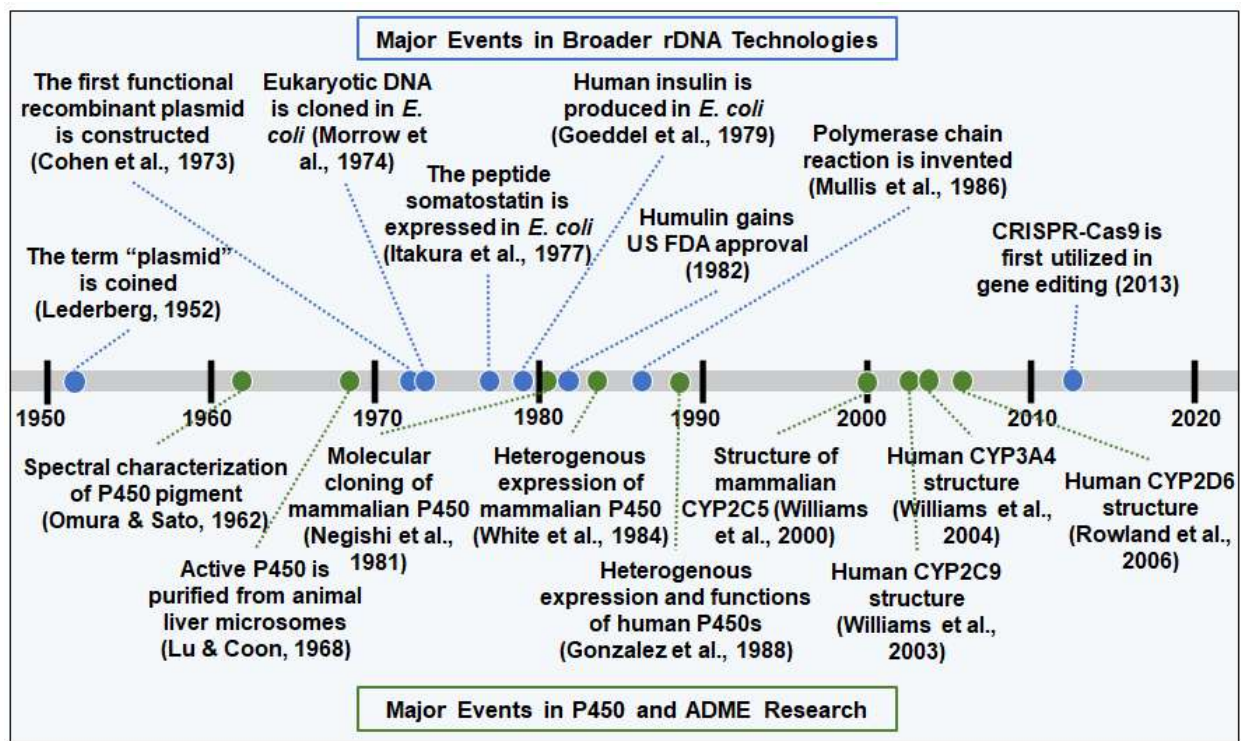


Figure 1. Milestones of recombinant DNA technologies and relevant applications to P450 and ADME research

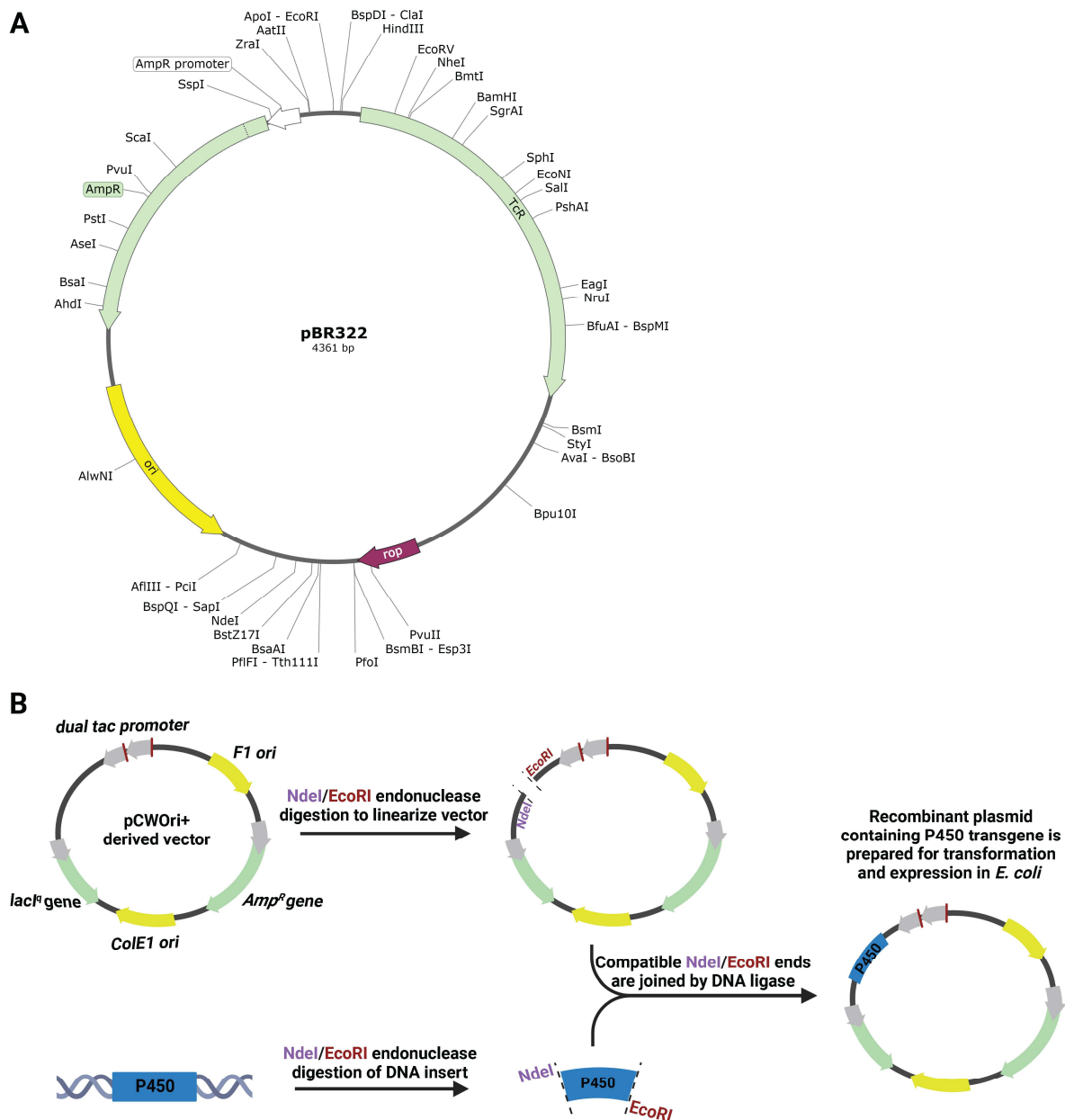


Figure 2. Recombinant Protein Expression Vectors: Characteristics and Examples in P450 Research. (A) First generation vector pBR322 contains an ampicillin resistance gene (AmpR) and a tetracycline resistance gene (TcR), both shown in green. These genes serve as selection markers to identify positive transformants upon antibiotic treatment. The pBR322 plasmid contains a ColE1 origin of replication (ori) site which is illustrated in yellow. This ori site is recognized by *E. coli* host machinery to enable replication of the in this model organism. The repressor of primer (rop) gene illustrated in red encodes the rom protein, which limits the copy number of this vector. (B) An optimized expression vector derived from pCWori+ is utilized to construct target plasmid towards heterologous P450 expression in *E. coli*¹²². This vector contains two tac promoter sequences that are under transcriptional control of the lac repressor protein which is illustrated in red²⁴. The lac repressor is encoded by the lacI^q gene shown in green, which suppresses transcription until an inducing agent such as isopropyl β -D-thiogalactopyranoside (IPTG) is added. The vector and recombinant P450 gene insert are digested with endonucleases NdeI and EcoRI, enabling insertion and ligation of the transgene downstream of the two inducible tac promoters. This pCWori+ derived expression vector also possesses two origin of replication sites shown in yellow, which include the previously discussed ColE1 ori as well as an F1 ori that provides a recognition site for M13 bacteriophages.

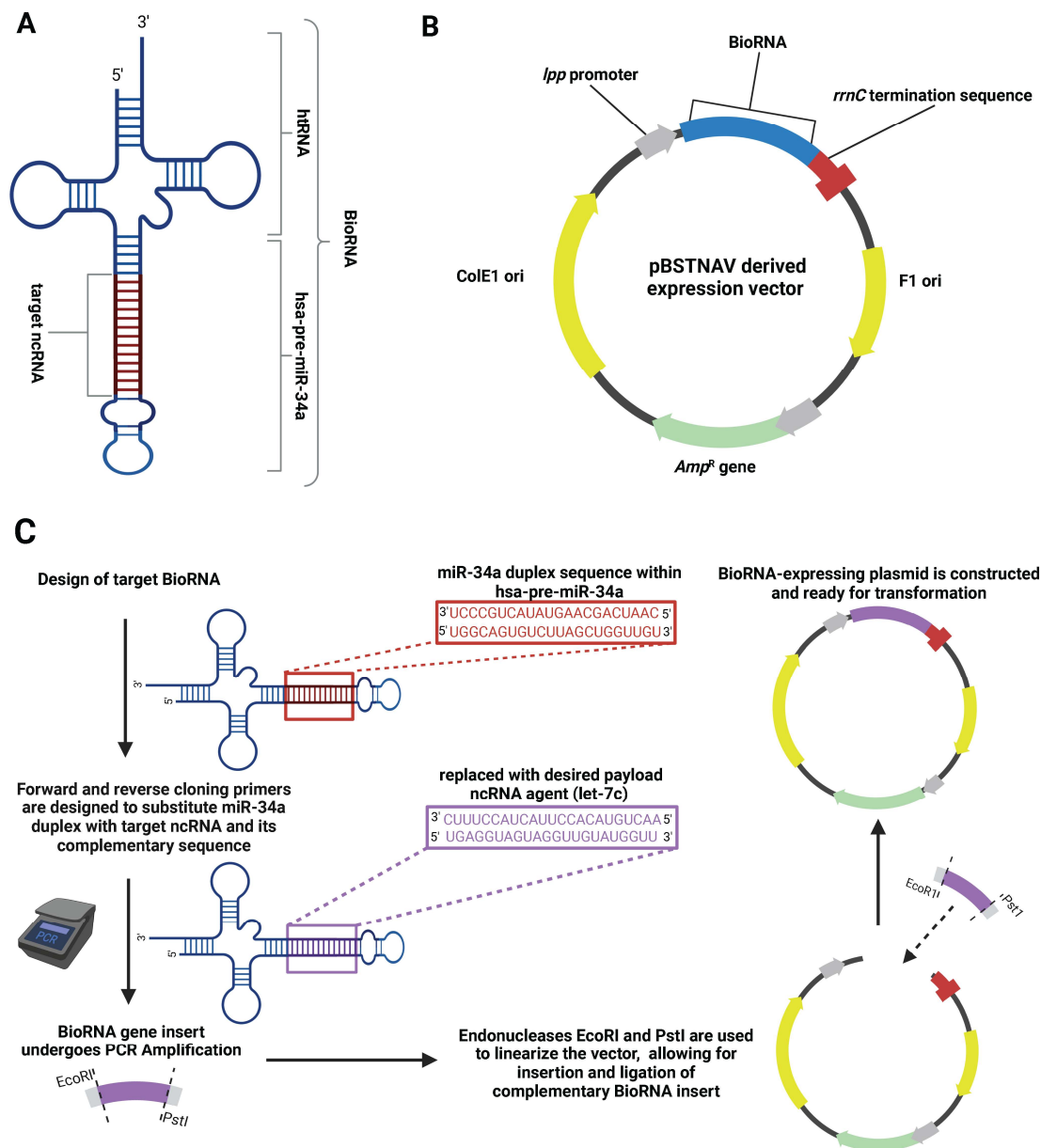


Figure 3. Design of Recombinant RNA Expression Plasmids for Fermentation Production in *E. coli*. (A) Recombinant or bioengineered RNA (BioRNA) agents are expressed with hybrid tRNA/pre-miRNA carriers, protecting them from RNase degradation and generating consistently high yield of desired BioRNA from bacterial culture. The miR-34a duplex highlighted in red can be replaced with a payload ncRNA of interest to be recombinantly expressed as part of this hybrid carrier. (B) An expression vector derived from the pBSTNAV plasmid is utilized for recombinant BioRNA expression. This vector contains a strong lipoprotein gene promoter (*lpp*) as well as a ribosomal RNA operon transcription terminator (*rrnC*) to ensure efficient expression of the BioRNA insert. This vector also contains ColE1 and F1 ori sites, which are recognized by *E. coli* and M13 bacteriophages respectively. The gene encoding ampicillin resistance (*Amp^R*) is also present in the vector, which enables positive selection of positive transformants through antibiotic treatment. (C) Payload ncRNA is identified and the resulting BioRNA sequence is defined accordingly. Restriction sites are added to the ends of the BioRNA coding sequence, and cloning primers are designed to replace mature miR-34a-5p and its complementary strand with the desired payload ncRNA. This BioRNA insert is then amplified by polymerase chain reaction (PCR) in preparation for vector insertion. The vector is then linearized by endonucleases EcoRI and PstI, allowing insertion and ligation of the recombinant BioRNA gene insert. Once verified, the BioRNA expression plasmid is then ready for transformation into HST08 *E. coli* for recombinant BioRNA expression.

Chapter 2: Small RNA or Oligonucleotide Drugs and Challenges in Evaluating Drug-Drug Interactions (DDIs)

Abstract

Small RNA or oligonucleotide therapeutics represent a unique modality outside the traditional treatment paradigm of small molecule and protein-based drugs that have historically only targeted a small fraction of the proteome. Innovations in the structural design and chemical modification have been invaluable for recent oligonucleotide therapeutics, greatly improving their biological stability, intracellular delivery, and targeting. Widespread adoption of these strategies has further enabled the application of oligonucleotides as viable drugs and expanded the class of RNA therapeutics, with thirteen antisense oligonucleotides (ASOs) (fomiversen, mipomersen, nusinersen, inotersen, eteplirsen, golodirsen, casimersen, viltolarsen, tofersen, eplontersen, olezarsen, and donidalorsen), seven small interfering RNAs (siRNAs) (patisiran, givosiran, lumasiran, inclisiran, vutrisiran, nedosiran, and fitusiran), and two aptamers (pegaptanib and avacincaptad pegol) that have been approved by the United States Food and Drug Administration (FDA). RNA therapeutics have expanded the druggable space and provide a novel treatment strategy, they do not fit within the framework of our current methodology in evaluating risk of drug-drug interactions (DDIs) and assessing pharmacokinetic/pharmacodynamic (PK/PD) relationships. This article provides an overview of FDA-approved oligonucleotide therapies, emphasizing chemical modifications, molecular targets for mechanistic actions, and available ADME and PK/PD properties, followed by the discussion of critical needs for risk assessment strategies suited for this unique modality that focuses on possible DDIs with concomitant drugs. The latter may involve direct competition for the endogenous RNA interference machinery to alter ADME or relevant PD gene expression, rather than uncommon binding or interactions with drug-metabolizing enzymes or transporters found and recommended for small molecule drugs.

Introduction

Conventional therapeutic strategies centered around protein-targeted drugs have been constrained by our limited understanding of RNAs as simply a set of messengers relay the genetic code into functional protein. With only a small percentage of messenger RNA (mRNA) encoded proteins being considered druggable, research into protein-targeted therapies was limited to a fraction of the proteome. The discovery of many functional RNA species that did not encode proteins provided a perspective of protein expression and RNA function that was much more complex, but quickly showed therapeutic potential when a single-stranded oligonucleotide was found to target mRNA and control gene expression¹⁵⁷. This continued with the discovery of small non-coding microRNAs (miRNAs), which were naturally expressed within *C. elegans* to elicit post-transcriptional gene regulation (PTGR) of target mRNA via antisense interaction^{174,175}. Soon after, exogenous double-stranded RNAs showed regulation of complementary mRNA, a mechanism referred to as RNA interference (RNAi) that set the stage for future therapeutic use of small interfering RNAs (siRNAs)^{176,177}.

These discoveries gave rise to RNA-based therapeutics, a novel class of therapies that are fundamentally distinct from traditional small molecules and protein biologics. This class encompasses a diverse set of therapeutic strategies including aptamers, antisense oligonucleotides (ASOs), and siRNAs, which modulate protein expression or activity of their specific targets^{178,179}. These therapies exhibit unique chemical structures and diverse mechanisms of action that are critically different from small molecule and biologic protein drugs. RNA therapeutics' unique targeting and mechanistic actions expand the range of druggable molecules within the genome, but

they also differ in their pharmacokinetic (PK) properties compared to well-understood small molecule and protein-based drugs. Oligonucleotide drugs are naturally unstable in biological systems, being readily degraded by natural human endo- and exonucleases that cleave phosphodiester bonds at unique locations within the molecule¹⁸⁰⁻¹⁸³. Additionally, the charged backbone, hydrophilic nature, and large size of many oligonucleotide drugs prevent them from crossing cell membranes, posing significant delivery challenges, a major concern affecting clinical drug administration, distribution, metabolism, and excretion (ADME) processes. These challenges are commonly addressed by chemical modification of the phosphodiester linkages, ribose sugar rings, and nucleobases, as well as through the conjugation of large polymers or ligands. These modifications confer nuclease resistance, improve oligo delivery and PK, and enable specific targeting to hepatocytes through the use of ligands like *N*-acetylgalactosamine (GalNAc)^{182,184-186}.

Distinct from small molecule and protein drugs, oligonucleotides are not major substrates of cytochrome P450 enzymes but subject to cleavage by various nucleases and hydrolases, nor are they typical substrates for efflux transporters, hepatic uptake transporters, or renal uptake transporters but endocytosis or receptor mediated uptake, and thus exhibiting nontraditional PK properties. Further, systemic PK parameters of RNA therapies do not always reflect target tissue distribution and are not often reflective of pharmacodynamic (PD) outcomes, highlighting the need for additional pharmacodynamic endpoints in multiple-dose studies such as target mRNA/protein or other relevant PD biomarkers¹⁸⁷.

RNA therapeutics' sequence driven specificity offers novel therapeutic opportunities, but they also present unique challenges in evaluating risk of drug-drug interactions (DDIs) and adverse events,

from both PK and PD perspectives. With these factors in mind, the U.S. Food and Drug Administration (FDA) recommends several key risk evaluations including assessing potential for QTc interval prolongation, as well as assessing the impact of hepatic and renal impairment on pharmacodynamic endpoints and biomarkers independent of PK, with participants across a range of organ function. The distinct structures, non-natural chemical modifications, unique mechanistic actions, and nebulous PK/PD relationships of oligonucleotide therapeutics underscore the needs for risk assessment strategies uniquely tailored to this modality, including immunogenicity, on/off-target effects, and PD-based DDI with drugs or endogenous RNA that share mechanistic action or pathways. Indeed, RNAi or ASO drugs would likely compete with the genome-derived microRNAs (miRs or miRNAs) for the intrinsic RNAi components and subsequently, alter the PTGR of ADME or related PD genes and then lead to possible DDIs^{172,188-190}.

This review will first introduce the U.S. Food and Drug Administration (FDA) approved oligonucleotide drugs, their molecular targets, mechanistic actions, therapeutic indications, known ADME/PK properties, and discuss the challenges in the evaluation of DDIs for RNA therapeutics¹⁸⁷.

Common Mechanistic Actions of RNA Therapeutics

Fundamental scientific research uncovering the diverse forms and functions of natural RNAs beyond encoding protein has provided valuable inspiration in the design and application of RNA therapeutics. RNA has since emerged as a versatile treatment modality, employing several different mechanisms of action across FDA-approved therapies. While there are many exciting therapeutic applications of different forms of RNAs under clinical and preclinical development, this review will focus on the FDA-approved aptamers, ASOs, siRNAs (**Figure 1**).

Aptamers are single-stranded oligonucleotides optimized for high-affinity binding to a specific protein sequence, commonly acting as inhibitors or antagonists of their target to reduce protein activities or functions⁷⁷ (**Figure 1**). Aptamers that function as agonists and allosteric modulators of their target have also been developed, although none have yet reached the clinic^{191,192}. Aptamer drugs assume complex tertiary structures that allow binding of their target with a high degree of specificity. Oligonucleotide sequences form a diverse range of structural motifs that are utilized in aptamer design to target a variety of molecules beyond just proteins, including nucleic acids, small molecules, and heavy metals^{77,193}. While other strategies are promising, both FDA-approved aptamer therapies function through protein targeting (**Figure 1**)^{194,195}.

ASOs are short, single-stranded oligonucleotides that engage with specific RNA sequences through Watson-Crick base pairing^{77,178}. FDA-approved ASO therapies bind target mRNA and regulate protein expression through several possible mechanisms, including cleavage of the target transcript by RNase H, and inhibition of its translation through steric blockage of ribosome

binding. ASO therapies can also act through alternative splicing of pre-mRNA to promote inclusion or exclusion of specific exons, altering the identity of the corresponding protein (**Figure 1**). These mechanisms encompass all FDA-approved ASOs, with other types of remedies, such as ASO against disease-associated miRNAs, showing limited success in the clinic^{196,197}.

SiRNAs are double stranded RNA molecules that form complementary base pairings with target mRNA transcripts, modulating the levels of corresponding proteins through RNAi pathway^{77,198,199}. SiRNA therapies contain an antisense/guide strand designed to recognize a sequence within the coding region or 3' untranslated region (UTR) of a target transcript, where they bind with (near-)perfect complementarity. Administered siRNA duplexes within the cytoplasm are unwound and the antisense/guide strand is preferentially loaded into the RNA induced silencing complex (RISC). The guide identifies complementary mRNA sequences to direct RISC-mediated cleavage of target transcripts, leading to the reduction of protein levels (**Figure 1**). The therapeutic action of siRNA cannot happen without the key machinery that comprises the RISC, with Argonaute (AGO) protein serving as a critical and necessary component. The four AGO isoforms in humans serve similar functions in RNAi, but only AGO2 possesses the catalytic activity needed for nucleolytic cleavage of RISC-bound target mRNA. This is key for siRNA drugs as they possess highly complementary guide strands, form ideal interactions for mRNA cleavage, and preferentially engage with AGO2/RISC. By contrast, endogenous miRNAs acting through RNAi machinery typically binds via the seed region spanning 2-7 nt and can interact with AGO1-4/RISC, forming shorter complementary interactions that are less specific but allowing targeting of multiple unique mRNA transcripts. This seed-mediated target binding by miRNA does not rapidly cleave the mRNA but instead inhibits its translation and sometimes

promotes mRNA degradation. Despite this distinction, siRNA and miRNA may compete for key parts of the RISC, with AGO2 serving as a major example. Later in this review, we will discuss the unique challenges this poses in evaluating DDI risk and provide perspective for the future study and intelligent design of RNAi therapeutics.

Aptamer Drugs

Pegaptanib (Macugen) was the first FDA-approved aptamer drug and the first therapy to target vascular endothelial growth factor (VEGF), being approved in 2004 for the treatment of age-related macular degeneration (AMD) (**Table 1**)^{77,195,200}. Pegaptanib treats the neovascular (wet) form of AMD, where abnormal blood vessel growth causes loss of vision. The 28-nucleotide RNA sequence of pegaptanib specifically targets the heparin binding domain of extracellular VEGF, preventing efficient activation of VEGF receptors and inhibiting abnormal blood vessel growth within the eye to halt vision loss. Pegaptanib's tertiary structure provides isoform-selective targeting of VEGF, binding the extracellular isoform most relevant to AMD progression (VEGF-165) while sparing other biologically active isoforms important in healthy function (VEGF-121)²⁰¹⁻²⁰³. Pegaptanib proved effective in two concurrent clinical trials, where 70% of patients lost less than 15 letters of visual acuity compared to 55% in control groups over 54 weeks. This was also reflected in reduced risk of severe vision depreciation (loss of 30 letters of visual acuity or more) being twice as likely in the sham-injection group compared to pegaptanib treatment group²⁰⁰. Significant benefit was evident across all doses assessed (0.3 mg, 1 mg, 3 mg).

Pegaptanib contains extensive chemical modifications that protect it from nuclease degradation, improve its PK properties, and improve target binding. All pyrimidines are 2'-deoxy-2'-fluoro nucleosides, while all purine nucleosides contain 2'-*O*-methyl modifications with the exception of two adenosines (positions 4 and 5) (**Table 1**)¹⁹⁵. Additionally, the 3' terminal end is an inverted deoxythymidine bound through a 3'-3' phosphodiester linkage, further improving the aptamer's metabolic stability. The 5' end of pegaptanib is conjugated to two 20 kDa polyethylene glycol (PEG) moieties via aminohexyl linkage, greatly increasing its size to support a long half-life within the vitreous humor and limit its systemic clearance by renal filtration^{180,204}. Renal clearance was the major route of elimination identified for pegaptanib, and patients with impaired renal function were assessed in PK studies^{205,206}. However, these patients did not require dose adjustment – likely because of pegaptanib's slow absorption to the plasma following intravitreal (IVT) injection.

Many years later in 2023, Avacincaptad pegol (Izervay) would become the second aptamer drug approved by the FDA, indicated for the treatment of geographic atrophy (GA) secondary to AMD (**Table 1**)¹⁹⁴. GA is the advanced stage of dry AMD, where chronic inflammation and oxidative stress lead to the irreversible loss of retinal cells and eventually the loss of central vision. Avacincaptad pegol slows the progression of GA by inhibiting the complement system, an inflammatory signaling cascade of the immune system²⁰⁷. Avacincaptad pegol specifically binds to complement protein C5 and prevents its cleavage to C5a and C5b, lessening subsequent inflammatory signaling that drives cell death and GA progression²⁰⁸⁻²¹⁰. In the GATHER1/2 trials, 3.4% of treated patients exhibited persistent loss of visual acuity (>15 letters) compared to 7.8% in the sham group over 12 months²¹¹. Both pegaptanib and avacincaptad were developed by systematic evolution of ligands by exponential enrichment (SELEX) which screens large pools of

nucleotide sequences for affinity to a specific target, identifying the highest-affinity oligonucleotide ligands in many subsequent rounds of enrichment²¹².

Avacincaptad pegol is a 39 nucleotide RNA aptamer with extensive chemical modifications that are heavily inspired by the success of pegaptanib. Similarly, all present pyrimidines are 2'-deoxy-2'-fluoro nucleosides, the 3' terminal end is capped with an inverted deoxythymidine bound via 3'-3' phosphodiester linkage, and the 5' end is bound to a 43 kDa PEG moiety via aminohexyl linker. All purines are 2'-*O*-methyl substituted, with the exception of two unmodified guanosines (positions 5 and 17) and one unmodified adenosine (position 33) (**Table 1**)¹⁹⁴. Echoing the strategy of pegaptanib, these structural features provide nuclease resistance and extend avacincaptad pegol's half-life to support monthly dosing. Dose adjustment is similarly not required for renal or hepatic impairment, but treatment was associated with increased rates of neurovascular (wet) AMD.

ASO Drugs

Fomivirsen (Vitravene), an ASO indicated for the local intravitreal treatment of cytomegalovirus (CMV) retinitis in patients with acquired immunodeficiency syndrome, became the first FDA-approved RNA therapeutic in 1998 (**Table 2**)^{213,214}. CMV retinitis is an opportunistic infection characterized by formation of lesions in the retina that progress to loss of vision. Fomivirsen binds to its target, human CMV immediate early region 2 (IE2) mRNA, via complementary base pairing and prevents translation of several proteins critical to viral replication²¹⁵. Fomivirsen inhibits translation through antisense hybridization of target mRNA, creating a steric blockade that limits ribosomal interaction. This binding also enables RNase H-mediated degradation of target mRNA, though less efficiently than later-generation ASOs²¹⁶. Clinical trials utilized fundus photography of the retina to assess CMV retinitis lesions and progression, with only 44% of patients in the immediate treatment group exhibiting disease progression compared to 70% in the group where treatment was deferred²¹⁷. Fomivirsen is a 21-mer ASO with a full phosphorothioate (PS) backbone, a modification that would become standard practice in future ASO design. PS linkages protect ASOs from nuclease degradation and greatly enhance plasma protein binding (>90%) to limit renal clearance and dramatically prolong their half-life²¹⁸⁻²²⁰. While extensive plasma protein binding allows PS ASOs to escape filtration by the kidneys, it also promotes broad tissue distribution that can increase off-target exposure and potential for associated toxicities. PS backbone modifications can also affect potency of mRNA target regulation, with high-affinity binding to plasma proteins like α -2-macroglobulin serving as a key example^{220,221}.

Mipomersen (Kynamro) is a 20-mer PS ASO that targets the apolipoprotein B-100 (Apo B-100) transcript, approved in 2013 to treat homozygous familial hypercholesterolemia (HoFH) via subcutaneous (SQ) injection (**Table 2**). HoFH is characterized by dangerous accumulation of low-density lipoprotein cholesterol (LDL-C), of which Apo B-100 is a key precursor^{222,223}. Mipomersen hybridizes with target Apo B-100 mRNA, preventing its translation through steric inhibition of ribosome interaction and RNase H-mediated transcript cleavage. Preclinical animal studies demonstrated reduction of liver Apo B-100 mRNA levels, as well as serum levels of Apo B-100, total cholesterol, and LDL-C²²⁴. Clinical assessment of mipomersen showed significant reduction of baseline LDL-C levels (36%) which served as the primary endpoint, but also demonstrated significant reduction of secondary endpoints Apo B-100 and Apolipoprotein A²²⁵⁻²²⁷. Mipomersen was the first ASO to use a “gapmer” design, featuring a central DNA gap of ten nucleotides flanked by “wings” of five 2'-O-(2-methoxyethyl) (2'-MOE) modified nucleosides on each side. The 2'-MOE modified wings provide exonuclease resistance, reduce immune stimulation, and increase target affinity, while the central DNA gap enables RNase-mediated cleavage of target mRNA, a process which is otherwise inhibited by 2'-sugar modifications. Mipomersen was also the first ASO to utilize 5-methylcytosine and 5-methyluracil nucleobases, naturally occurring pyrimidine modifications that reduce immunogenicity and improve hybridization to target mRNA²²⁸⁻²³¹. Mipomersen pioneered PS 2'-MOE gapmer design and would serve as a valuable example in the development of future ASO therapies. Unfortunately mipomersen received multiple black-box warnings from the FDA due to hepatotoxicity risk, with common adverse reactions including hepatic steatosis and elevations in serum transaminases like alanine aminotransferase (ALT) and aspartate aminotransferase (AST)²²³. Because of these risks,

mipomersen was contraindicated in patients with hepatic impairment and eventually pulled from the market in 2019^{227,232}.

Nusinersen (Spinraza) is a 18-mer PS 2'-MOE ASO approved in 2016 for the treatment of spinal muscular atrophy (SMA) (**Table 2**)²³³. SMA is characterized by survival motor neuron (SMN) protein deficiency, leading to spinal motor neuron degeneration and death. Nusinersen works by alternative mRNA splicing, binding intronic splicing silencer N1 (ISS-N1) within the SMN2 pre-mRNA to promote exon 7 inclusion and enable production of functional SMN protein²³⁴⁻²³⁶. In addition to the PS linkages and 2'-MOE sugar modifications mentioned, all pyrimidine nucleobases in nusinersen are 5-methyl substituted. While these modifications benefit ASO stability, potency, and tissue distribution as being discussed, they did not enable crossing of the blood-brain barrier when nusinersen was administered systemically. However, nusinersen would prove to be a great success in the treatment of SMA when administered locally via intrathecal injection.

Inotersen (Tegsedi) is a 20-mer PS ASO gapmer that was approved in 2018 for the treatment of hereditary transthyretin-mediated amyloidosis (hATTR) (**Table 2**), a rare genetic disorder that causes misfolding of transthyretin (TTR) protein. Misfolded TTR proteins manifest as dangerous amyloid deposits that commonly lead to cardiomyopathy and neuropathy, with kidney dysfunction also being a secondary concern. Inotersen hybridizes with TTR mRNA, promoting its degradation by RNase H. Inotersen heavily mirrors the design of mipomersen, containing the same 5-10-5 gapmer design with 2'-MOE wings, a central DNA gap, and 5-methylcytosine and 5-methyluracil nucleobases. Inotersen is similarly administered SQ and exhibits extensive plasma protein binding

and widespread distribution, with the highest concentrations depositing to the liver and kidneys. Inotersen exhibited significant toxicities, receiving two black box warnings for thrombocytopenia (drastic reduction in platelet count that causes severe bleeding), and glomerulonephritis (inflammation of the kidneys, additive to the toxicity of amyloid protein buildup and consistent with severe immune response). Inotersen treatment suppresses natural vitamin A levels, explaining some of its potential toxicities and necessitating vitamin A supplementation during treatment. Overall, inotersen's variety of potential adverse events led to very strict exclusion criteria for patient enrollment and eventually its discontinuation in 2024, with another ASO and multiple RNAi therapies for hATTR being better tolerated by patients,²³⁷

Eteplirsen (Exondys 51) is a 30-mer oligonucleotide that was approved in 2016 for the treatment of Duchenne muscular dystrophy (DMD) that is exon 51 amenable (**Table 2**). In DMD the gene encoding the dystrophin protein is not functional, with several different possible mutations capable of causing the disease. Dystrophin is a critical protein in motor function and those without a functional copy have reduced life expectancy and quality of life. Eteplirsen binds to exon 51 of dystrophin pre-mRNA, causing this exon to be skipped during mRNA splicing and resulting in a functional dystrophin protein for patients with the corresponding mutation. Eteplirsen incorporates two unique modifications, featuring uncharged phosphorodiamidate backbone linkages as well as six-membered morpholino rings replacing the natural five-membered sugar rings present in DNA/RNA. Eteplirsen was the first of four phosphorodiamidate morpholino oligonucleotides (PMOs) to be approved for DMD, followed by golodirsen (Vyondys 53) in 2019, viltolarsen (Viltepso) in 2020, and casimersen (Amondys 45) in 2021 (**Table 2**). All approved PMO therapies work via the same mechanism and are administered via intravenous (IV) infusion, with golodirsen

and viltolarsen indicated for DMD amenable to exon 53 skipping and casimersen for DMD amenable to exon 45 skipping (**Table 2**)²³⁸⁻²⁴¹. These PMO therapies' unique structural features confer exceptional metabolic stability, each being excreted in the urine as the parent drug without any known metabolites. Distinct from PS linked ASOs, PMOs exhibit modest plasma protein binding (<40%) and are more readily cleared from the body. The PMO subclass is generally well tolerated, with renal toxicity being the primary concern. Patients with renal impairment exhibit reduced clearance of PMO drugs, but specific recommendations for dose adjustment can be challenging in DMD due to reduced muscle mass and creatinine levels.

Tofersen (Qalsody) is a 20-mer ASO gapmer with mixed PS/PO linkages, approved to treat amyotrophic lateral sclerosis (ALS) with superoxide dismutase 1 (SOD1) mutation in 2023 (**Table 2**)²⁴². Tofersen's structure is consistent with other ASO gapmers, containing the same 5-10-5 design with 2'-MOE wings, a central DNA gap, and 5-methylcytosine and 5-methyluracil nucleobases. Tofersen is primarily metabolized by exonucleases consistent with other ASOs but is uniquely administered via intrathecal injection. Tofersen promotes degradation of SOD1 mRNA through antisense binding, preventing accumulation of plasma neurofilament light chains that promote ALS progression.

Eplontersen (Wainua) was approved by the FDA to treat polyneuropathy of hATTR in 2023 (**Table 2**), and it was the first ASO drug that utilized conjugation of GalNAc ligands to achieve hepatocyte-specific delivery^{243,244}. Eplontersen is a 20-mer ASO gapmer with mixed PS/PO linkages and a 5-10-5 design consistent with other discussed therapies. Eplontersen targets the TTR transcript and promotes its degradation via RNase H following antisense hybridization.

Eplontersen is conjugated to a tri-antennary GalNAc ligand (GalNAc₃) via a trishexylamino linkage. The conjugated GalNAc moieties specifically bind to asialoglycoprotein receptors (ASGPRs) on the surface of hepatocytes, promoting ASO internalization at the target tissue. This approach has proven invaluable in the liver-targeted delivery of RNA drugs. In addition to improving target tissue specificity, GalNAc conjugation also greatly improves elimination half-lives of RNA therapies to enable less frequent dosing. Individual GalNAc ligands are cleaved by *N*-acetyl- β -glucosaminidase, creating several possible metabolites possessing different lengths and remaining number of GalNAc sugars (**Figure 2**)¹⁸⁶. However, GalNAc₃-conjugated ASO is the most common form in plasma following SQ administration. Within target tissue, unconjugated ASO undergoes degradation by endo- and exonucleases consistent with the unconjugated ASOs discussed. Similarly, GalNAc-conjugated ASOs are extensively plasma protein bound, limiting their renal elimination. Multiple ASO therapies have also employed this GalNAc₃ conjugation strategy to achieve liver-specific ASO delivery and improve tolerability of therapy²⁴⁵.

Imetelstat (Rytelo) is an ASO therapeutics possessing a unique design, approved in 2024 for the treatment of myelodysplastic syndromes (MDS) with transfusion-dependent anemia (**Table 2**)²⁴⁶. Imetelstat binds to the template region of Human telomerase's RNA component. This binding elicits competitive inhibition of telomerase enzymatic activity that is overactive in malignant stem and progenitor cells and key to MDS progression²⁴⁷. Imetelstat is a 13-mer ASO uniquely consisting of 3'-amino-3'-deoxy nucleotides, where the natural 3'-oxygen is replaced by a nitrogen that forms a 3'-amino group. joined with PS linkages. Imetelstat is uniquely conjugated to a palmitoyl lipid moiety that enhances its permeability and potency of telomerase inhibition (**Table**

2)^{248,249}. However, IV administration of ASOs conjugated to lipophilic moieties is still often required, distinct from GalNAc conjugation that enables SQ administration²⁵⁰.

One such therapy is Olezarsen (Tryngolza), approved in 2024 adjunct to low fat diet for familial chylomicronemia syndrome (FCS) (**Table 2**)^{179,251}. Olezarsen targets the mRNA transcript of Apolipoprotein C-III, promoting its degradation. This improves the capability of patients to clear triglycerides and low-density cholesterol from the plasma that drive FCS progression. GalNAc₃ conjugation is also employed in the most recent ASO drug, Donidalorsen (Dawnzera), which was approved in 2025 for the treatment of hereditary angioedema (HAE) (**Table 2**). Donidalorsen specifically targets prekallikrein mRNA, promoting its degradation through RNase H1. This reduces plasma prekallikrein protein levels, lowering incidence of swelling episodes characteristic of HAE²⁵². GalNAc₃-linked ASO gapmers like eplontersen, olezarsen, and donidalorsen exhibit superior tolerability and tissue specificity compared to their unconjugated predecessors and represent a significant advancement in ASO design. However, there are still limited studies assessing the use of such drugs in patients with hepatic and renal impairments, with no specific dose adjustments available based on these parameters.

RNAi Drugs

Patisiran (Onpattro) represents a significant achievement in the field as the first RNAi therapy, gaining FDA approval to treat polyneuropathy of hATTR in 2018 (**Table 3**)^{253,254}. Patisiran is a 21-mer/21-mer siRNA duplex containing an antisense strand that targets the 3'UTR of TTR mRNA with near-perfect complementarity and promotes its degradation through the RISC. Unlike later

RNAi therapies, patisiran employs a lipid nanoparticle (LNP) formulation that requires administration by IV infusion. Patisiran exclusively contains natural PO backbone linkages, as well as several 2'-*O*-methyl substituted cytosine and uridine bases that limit nuclease-mediated degradation in plasma and tissues. Short chain oligonucleotide metabolites produced via nuclease degradation undergo renal elimination. Consistent with other RNA drugs, patisiran was not found to inhibit or induce major cytochrome P450 enzymes. Population studies showed no impact on the PK from mild renal and hepatic impairment, while moderate to severe impairment was not assessed.

Givosiran (Givlaari), a 21-mer/23-mer siRNA to treat acute hepatic porphyria (AHP), was approved by the FDA in 2019 ²⁵⁵ (**Table 3**). The antisense strand of givosiran targets the coding region of aminolevulinate synthase 1 (ALAS1) mRNA with near-perfect complementarity and promotes its degradation via RISC¹⁹⁸. Givosiran contains extensive 2'-fluoro and 2'-*O*-methyl substituted ribonucleosides. Givosiran's backbone contains primarily PO linkages, with PS linkages present on the 5' terminal end of the sense strand and both terminal ends of the antisense strand (**Figure 2 and Table 3**)²⁵⁵. Critically, givosiran is conjugated to a tri-antennary GalNAc moiety termed L96 that provides enhanced stability and specific delivery to hepatocytes, enabling SQ administration. Within the liver, *N*-acetyl- β -glucosaminidase generates several possible metabolites possessing different lengths and remaining number of GalNAc sugars while nucleases generate shortened oligonucleotide sequences that can be excreted renally (**Figure 2**)^{182,256}. The L96 ligand is conjugated to the 3' terminal end of the sense strand, improving its thermodynamic stability. This improved stability and large steric bulk provided by the L96 moiety can influence the strand-selection process, where the more thermodynamically unstable strand of the duplex is

preferentially incorporated to the RISC²⁵⁷⁻²⁵⁹. It is also shown that, when assessing RNAi drug metabolism in preclinical models, it is worth considering the impact of pH on the stability of individual sense or antisense strands, including multiple physiological pH conditions for *in vitro* testing can provide insight into how metabolism and stability may change during circulation and distribution to certain tissues and subcellular components¹⁸³.

Another key structural element present in givosiran is the two-nucleotide overhang present at the antisense 3' terminus. In addition to providing further nuclease resistance, asymmetric design of siRNA duplexes influence the incorporation of the antisense/guide strand to the RISC²⁶⁰. The conjugation of L96 ligand to the sense 3' terminus and presence of a two-nucleotide overhang at the 3' antisense terminus represent key advancements in the design of RNAi therapeutics that would be utilized extensively in later therapies (**Figure 2**). Several other siRNA drugs that mirror these structural innovations are detailed herein.

Lumasiran (Oxlumo) is a 21-mer/23-mer siRNA that was approved by the FDA in 2020 for the treatment of primary hyperoxaluria type 1 (PH1)²⁶¹(**Table 3**). The antisense strand of lumasiran targets the mRNA of hydroxyacid oxidase 1 (HAO1), hybridizing to the 3'UTR with perfect complementarity and promoting RISC-mediated degradation of the transcript^{261,262}. Knockdown of HAO1 by lumasiran prevents harmful accumulation of calcium oxalate crystals in the urine and plasma, which are overproduced in patients with PH1 and are the underlying cause of resulting organ damage.

Inclisiran (Leqvio) is a 21-mer/23-mer siRNA that was approved by the FDA in 2021 to treat heterozygous familial hypercholesterolemia (HeFH) or existing atherosclerotic cardiovascular disease (ASCVD) (**Table 3**). While originally approved for use alongside statin therapy, its indication was recently expanded for use as a monotherapy²⁶³. Inclisiran targets the 3'UTR of the proprotein convertase subtilisin/kexin type 9 (PCSK9) transcript, binding with perfect complementarity and promoting its RISC-mediated degradation^{183,263-266}.

Vutrisiran (Amvuttra) was approved by the FDA in 2022 to treat polyneuropathy and cardiomyopathy related to hATTR (**Table 3**). Vutrisiran targets the 3'UTR of the TTR transcript with perfect complementarity, promoting its degradation via RNAi^{267,268}. The most recent RNAi therapy to utilize this design strategy is Fitusiran (Qfitlia), which was approved by the FDA in 2025 as a prophylactic treatment for Hemophilia A and B^{269,270} (**Table 3**). Hemophilia A and B are genetic bleeding disorders characterized by insufficient levels of pro-clotting factors VIII and IX respectively. Fitusiran aims to rebalance the coagulation cascade by suppressing antithrombin (AT), a key anticoagulant that mediates hemostasis through inhibition of several enzymes critical to clot formation, including factor IIa (thrombin), factor Xa, and factor IXa. Fitusiran hybridizes to the coding region of the antithrombin transcript with perfect complementarity, promoting its degradation to suppress AT levels and reduce bleeding risk²⁷¹. Clinical efficacy of fitusiran was assessed through annualized bleeding rate (ABR), with integrated analysis of multiple studies showing significant reductions in mean ABR compared to on-demand clotting factor replacement (71%), as well as on-demand and prophylactic treatment with bypassing agents (73%, 70%)²⁷². Fitusiran has not been evaluated in a head-to-head trial against prophylactic antibody treatments like emicizumab, which similarly has a long PD half-life and exhibits sustained suppression of AT

levels. However genetic or acquired deficiency of AT can increase risk of thrombotic events, highlighting the importance of an informed dosing schedule for fitusiran which was optimized to target AT activity levels between 15% and 35% based on monthly patient blood sampling²⁷². Even with a detailed and flexible dosing strategy informed by this additional endpoint, fitusiran carries an FDA black box warning for thrombotic events as well as acute and persistent gallbladder disease²⁶⁹.

These RNAi drugs all similarly consist of a 21-mer/23-mer duplex with a 2-nucleotide antisense overhang, extensive 2'-fluoro and 2'-*O*-methyl substituted ribonucleosides, and a L96-conjugated sense strand (**Figure 2A**). The backbone of each duplex are primarily PO linkages, with PS linkages present on the 5' terminal end of the sense strand and both terminal ends of the antisense strand. Each of these approved siRNA therapeutics undergo some common metabolic pathways, where shortened nucleotide products are generated through endogenous nuclease cleavage (**Figure 2**)²⁷³. While these GalNAc-conjugated siRNA therapies exhibit nontraditional PK/PD profiles distinct from small molecule drugs, their unique ADME properties are predictable and highly conserved across species, aiding efforts translating preclinical findings to humans¹⁸⁴.

On the other hand, Nedosiran (Rivfloza), approved by the FDA in 2023 for the treatment of PH1²⁷⁴⁻²⁷⁶, represents a unique strategy in siRNA design, consisting of a 36-mer/22-mer siRNA duplex (**Table 3**). Nedosiran contains primarily PS linkages, with a sense strand that binds itself in a hairpin-like structure. Rather than utilizing the GalNAc₃ L96-conjugation strategy used in other approved siRNA drugs, Nedosiran uniquely employs adenosine and guanosine nucleobases that are conjugated to GalNAc sugars to enable targeted hepatocyte delivery. Nedosiran binds the

3'UTR of lactate dehydrogenase A (LDHA) mRNA, hybridizing with near-perfect complementarity to promote RISC-mediated degradation of the transcript. Knockdown of the LDHA transcript in the liver reduces lactate dehydrogenase protein levels and subsequent production of oxalate crystals in PH1 patients.

Challenges in the Evaluation of DDI of RNA Drugs

With diverse mechanistic actions, versatile chemistry, and distinct PK properties, RNA therapeutics provide unique strategies that have been leveraged to invaluable treatments for unaddressed diseases but also demand their own tailored strategies in assessing DDI separate from conventional approaches optimized for small molecule and protein drugs. Recent FDA guidance provides recommendations to support the development of oligonucleotide drugs, with considerations specific to this therapeutic modality including assessments of QTc interval prolongation, immunogenic risk, and possible impact of hepatic and renal impairment as well as DDIs^{187,277}. Consistent with traditional therapeutic modalities, single and multiple-dose studies are recommended to assess the PK properties of oligonucleotide drugs in early development and determine resulting target tissue distribution, PD efficacy (e.g., reductions in target mRNA or corresponding protein, biomarker of treatment efficacy specific to disease), and DDI risk. Analytical techniques employed in determining oligonucleotide biodistribution and metabolic fate include quantitative whole body radiography (QWBA), mass-spectrometry based approaches, and stem-loop reverse transcription quantitative polymerase chain reaction^{181,184,186,278,279}. Traditional approaches in preclinical characterization of PK properties and PK-related DDI potential (e.g., assessing their potential as substrates, inhibitors, or inducers of cytochrome P450 enzymes or

major drug transporters hepatocyte, recombinant, or microsomal approaches paired with probe substrates) are generally not major contributors to oligonucleotide drug metabolism, exposure, or DDI²⁸⁰⁻²⁸². Oligonucleotide drugs are instead metabolized to shortened oligonucleotide chains by endogenous endo- and exonucleases that are generally well-conserved between species, reducing the likelihood of human-specific metabolites with potential for toxicity. PK and DDI assessments must consider all components of the therapeutic agent including any conjugated ligands, LNP-based delivery components, and full oligonucleotide molecule²⁸³. This should also include major metabolites of examined drugs, most commonly being the parent oligonucleotide sequence shortened by 1 nucleobase, which may have hybridization-based effects of its own at the intended target or off-targets²⁸⁴.

PK-based DDIs were shown uncommon across oligonucleotide drugs, with adverse events due to organ-specific PD interactions being more typical. Individual classes of RNA therapeutics (aptamers, ASOs, RNAi) and their chemical subgroups often exhibit PK properties and organ distribution very similar to one another, due in large part to the LNP or polymer formulations and ligand-conjugation strategies (e.g., GalNAc) to overcome challenges in RNA delivery and achieve preferential or targeted tissue distribution. The advancement of these delivery strategies and especially GalNAc have provided great benefit in the treatment of liver-based diseases, but there are many therapeutic targets and disease states for which this is not suitable. In these instances, local administration to target tissues using organ-suitable formulations (e.g. IVT administration of aptamers and fomivirsen, IT administration of nusinersen and tofersen) has been utilized. These strategies however carry their own unique challenges in assessing PK/PD relationships within the target tissue and organ-specific off target effects that contribute to DDI risk.

LNP platforms for RNA delivery are customizable, employing several different components that can be functionally optimized to preferentially target the tissue or subcellular compartment of interest. These are often lipid components, polymers, and biochemicals (PEG, lipids, cholesterol), generally well distributed to the liver and kidneys. The utility of LNP formulations extend past the RNA therapies discussed herein, with the mRNA vaccines for COVID-19 being a major example. Therapeutic investigations of miRNA mimics have also utilized LNP and extracellular vesicle-based encapsulation approaches as well as several viral expression vectors – with concern to minimize immunogenic potential in these tools^{77,199}. LNP formulations have also demonstrated their flexibility and utility in clinical trial of small activating RNA (saRNA) drug candidate that target specific transcription factors within the nucleus to promote expression of endogenous therapeutic proteins. One example is MTL-CEPBA, a saRNA that utilizes a liposomal LNP SMARTICLES formulation to achieve saRNA delivery to the nuclear compartment of target myeloid cells²⁸⁵. Customizable LNP formulations use a range of ingredients including biomolecules specific to the target of interest, synthetic polymers, and even metals. While these solutions to achieve tissue- and compartment-specific delivery of RNA drugs might enable treatment of new diseases, the variety of combined excipients utilized in these technologies highlight the importance of assessing all formulation ingredients along with the parent drug early in development to identify potential DDIs and organ-specific toxicities.

While *in vitro* assessment of enzyme and transporter-mediated DDI is necessary for oligonucleotide drugs, those induction or inhibition studies commonly established for small molecule therapeutics may not be sufficient or suitable for RNA medications. Indeed, the clinical

discovery on 2- to 3-fold increase in systemic exposure to dextromethorphan (CYP2D6) and caffeine (CYP1A2) by co-administered givosiran among subjects with acute intermittent porphyria²⁸⁶ was not predicted by *in vitro* findings on its lack of effects on CYP enzymes. The proposed mechanism involving the interference with hepatic heme content due to on-target ALAS1 suppression by givosiran²⁸⁶ cannot explain the selective impact on CYP1A2 and CYP2D6 mediated metabolism versus other CYP3A4, CYP2C9, and CYP2C19 as examined, whereas this notion seems taken in the FDA guidance¹⁸⁷. Given the fact that many miRNAs have been identified to control PTGR of specific ADME genes^{171,287}, an inevitable competition for the RNAi machinery by siRNA drugs may significantly alter the protein levels of particular drug-metabolizing enzymes or transporters, causing possible DDIs **(Figure 3)**^{172,188-190}. Likewise, potential for PD-mediated DDI and adverse events due to on-target activity should also be investigated where appropriate. One example of on-target effects leading to exaggerated PD response is fitusiran, which required additional safety assessment ahead of Phase 3 to optimize dosing schedule and mitigate risk of excessive AT suppression through using patient AT activity levels as an additional endpoint²⁷². This additional study was critical to minimize DDI risk and provide guidance for modified dosing of on-demand factor treatments in the instance of breakthrough bleeds. This serves as an example of how oligonucleotide drugs may increase DDI risk through PD-based effect on their intended target for concomitant drugs that do not share the same target but affect the same physiological pathway.

Oligonucleotide drugs possess distinct features from other modalities and between individual classes of RNA therapies (e.g., nucleobase sequence, molecular size, chemical modifications, conjugation of varying ligands, mechanistic action) that contribute to varying tissue distribution,

plasma protein binding, and potential DDI mechanisms. While aptamer drugs may assess DDI likelihood through off-target protein binding, ASO and siRNA drugs should assess potential of full or partial hybridization interactions with non-target mRNA transcripts as well as other potential endogenous DNA/RNA like mitochondrial and nuclear genomes. In particular, organ-specific off-target PD effects (e.g. miRNA-like seed hybridization at non-target transcripts by siRNA) are critical in overall safety assessments and to determine risk of DDIs with concomitant drugs. Identification and assessment of these miRNA-like interactions at non-target transcripts are routinely performed for siRNA drugs and often included in new drug application filings to the FDA prior to clinical development. It is also worth considering the impacts of RNA therapeutics across multiple organ systems, such as those targeting lipid and lipoprotein pathways (e.g., inclisiran). Modulations of lipid and lipoprotein levels across different organ systems could potentially influence the distribution and exposure of lipophilic drugs. Species differences in pharmacologically relevant tissue function and target sequence must also be addressed, with one useful approach being the use of surrogate RNA molecules possessing appropriate sequence or structure modifications that reflect species differences to allow assessment of equivalent PD endpoint in model organism. Species differences in drug effect and DDI may also be related to differences in adjacent factors within the same pathway or physiological process, as observed in non-human primate studies that over-predicted risk of complement activation from 2'MOE ASO treatment due to inhibition of factor H^{288,289}. Species differences must also be considered in plasma protein binding, RNA binding proteins, and when determining ideal route of administration in nonhuman species to best recapitulate human metabolism and DDI risk²²⁰.

With many RNA drugs being widely distributed and accumulating in the liver and kidneys, early preclinical and Phase 1 efforts must assess the impact of hepatic and renal impairment on PK/PD relationships and resulting DDI safety risk^{265,274,275,290}. Specifically, further assessment in renal impairment is recommended for RNA drugs that are highly cleared by the kidneys (>30%), a common characteristic of many oligonucleotide therapeutics with PS backbones that promote plasma protein binding. With prolonged PD effects and effects on organ function not always reflective of observed PK, PD-based endpoints relevant to the target organ are recommended when possible and provide valuable perspective in establishing dose-response relationships for efficacy and potential DDI¹⁸⁷. Many animal species were reported to exhibit similar oligonucleotide PK properties to humans and are well suited for translational study, providing invaluable information for how target tissue PK relates to sustained PD outcomes. Population PK/PD modeling can be valuable in relating this information to organ-specific toxicity endpoints or other biomarkers to inform proper schedules and ideal doses for first-in-human studies^{182,184,245,284,291}. In general, clinical trial enrollment should emphasize patient inclusion across a substantial range of organ functions when appropriate.

Aforementioned it is critical to assess DDI risk of RNA drugs by considering the general RNAi mechanism shared by the genome derived miRNAs and exogenously introduced siRNAs (or other forms of RNAi agents), i.e., competing for the RNAi machinery that may alter endogenous miRNA function and homeostasis governing the PTGR of ADME and relevant PD genes (**Figure 3**). Endogenous machinery that governs the processes of miRNA duplex unwinding, RISC loading, and RNAi-based regulation may be subject to competition by exogenous RNA molecules. Natural PTGR by endogenous miRNAs is a critical determinant of protein outcomes for both PK and PD

genes^{153,171,287,292}. By competing for critical RNAi machinery integral to RISC function such as AGO proteins, exogenous siRNA administration can disrupt natural PTGR by miRNA (**Figure 3A**)²⁹³. Competition for RNAi machinery by exogenous RNAs can therefore alter the protein expression of miRNA-controlled genes to impact ADME/PK or PD function of co-administered drugs, leading to adverse DDIs^{171,172,189}.

In addition to exogenous RNA-mediated disruption of natural PTGR by miRNA, transcribed or transfected short hairpin RNAs (shRNAs) may also contribute to potential DDI risk through competitive saturation of elements critical to endogenous miRNA biogenesis and transport (**Figure 3B**). One such element is exportin-5 (XPO5), a protein that mediates the nuclear export of precursor miRNAs (pre-miRNAs) and is critically important to miRNA biogenesis. Without functional XPO5 available to facilitate this transport, pre-miRNAs accumulate in the nucleus, unable to be processed to their mature miRNA duplexes by the cytoplasmic ribonuclease Dicer²⁹⁴⁻²⁹⁶. Nuclear export by XPO5 and cytoplasmic processing by Dicer are critical steps in endogenous miRNA biogenesis, necessary for the natural PTGR of cytoplasmic miRNA targets. ShRNAs expressed within the nucleus are also substrates of XPO5, competing with endogenous pre-miRNAs for nuclear export (**Figure 3B**). XPO5-mediated nuclear shuttling is a rate-limiting step in endogenous miRNA biogenesis, with limited XPO5 protein being susceptible to competitive saturation by nuclear shRNA²⁹⁷. Competitive saturation of XPO5 and resulting global disruption of miRNA biogenesis has significant consequences in terms of toxicities and DDI risk, demonstrated across multiple mammalian species and organ systems²⁹⁸⁻³⁰¹. In addition to competitive saturation of XPO5, alteration of XPO5 activity through phosphorylation or other processes must also be considered in assessing potential toxicity and DDI risk of RNA therapies³⁰².

In addition to these risks posed by competitive saturation or phosphorylation of XPO5, exogenous RNA molecules may also disrupt miRNA biogenesis by competing for key cytoplasmic enzyme Dicer. Many RNA molecules may be substrates of Dicer, including shRNAs exported from the nucleus as well as transfected synthetic shRNAs or siRNAs (**Figure 3B**). While the predominant 21-mer/23-mer duplex design strategy employed in approved RNAi therapeutics (**Figure 2 and Table 3**) are not canonical substrates of Dicer, this is a critical factor that must be addressed when assessing DDI risk of new oligonucleotide drug candidates. Considering this critical impact on endogenous miRNA abundance and function, new RNA therapies must be assessed for potential XPO5/Dicer competition and modulation to minimize DDI risk and ensure treatment tolerability.

Assessment of DDI potential related to the disruption of endogenous small RNA pathways may also extend to other classes such as PIWI-interacting RNAs (piRNAs), which are expressed in germline cells across many animal species. This family of noncoding RNAs act in concert with PIWI proteins, impacting biological function and even drug resistance through several mechanisms including the silencing of transposable elements and epigenetic modifications. Both PIWI and AGO proteins are part of the same evolutionarily conserved family, participating in critical processes underlying the function of endogenous piRNAs, miRNAs, as well as siRNA drugs^{303,304}. To support assessments of DDI risk due to perturbation of these pathways, further advancements in our research tools to investigate the functions and spatiotemporal characteristics of related RNA/protein complexes are needed³⁰⁵⁻³⁰⁷. Current approaches including cross-linking immunoprecipitation (CLIP) and fluorescence in situ hybridization (FISH) have been applied to interrogate ncRNA/AGO protein complexes but still carry significant challenges in spatial or temporal resolution, necessitating rigorous fractionation controls to interrogate nuclear vs

cytoplasmic interaction, for example. Additionally, UV cross-linking methods applied in CLIP and its variations may produce results that are not always representative of true cellular RNA/protein interactions, stabilizing complexes that may in fact be transient interactions. FISH methods also exhibit their own challenges, providing valuable spatial evidence for RNA but requiring concurrent immunofluorescent imaging for the protein of interest. While co-localization of these signals can provide evidence of RNA/protein proximity, this does not necessitate complex formation and can be challenging for ubiquitous proteins like AGO2 due to high background fluorescence. As siRNA therapies and their delivery strategies advance to tackle new targets and reach intracellular compartments specific to their mechanistic function, so too must our tools to interrogate the spatial and temporal aspects of these drugs and their critical partners like the AGO/PIWI family. Innovations in these methods will be critical to assess the potential impacts and DDIs of oligonucleotide drugs related to endogenous RNA pathways moving forward.

Another way oligonucleotide drugs could affect the abundance of endogenous noncoding RNAs is through disruption of target directed miRNA degradation (TDMD), which depends on AGO proteins and ubiquitin ligases like ZSWIM8 to regulate overall miRNA levels^{308,309}. Although siRNAs are usually protected from these pathways because they are designed for preferential AGO2 loading³¹⁰⁻³¹³, therapeutic concentrations could possibly saturate these shared systems. This competition could disrupt the natural degradation of endogenous miRNA, potentially altering the expression of the many genes they regulate. While the inherent stability of AGO2-bound siRNAs benefits their duration of action, it also prolongs the potential for interference with endogenous miRNA function. Continued efforts in RNAi therapeutics must prioritize the design of candidates

that minimize the risk of pathway competition and make efforts to ensure their effects on the endogenous miRNA network do not become a source of adverse DDIs.

As such, it is recommended to assess the risk of DDI for RNA therapeutics by employing relevant preclinical model systems (e.g., human hepatocytes) to directly examine the effects of RNAi drugs on the activities of important drug-metabolizing enzymes and transporters (e.g., relevant probes or target drugs to be co-administered) as well as related therapeutic targets, or their protein levels. It is also noteworthy that the apparent impact could be suppression of ADME/PK/PD gene expression (protein levels; and thus function) instead of so called “induction” of enzymes or transporters applied to small molecule drugs. The latter is usually a result of the activation of nuclear receptors or transcription factors upon ligand binding, whereas oligonucleotides are unlikely to act on the ligand binding sites directly, with some chances to interfere with DNA binding domains theoretically. On the other hand, small molecule drugs have been shown to alter the levels of specific miRNAs in cells (e.g., streptomycin on miR-21, and methadone on miR-124) through interfering with miRNA biogenesis machinery or RNAi elements^{314,315}, indicating the needs for evaluating possible DDIs with appropriate assays in proper preclinical models where the RNAi drugs are the victims of co-administered drugs. Rather, clinical investigations properly designed and completed would provide direct evidence regarding to what degrees the ADME/PK/PD or safety profiles of co-administered drugs are altered by RNA therapeutics, and *vice versa*, among patients.

Conclusions and Perspectives

Fundamental scientific advancements pave the way for innovative therapeutic approaches, expanding the molecular toolkit of drug design to reach targets previously thought impossible. This is exemplified by the emergence of RNA or oligonucleotide therapeutics, which can harness our intrinsic biological machinery to manage human disease in novel ways and greatly improve the lives of underserved patient populations. With these innovations, however, also comes a new layer of complexity and many unanswered questions that must be addressed if we are to predict DDIs and ensure the safety of this promising treatment modality.

The distinct structural characteristics and functionalities of oligonucleotide drugs that make them uniquely useful therapeutic tools also present unique challenges in assessing their ADME/PK properties such as plasma protein binding, metabolic stability, biotransformation, and tissue distribution, requiring a specialized set of analytical techniques and risk assessment strategies. Traditional approaches utilized in the development of small molecule and protein drugs are not sufficient to predict DDI risk or elucidate PK/PD relationships for oligonucleotide drugs, with recent FDA guidance providing some recommendations. Valuable advancements have been made in the strategies to assess DDI risk of aptamers, ASOs, and siRNAs based on these features, as well as based on their distinct mechanisms of action and PD effects. However, an overlooked element is that RNA therapeutics would likely compete with endogenous miRNAs for the intrinsic RNAi components and thus alter the protein levels of miRNA-regulated ADME/PK/PD genes, leading to possible DDIs. This warrants more transparent communications among regulatory agencies and pharmaceutical industries along with neutral academic communities to provide further clarity in proper identification of the risks and avoid unwanted DDIs of novel RNA therapeutics with co-administered medications.

Tables

Table 1. FDA-approved aptamer drugs. Abbreviations: (3'~3')dT, inverted thymidine; A, adenosine; Am, 2'-*O*-methyladenosine; AMD, age-related macular degeneration; C, cytidine; C5, complement component 5; Cf, 2'-fluorocytidine; d, deoxyribonucleoside; G, guanosine; GA, geographic atrophy; Gm, 2'-*O*-methylguanosine; *IVT*, intravitreal; PEG, polyethylene glycol; PO (~), phosphodiester linkage; *RoA*, route of administration; T, thymidine; U, uridine; Uf, 2'-fluorouridine; VEGF, vascular endothelial growth factor. In the absence of any other label, 2'-deoxynucleosides are assumed.

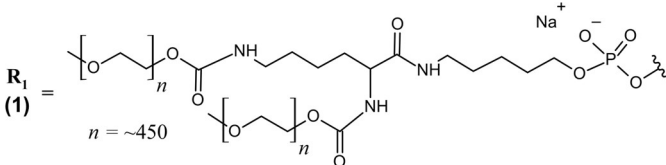
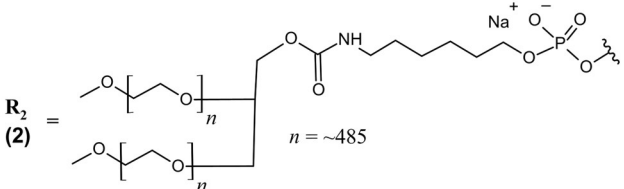
Aptamer drug <i>RoA</i>	Chemistry	Target Protein	Indication	Year approved
Pegaptanib (Macugen®) <i>IVT</i>	PEGylated 28-nt RNA aptamer; all PO (~) linkages; NaCl/NaPO₄ solution for <i>IVT</i> injection; MW = ~50 kDa (sodium salt bound to two 20 kDa PEG moieties) R₁ — 5'-Cf~Gm~Gm~A~A~Uf~Cf~Am~Gm~Uf~Gm~Am~Am~Uf~Gm~Cf~Uf~Uf~Am~Uf~Am~Cf~Am~Uf~Cf~Cf~Gm~(3'~3')dT-5' R₁ (1) = 	VEGF	AMD	2004
Avacincaptad pegol (Izervay™) <i>IVT</i>	PEGylated 39-nt RNA aptamer; all PO (~) linkages; NaCl/NaPO₄ solution for <i>IVT</i> injection; MW = 56 kDa (free acid bound to one 43 kDa PEG moiety) R₂ — 5'-Cf~Gm~Cf~Cf~G~Cf~Gm~Gm~Uf~Cf~Uf~Cf~Am~Gm~Gm~Cf~G~Cf~Uf~Gm~Am~Gm~Uf~Cf~Uf~Gm~Am~Gm~Uf~Uf~Uf~A~Cf~Cf~Uf~Gm~Cf~Gm~(3'~3')dT-5' R₂ (2) = 	Complement protein C5	GA/AMD	2023

Table 2. FDA-approved ASO drugs. Abbreviations: *, 2'-O-(2-methoxyethyl) nucleoside; aCSF, artificial cerebrospinal fluid; Apo B-100, apolipoprotein B-100; Apo C-III, apolipoprotein C-III; ALS, amyotrophic lateral sclerosis; ASO, antisense oligonucleotide; DMD, Duchenne muscular dystrophy; GalNAc, N-acetylgalactosamine; CMV IE2, cytomegalovirus immediate early region 2; FCS, familial chylomicronemia syndrome; HAE, hereditary angioedema; hATTR, hereditary transthyretin-mediated amyloidosis; hATTR-PN, hereditary transthyretin-mediated amyloidosis with polyneuropathy; HoFH, homozygous familial hypercholesterolemia; ISS-N1, intronic splicing silencer N1; *Italicized*, 3'-amino-3'-deoxy nucleoside; *IT*, intrathecal; *IV*, intravenous; *IVT*, intravitreal; m⁵C, 5-methylcytosine; m⁵U, 5-methyluridine; MDS, myelodysplastic syndromes; PBS, phosphate buffered saline; PKK, prekallikrein PMO, phosphorodiamidate morpholino oligomer; PN (»), phosphorodiamidate linkage; PO (~), phosphodiester linkage; PS (-), phosphorothioate linkage; *RoA*, route of administration; SMA, spinal muscular atrophy; SOD1, superoxide dismutase 1; *SQ*, subcutaneous; THA, tris-hexylamino linker; TTR, transthyretin; underlined, morpholino nucleoside; WFI, water for injection. In the absence of any other label, 2'-deoxynucleosides are assumed. Molecular weights in kDa were rounded to the nearest thousandth.

ASO Drug <i>RoA</i>	Chemistry	Target RNA	Indication	Year approved
Fomivirsen (Vitravene®) <i>IVT</i>	21-mer ASO; PS (-) linkages; carbonate-buffered saline solution for <i>IVT</i> injection; MW = 7.122 kDa (sodium salt) 5'-dG-dC-dG-dT-dT-dT-dG-dC-dT-dC-dT-dT-dC-dT-dT-dG-dC-dG-3'	CMV IE2 mRNA	CMV retinitis	1998
Mipomersen (Kynamro®) <i>SQ</i>	20-mer ASO Gapmer (5*-10-5*); PS (-) linkages; unbuffered aqueous solution for <i>SQ</i> injection; MW = 7.595 kDa (sodium salt) 5'-G*-m ⁵ C*-m ⁵ C*-m ⁵ U*-m ⁵ C*-dA-dG-dT-m ⁵ dC-dT-dG-m ⁵ dC-dT-dT-m ⁵ dC-G*-m ⁵ C*-A*-m ⁵ C*-m ⁵ C*-3'	Apo B-100 mRNA	HoFH	2013
Nusinersen (Spinraza®) <i>IT</i>	18-mer ASO; PS (-) linkages; aCSF solution for <i>IT</i> injection; MW = 7.501 kDa (sodium salt) 5'-m ⁵ U*-m ⁵ C*-A*-m ⁵ C*-m ⁵ U*-m ⁵ U*-m ⁵ U*-m ⁵ C*-A*-m ⁵ U*-A*-A*-m ⁵ U*-G*-m ⁵ C*-m ⁵ U*-G*-G*-3'	SMN2 pre-mRNA (ISS-N1)	SMA	2016
Inotersen (Tegsedi®) <i>SQ</i>	20-mer ASO Gapmer (5*-10-5*); PS (-) linkages; unbuffered aqueous solution for <i>SQ</i> injection; MW = 7.601 kDa (sodium salt) 5'-m ⁵ U*-m ⁵ C*-m ⁵ U*-m ⁵ U*-G*-dG-dT-dT-dA-m ⁵ dC-dA-dT-dG-dA-dA-A*-m ⁵ U*-m ⁵ C*-m ⁵ C*-m ⁵ C*-3'	TTR mRNA	hATTR	2018
Eteplirsen (Exondys 51®) <i>IV</i>	30-mer <u>PMO</u> ; PN (») linkages; PBS concentrate diluted in saline for <i>IV</i> infusion; MW = 10.306 kDa <u>R</u> » 5'-C»T»C»C»A»A»C»A»T»C»A»A»G»G»A»A»G»A»T»G»G»C»A»T»T»T»C» T»A»G-3' <u>R</u> (3) =	Dystrophin pre-mRNA (Exon 51)	DMD (Exon 51)	2016
Golodirsen (Vyondys 53™) <i>IV</i>	25-mer <u>PMO</u> ; PN (») linkages; PBS concentrate diluted in saline for <i>IV</i> infusion; MW = 8.647 kDa <u>R</u> » 5'-G»T»T»G»C»C»T»C»C»G»G»T»T»C»T»G»A»A»G»G»T»G»T»T»C-3'	Dystrophin pre-mRNA (Exon 53)	DMD (Exon 53)	2019

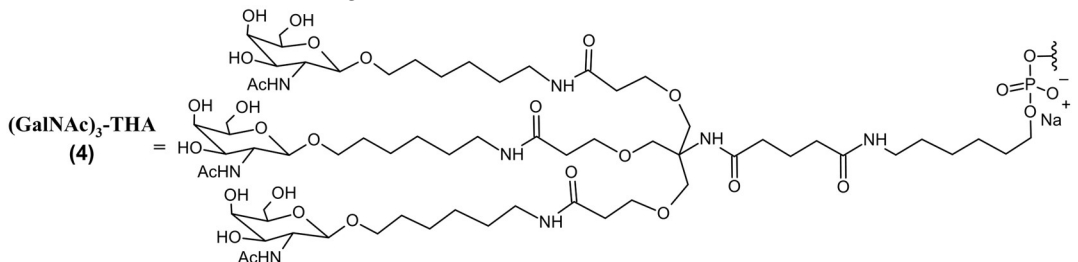
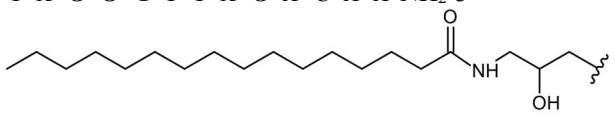
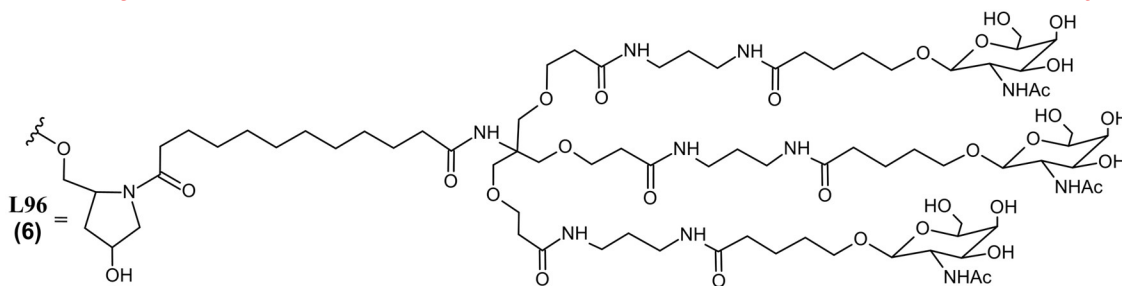
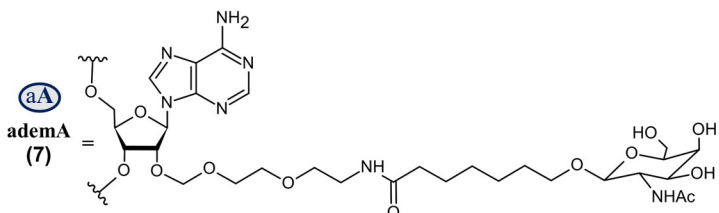
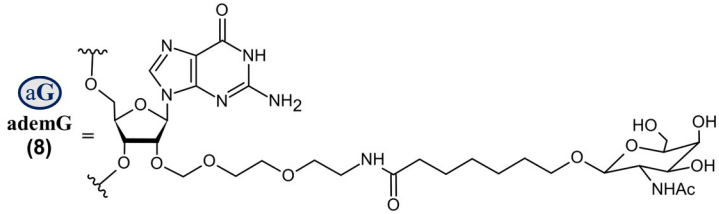
Casimersen (Amondys 45™) <i>IV</i>	22-mer PMO; PN (») linkages; PBS concentrate diluted in saline for <i>IV</i> infusion; MW = 7.585 kDa R » 5'-C»A»A»T»G»C»C»A»T»C»C»T»G»G»A»G»T»C»C»T»G-3'	Dystrophin pre-mRNA (Exon 45)	DMD (Exon 45)	2021
Viltolarsen (Viltepso™) <i>IV</i>	21-mer PMO; PN (») linkages; saline solution for <i>IV</i> infusion; MW = 6.925 kDa 5'-C»C»T»C»C»G»G»T»T»C»T»G»A»A»G»G»T»G»T»T»C-3'	Dystrophin pre-mRNA (Exon 53)	DMD (Exon 53)	2020
Tofersen (Qalsody™) <i>IT</i>	20-mer ASO Gapmer (5*-10-5*); PO (~) and PS (-) linkages; aCSF solution for <i>IT</i> injection; MW = 7.127 kDa 5'-m ⁵ C*-A*-G*-G*-A*-dT-dA-m ⁵ dC-dA-dT-dT-dT-m ⁵ dC-dT-dA-m ⁵ C*-A*-G*-m ⁵ C*-m ⁵ U*-3'	SOD1 mRNA	SOD1-ALS	2023
Eplontersen (Wainua™) <i>SQ</i>	20-mer GalNAc-linked ASO Gapmer (5*-10-5*); PO (~) and PS (-) linkages; PBS solution for <i>SQ</i> injection; MW = 9.046 kDa (sodium salt) (GalNAc) ₃ -THA~ 5'-m ⁵ U*-C*~m ⁵ U*~m ⁵ U*~G*~dG-dT-dT-dA- m ⁵ dC-dA-dT-dG-dA-dA-A*~m ⁵ U*~m ⁵ C*~m ⁵ C*~m ⁵ C*-3' 	TTR mRNA	hATTR-PN	2023
Imetelstat (Rytelo™) <i>IV</i>	13-mer 3'-amino-3'-deoxy ASO; PS (-) linkages; lyophilized solid reconstituted in saline for <i>IV</i> infusion; MW = 4.896 kDa (sodium salt) R_p - 5'-T-A-G-G-G-T-T-A-G-A-C-A-A-NH ₂ -3' R_p (5) = 	Telomerase template RNA	MDS with transfusion-dependent anemia	2024
Olezarsen (Tryngolza™) <i>SQ</i>	20-mer GalNAc-linked ASO Gapmer (5*-10-5*); PO (~) and PS (-) linkages; PBS solution for <i>SQ</i> injection; MW = 9.124 kDa (sodium salt) (GalNAc) ₃ -THA~ 5'-A*-G*-m ⁵ C*-m ⁵ U*-m ⁵ U*-m ⁵ dC-dT-dT-dG-dT-m ⁵ dC-m ⁵ dC-dA-dG-m ⁵ dC-m ⁵ U*-m ⁵ U*-m ⁵ U*-A*-m ⁵ U*-3'	Apo C-III mRNA	FCS	2024
Donidalorsen (Dawnzera™) <i>SQ</i>	20-mer GalNAc-linked ASO Gapmer (5*-10-5*); PO (~) and PS (-) linkages; PBS solution for <i>SQ</i> injection; MW = 9.112 kDa (sodium salt) (GalNAc) ₃ -THA~ 5'-m ⁵ U*-G*-m ⁵ C*~A*~A*~dG-dT-m ⁵ dC-dT-m ⁵ dC-dT-dT-dG-dG-m ⁵ dC-A*~A*~A*~m ⁵ C*-A*3'	PKK mRNA	HAE	2025

Table 3. FDA-approved siRNA drugs. Abbreviations: A, adenosine; AdemA, GalNAc aminosugar conjugated adenosine; AdemG, GalNAc aminosugar conjugated guanosine; Af, 2'-fluoroadenosine; AHP, acute hepatic porphyria; ALAS1, delta-aminolevulinate synthase 1; ASCVD, atherosclerotic cardiovascular disease; AT, antithrombin; CM, cardiomyopathy; Cm, 2'-*O*-methylcytidine; Gf, 2'-fluoroguanosine; Gm, 2'-*O*-methylguanosine; HAO1, hydroxyacid oxidase 1; hATTR, hereditary transthyretin-mediated amyloidosis; HeFH, heterozygous familial hypercholesterolemia; *IV*, intravenous; L96, tri-*N*-acetylgalactosamine (GalNAc); LDHA, lactate dehydrogenase A; LNP, lipid nanoparticle; PBS, phosphate buffered saline; PCSK9, proprotein convertase subtilisin/kexin type 9; PH1, primary hyperoxaluria type 1; PS (–), phosphorothioate linkage; *RoA*, route of administration; *SQ*, subcutaneous; TTR, transthyretin; Um, 2'-*O*-methyluridine; Um[^], 2'-*O*-methyl-4'-*O*-((methoxy)phosphoryl)methyluridine; WFI, water for injection. Sense (blue) and antisense (red) strand sequences were obtained from FDA approved product inserts. Vertical lines illustrate Watson-Crick base pairing. Molecular weights in kDa were rounded to the nearest thousandth.

siRNA Drug <i>RoA</i>	Chemistry	Target mRNA	Indication	Year approved
Patisiran (Onpattro™) <i>IV</i>	21-mer/21-mer siRNA; PO (~) linkages; LNP formulation for <i>IV</i> infusion; MW = 14.304 kDa Sense: 5'-G~Um~A~A~Cm~Cm~A~A~G~A~G~Um~A~Um~Um~Cm~Cm~A~Um~dT~dT-3' Antisense: 3'-dT~dT~C~A~Um~U~G~G~U~U~C~U~C~A~Um~A~A~G~G~U~A-5'	TTR	hATTR	2018
Givosiran (Givlaari™) <i>SQ</i>	21-mer/23-mer siRNA; Only PS (–) linkages illustrated, all others PO (~); unbuffered aqueous solution for <i>SQ</i> injection; MW = 17.246 kDa (sodium salt) Sense: 5'-Cm~Am~Gm~Am~Am~Gf~Am~Gf~Um~Gf~Um~Cf~Um~Cf~Am~Um~Cm~Um~Um~Am~L96-3' Antisense: 3'-Um~Gm~Gm~Uf~Cm~Uf~Um~Uf~Cm~Uf~Cm~Af~Cm~Af~Gm~Af~Gm~Uf~Am~Gf~Af~Af~Um-5'	ALAS1	AHP	2019
Lumasiran (Oxlumo™) <i>SQ</i>	21-mer/23-mer siRNA; Only PS (–) linkages illustrated, all others PO (~); unbuffered aqueous solution for <i>SQ</i> injection; MW = 17.286 kDa (sodium salt) Sense: 5'-Gm~Am~Cm~Um~Um~Cf~Am~Uf~Cf~Cf~Um~Gm~Gm~Am~Am~Am~Um~Am~Um~Am~L96-3' Antisense: 3'-Am~Cm~Cm~Um~Gm~Am~Am~Af~Gm~Uf~Am~Gm~Gm~Am~Cf~Cf~Um~Uf~Um~Am~Um~Af~Um-5'	HAO1	PH1	2020



Inclisiran (Leqvio®) <i>SQ</i>	21-mer/23-mer siRNA; Only PS (–) linkages illustrated, all others PO (~); unbuffered aqueous solution for <i>SQ</i> injection; MW = 17.285 kDa (sodium salt)			
	Sense: 5'-Cm-Um-AmGmAmCmCf UmGfUm dT UmUmGmCmUmUmUmUm Gm Um-L96-3' Antisense: 3'-Am-Am-Gm Am Um Cf UmGfGmAf CmAf Am Af Am Cf Gm Af Af Af Am-Cf-Am-5'	PCSK9	HeFH + ASCVD	2021
Vutrisiran (Amvuttra®) <i>SQ</i>	21-mer/23-mer siRNA; Only PS (–) linkages illustrated, all others PO (~); PBS solution for <i>SQ</i> injection; MW = 17.290 kDa (sodium salt)			
	Sense: 5'-Um-Gm-GmGmAmUmUf Um Cf Af Uf GmUmAmAmCm CmAmAm Gm Am-L96-3' Antisense: 3'-Cm-Um-AmCm CmCmUm Af AmAf GmUmAmCmAf UmUmGf GmUmUm-Cf-Um-5'	TTR	hATTR CM + PN	2022
Nedosiran (Rivfloza®) <i>SQ</i>	36-mer/22-mer siRNA; All PS (–) linkages illustrated, all others PO (~); unbuffered aqueous solution for <i>SQ</i> injection; MW = 22.238 kDa (sodium salt)			
	Sense: 5'-Am-UmGfUmUfGmUmCf Cf Uf UfUmUfUmAfUmCf UmGm Am ~~~ Gm Cm AmGmCmCm (aG) (aA) Antisense: 3'-Gm-Gm-Um AmCfAmAfCm AfGmGmAmAfAfAmAf UmAfGf-Af-Cf-Um-5' 3'-CmGmUmCmGmGm (aA) (aA)			
	 	LDHA	PH1	2023
Fitusiran (Qfitlia®) <i>SQ</i>	21-mer/23-mer siRNA; Only PS (–) linkages illustrated, all others PO (~); PBS solution for <i>SQ</i> injection; MW = 17.193 kDa (sodium salt)			
	Sense: 5'-Gf-Gm-Uf Um Af AmCfAmCf Cf Af UmUf UmAf CmUf UmCf Am Af-L96-3' Antisense: 3'-Gm-Am-Cm Cf AmAf Um UfGmUfGmGmUmAf AmAf UmGf AmAf Gm-Uf-Um-5'	AT	Hemophilia A + B	2025

Figures and Legends

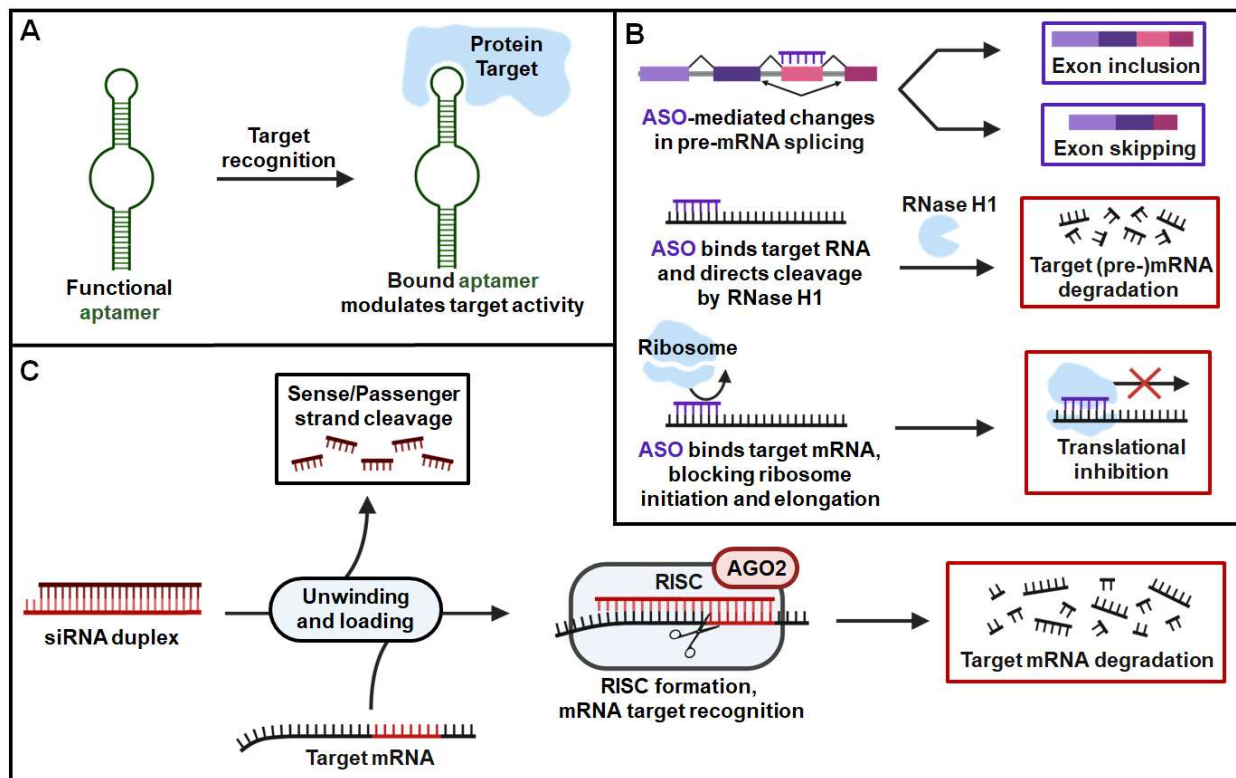


Figure 1. General pharmacological actions of FDA-approved RNA therapeutics. (A) Aptamers are single-stranded oligonucleotides that fold into unique structures to specifically bind their protein target and modulate its activity. (B) ASOs are short, single-stranded oligonucleotides that target specific RNA sequences via Watson-Crick base pairing and alter expression of their corresponding protein. ASOs can modulate target expression by several distinct mechanisms such as alternative splicing of pre-mRNA, induction of RNase H1-dependent cleavage of target RNA, and inhibition of target RNA translation through steric blockage of ribosome interaction. (C) SiRNAs are double stranded RNAs containing a guide/antisense strand designed to target a specific mRNA sequence by (near-)perfect complementary base pairing with target transcript. After the siRNA is unwound, the antisense strand is uploaded into the RISC where it identifies and cleaves complementary target mRNA sequence to modulate protein outcome.

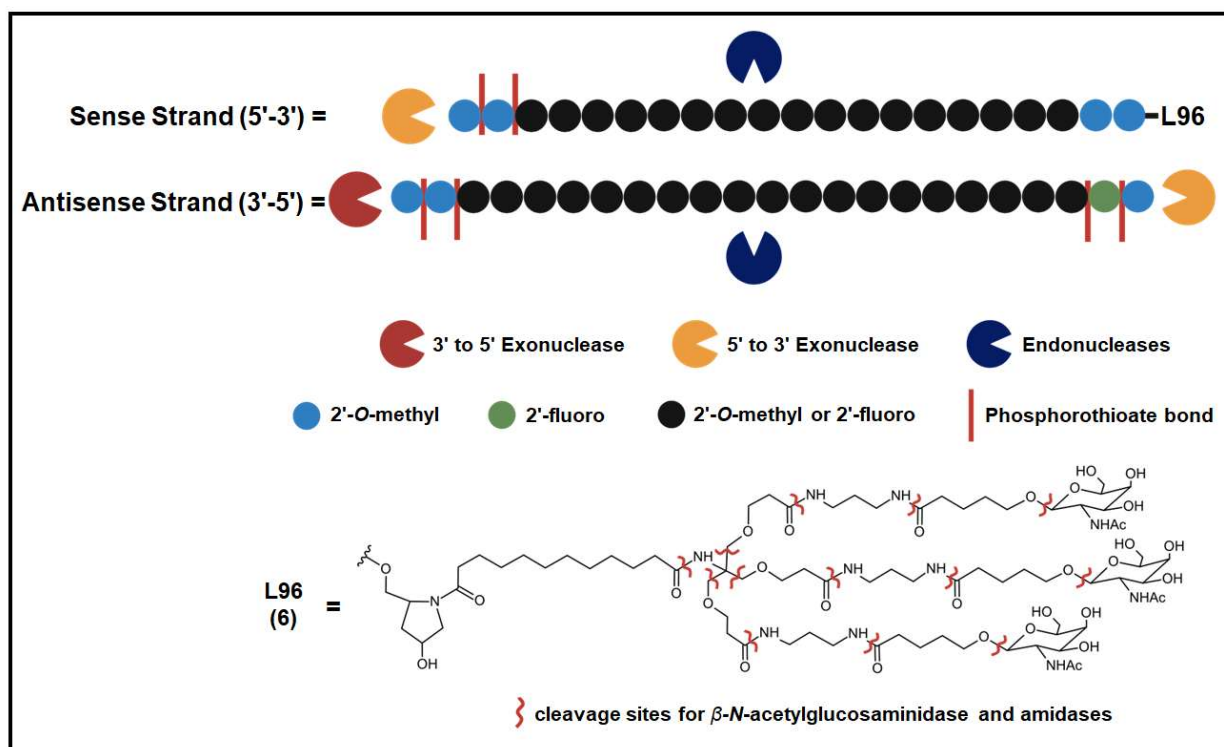


Figure 2. Common metabolic pathways of GalNAc-conjugated siRNA drugs. Some endogenous nucleases may cleave the sense and antisense strands at unique locations while exonucleases metabolize an RNA strand from the 5' or 3' end. Illustrated chemical modifications of ribonucleosides and the linkages are critical in preventing nuclease degradation, and they are commonly found amongst the FDA-approved siRNA drugs. GalNAc conjugation of the sense 3' terminus is widely employed to improve nuclease resistance and hepatocyte uptake, found among five out of seven FDA-approved siRNA drugs. L96 and similar GalNAc moieties, however, are subject to metabolism by β -N-acetylglucosaminidase and amidases. These enzymes can cleave several points within the L96 structure, giving rise to a variety of metabolites possessing different lengths and remaining number of GalNAc moiety.

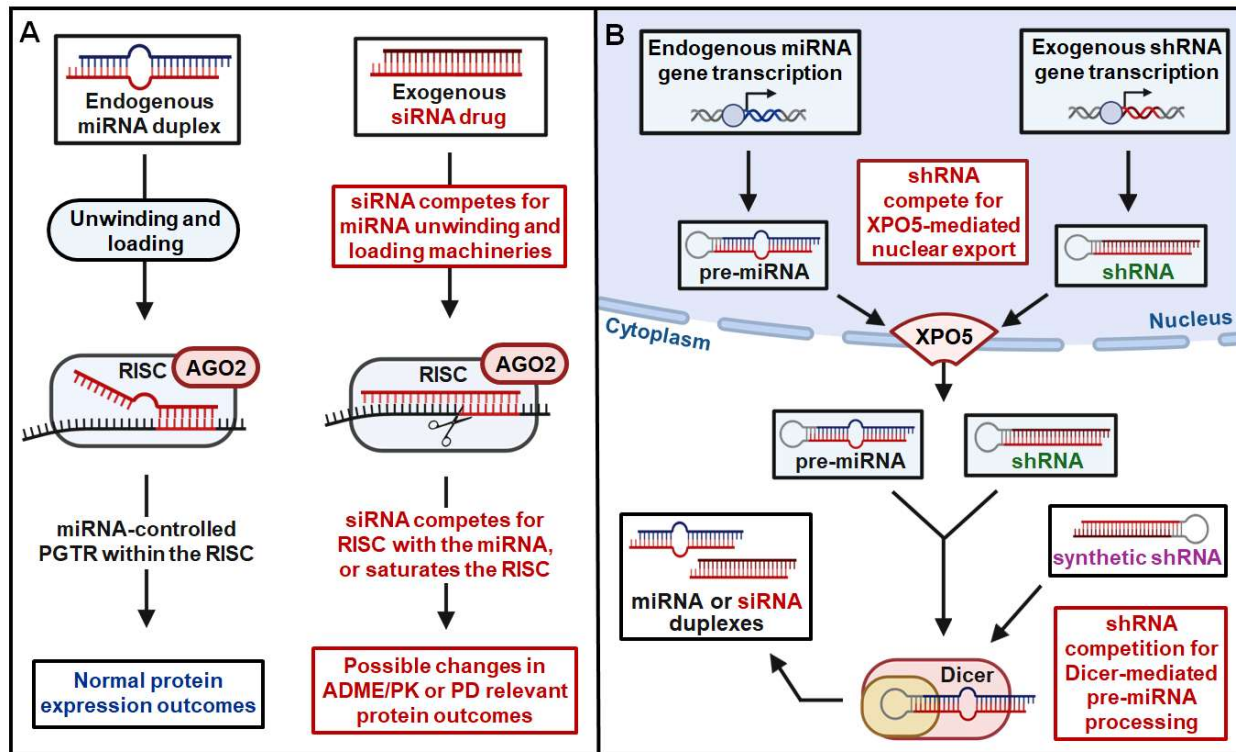


Figure 3. RNAi drugs may lead to risk of DDI through competition for endogenous RNAi machinery. (A) Competition with endogenous miRNAs for the unwinding, loading, and slicing/RISC components by siRNAs within the cytoplasm may alter the PTGR of ADME/PK or PD genes to cause possible DDI. (B) A shRNA, expressed within the nucleus or introduced directly into the cytoplasm, may compete with pre-miRNAs for the XPO5-mediated intracellular translocation or Dicer-mediated miRNA biogenesis, as well as the subsequent processes shown in panel (A), leading to potential DDI.

Chapter 3: Comparative Effectiveness of 7 Major Human let-7-5p Isoforms to Modulate Target Gene Expression in Liver Cell

Abstract

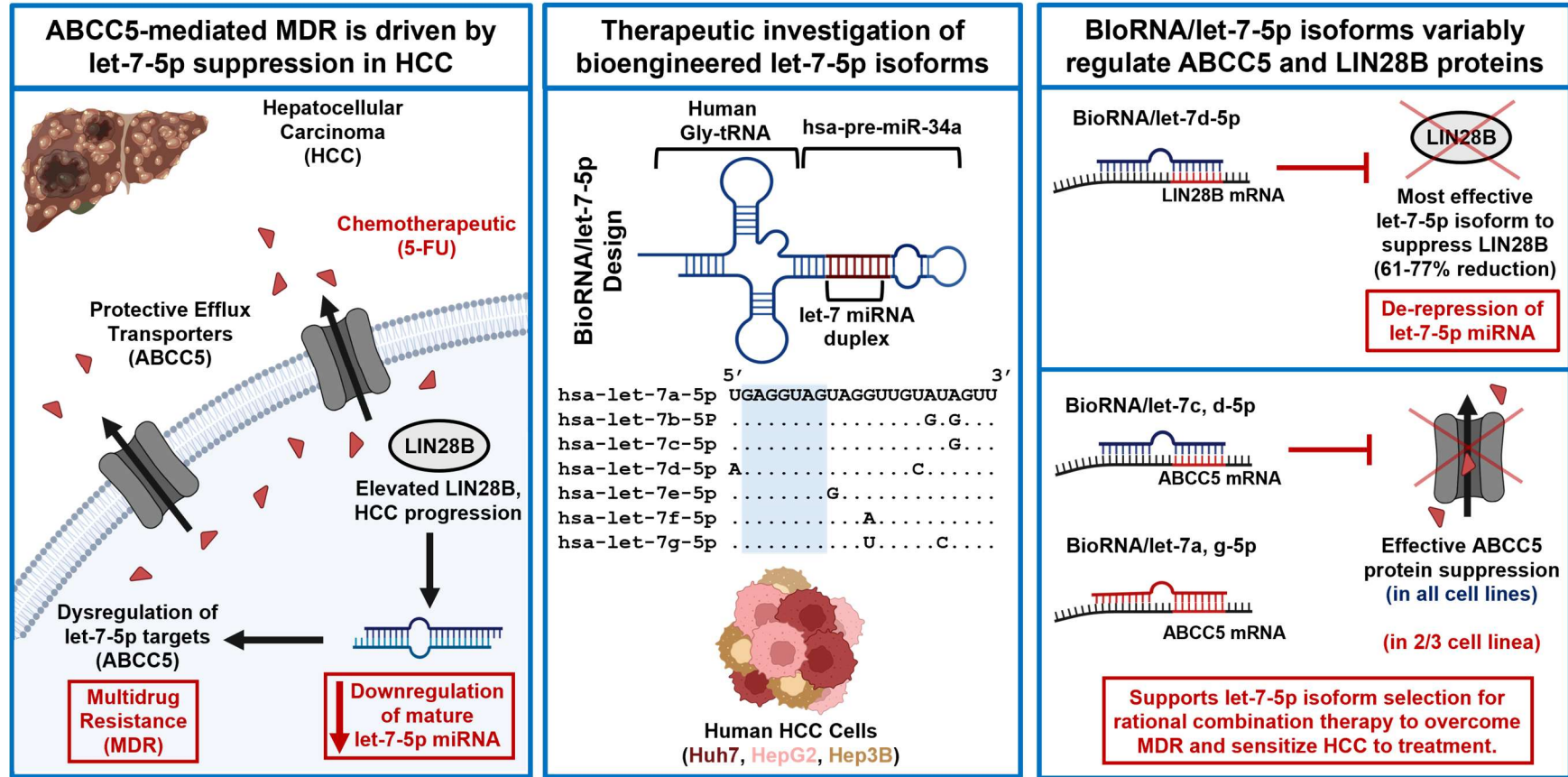
Dysregulation of efflux ABC transporters often confers multidrug resistance (MDR), presenting significant challenges in treating various diseases (e.g., hepatocellular carcinoma (HCC)). The let-7-5p microRNAs (miRNAs), commonly downregulated in HCC, have established roles in controlling post-transcriptional gene regulation of ABC transporters (e.g., multidrug resistance-associated protein 5 MRP5/ABCC5) and some oncogenes (e.g., RNA-binding protein LIN28B). Although previous research has demonstrated the potential of particular let-7-5p isoforms to regulate ABC transporters and inhibit HCC cell viability, the comparative efficacy of let-7-5p isoforms whose sequences differ in several nucleosides is unknown. This study was to compare effectiveness of 7 major let-7-5p isoforms (let-7a to let-7g) to regulate ABCC5 and LIN28B targets and inhibit HCC cell viability *in vitro* by using novel bioengineered RNA let-7-5p (BioRNA/let-7-5p) agents. Release of let-7-5p isoforms from individual BioRNA/let-7-5p molecules in Huh7, HepG2, and Hep3B cells was validated. Efficacy of BioRNA/let-7-5p isoforms to repress ABCC5/MRP5 and LIN28B protein levels was found to be target dependent, among them let-7c and 7d-5p exhibited broader regulatory efficacy against ABCC5/MRP5 while let-7d-5p emerged as the most potent suppressor of LIN28B, generally in accordance with let-7-5p abundance and target complementarity. By contrast, let-7-5p isoforms showed minimal impact on ABCC2/MRP2 and ABCC4/MRP4 protein levels. In addition, let-7-5p isoforms showed variable efficacy to inhibit the viability of different HCC cells. Together, our studies established the functional differences of let-7-5p isoforms in regulating target gene expression and inhibiting HCC cell viability, providing insights into intrinsic differences of miRNA isoforms to inform rational development of miRNA therapeutics or combination therapy.

Significance Statement

Using novel bioengineered RNA agents, this study established the functional differences of seven major human let-7-5p isoforms to control target gene expression and hepatocellular carcinoma cell viability *in vitro*. These findings demonstrate the potential of bioengineered RNA molecules to interrogate posttranscriptional gene regulation mechanisms, highlighting specific let-7-5p isoforms to modulate transporter and oncogene expression toward development of improved therapies.

Graphical Abstract

Comparative effectiveness of multiple human let-7-5p isoforms to modulate target gene expression in liver cells



Introduction

Multidrug resistance (MDR) is a critical challenge in anticancer and antimicrobial therapies, hindering the successful treatment of hepatocellular carcinoma (HCC), the most prevalent form of liver cancer³¹⁶. In HCC, MDR is often driven by the overexpression of efflux ATP-binding cassette (ABC) transporters or multidrug resistance-associated proteins (MRP), including ABCB1/MDR1, ABCC1-5/MRP1-5, and ABCC10/MRP7³¹⁷. These transporters allow cancer cells to pump out many chemotherapeutic drugs, reducing intratumoral drug exposure and thus therapeutic efficacy³¹⁸⁻³²⁰. Overexpression of these transporters is a hallmark of chemo-resistant HCC and has been linked to poor survival and therapeutic evasion, posing a major challenge for current HCC therapies³²⁰. Considering these challenges, creative interventions to overcome MDR are urgently needed for improving the treatment of HCC.

To develop effective therapies against resistant cancers, it is crucial to understand the underlying factors contributing to variable drug responses. Administered drugs are subject to absorption, distribution, metabolism, and excretion (ADME) processes that dictate a patient's exposure to the parent drug and its metabolites. Dysregulation of ADME processes in cancer can alter exposure to administered therapeutics, often resulting in reduced efficacy or likelihood of toxicity. Elucidating the factors contributing to aberrant ADME function, including transporter-mediated MDR common to HCC, provides valuable insights for drug development. Although factors like genetic polymorphisms and nuclear receptor activation have well-appreciated effects on ADME gene families, an important but often under-appreciated factor is the post-transcriptional gene regulation (PTGR) of ADME-related proteins by functional microRNAs (miRNAs) derived from the

genome^{171,320}. MiRNAs are double-stranded noncoding RNAs that function as key mediators of gene expression, controlling the protein outcomes during translation through interacting with specific miRNA response elements (MREs) within the 3'-untranslated region (3'UTR) of target mRNA transcripts³²¹. In particular, one strand of the miRNA duplex termed the guide strand (21-23 nt) is incorporated into an RNA-induced silencing complex (RISC) to identify target MRE sequences through complementary base-pairings, primarily by the miRNA seed sequence (nucleotides 2-8). Sequence-specific miRNA-MRE complementarity leads to the repression of translation or promotion of degradation of target transcripts.

PTGR by genome-derived miRNAs has been recognized as a critical mechanism behind large interindividual variations in ADME and pharmacotherapy^{171,320,322}, with the let-7 family serving as a good example. The human let-7-5p family miRNAs consist of several isoforms (from let-7a to let-7g plus let-7i) that vary slightly in their sequences (**Supplemental Table S1**). The let-7 family miRNAs have established roles in regulating genes involved in oncogenic processes, including c-MYC, RAS, CDK6, and LIN28B³²³⁻³²⁵. Meanwhile, several drug-metabolizing enzymes and efflux ABC transporters have been identified as PTGR targets of specific let-7-5p isoforms in human cells, including CYP2J2 by let-7b³²⁶, CYP19A1 by let-7f³²⁷, ABCC2/MRP2 by let-7c³²⁸, ABCC5/MRP5 by let-7a, and ABCC10/MRP7 by let-7a and -7e³¹⁷. The broad impact and tumor suppressive function make the let-7 family miRNAs promising candidates to rebalance oncogenic phenotypes and overcome MDR toward developing rational and improved HCC therapies.

Research efforts have highlighted miRNA dysregulation as an important factor in the pathogenesis and MDR of cancer, with the let-7-5p miRNA family being one of the most highly downregulated in HCC^{317,329}. This downregulation dampens their natural regulatory functions, promoting aberrant protein expression of let-7-5p targets integral to MDR, such as ABCC5/MRP5. Indeed, ABCC5/MRP5 gene expression is inversely correlated with let-7a-5p expression in HCC and is linked to poor prognosis³²⁹⁻³³³. Therefore, disruption of let-7-controlled PTGR is a major contributor to MDR in HCC, highlighting the potential of miRNA replacement therapy which seeks to reintroduce or restore let-7-5p species or function to overcome MDR.

To investigate miRNA-based PTGR mechanisms and therapies, the Yu laboratory has developed an innovative RNA bioengineering platform to produce recombinant miRNA molecules^{83,199,334-336}. Unlike RNA mimics chemically synthesized in vitro, these bioengineered RNA (BioRNA) agents are constructed in living cells and therefore more accurately capture the structures and modifications of endogenous miRNA^{78,199}. Most relevantly, a novel bioengineered let-7c-5p agent was effective to inhibit HCC viability and downregulate ABCC5/MRP5 protein levels in Huh7 cells, increasing intracellular accumulation and anticancer effect of fluorouracil (5-FU)^{160,167}. Recent efforts have optimized this design to create a new generation of fully humanized let-7-5p (BioRNA/let-7-5p) agents³³⁵.

While previous research has studied the PTGR functions and anticancer effects of some specific let-7-5p isoforms aforementioned, their comparative effectiveness remains largely unknown. Therefore, the aim of this study was to employ unparalleled BioRNA/let-7-5p agents to delineate the functional differences of seven major let-7-5p isoforms in the suppression of protein levels of

known let-7-5p targets ABCC5/MRP5 and LIN28B and inhibit human HCC cell viability *in vitro*. Our findings on the variations of different let-7-5p isoforms in controlling target gene expression and HCC cell viability, depending on let-7-5p abundance and its complementarity with specific targets, should provide valuable insights into their comparative effectiveness in PTGR and potential use to combat MDR in HCC.

Materials and Methods

Chemicals and materials

Fetal bovine serum (FBS; Cat# 26140079), Dulbecco's modified eagle medium (DMEM; Cat# 11965092), DMEM/F-12 medium (Cat# 1132033), RIPA lysis buffer (Cat# 89901), Pierce bicinchoninic acid (BCA) protein assay kit (Cat# 23227), opti-MEM reduced serum medium (Cat# 31985062), 0.05% trypsin EDTA 1X (Cat# 45000-660), TRIzol reagent (Cat# 15596018), recombinant human epidermal growth factor (Cat# PHG0311), Bronchial Epithelial Cell Growth Medium (BEGM) Bulletkit with SingleQuot growth supplements (Cat# 11675440), and Lipofectamine 3000 (Cat# L3000001) were purchased from Thermo Fisher Scientific (Waltham, MA). Protease inhibitor cocktail (Cat# P8340), human fibronectin (Cat# F2006), bovine serum albumin (BSA; Cat# A9576), *O*-Phosphorylethanolamine (Cat# P0503) and bovine collagen type I (Cat# CLS354231) were purchased from Sigma-Aldrich (St. Louis, MO). TGX Stain-Free FastCast Acrylamide Kit (10%; Cat# 1610183), Clarity Western Enhanced Chemiluminescence Substrates (Cat# 1705061), Blotting-grade blocker (Cat# 1706404), polyvinylidene difluoride (PVDF) membranes (Cat# 1620177), Precision Plus All Blue protein standards (Cat# 1610373), and iTaq Universal SYBR Green Supermix (Cat# 1725121) were purchased from Bio-Rad (Hercules, CA). Eagle's modified essential medium (EMEM; Cat# 89428-906), phosphate-buffered saline (PBS; Cat# 45000-446), Falcon nontreated tissue culture flasks (Cat# BD353133), Corning treated culture dishes (Cat# 25382-428), NxGen M-Mulv reverse transcriptase (Cat# 97065-184), and premixed dNTP solutions (Cat# 76081-616) were purchased from VWR International (Radnor, PA). Direct-zol RNA miniPrep kit (Cat# R2061) was purchased from Zymo

Research (Irvine, CA). CellTiter-Glo 2.0 Cell Viability Assay kit (Cat# G9241) was purchased from Promega (Madison, WI). Several isoform-specific stem-loop RT and qPCR primers were designed based on the mature hsa-let-7-5p sequences (**Supplemental Table S1**) and purchased from Integrated DNA Technologies (Coralville, IA). All other materials, chemicals, and solvents were purchased from VWR, Sigma-Aldrich, or Thermo Fisher Scientific.

Production of BioRNA/let-7-5p molecules

Recombinant or bioengineered miRNA let-7-5p isoforms and control RNA were produced using a human glycyl tRNA-fused hsa-pre-miR-34a carrier, as described most recently³³⁵. In brief, target BioRNA were cloned into target vector, overexpressed in *E. coli*, and purified by anion-exchange fast protein liquid chromatography. BioRNA/let-7-5p agents used in this study were highly pure, as determined by high-performance liquid chromatography method ($\leq 98\%$), and showed low endotoxin levels (≤ 5 EU/ μ g RNA).

Cell culture

Human Hep3B (HB-8064) and HepG2 (HB-8065), murine Hepa1-6 (CRL-1830) and AML12 (CRL-2254), and healthy human liver epithelial THLE-2 (CRL-2706) and THLE-3 (CRL-3583) cell lines were purchased from American Type Culture Collection (Manassas, VA). Human Huh7 cell line was graciously provided by Dr. Baitang Ning³³⁷. HepG2 and Hep3B cells were maintained in EMEM, Huh7 and Hepa1-6 cells were maintained in DMEM, and AML12 cells were maintained in DMEM/F-12 medium. For THLE-2 and THLE-3 cultures, untreated Falcon flasks were coated

overnight with bronchial epithelial cell basal medium (BEBM) supplemented with 0.01 mg/mL fibronectin, 0.03 mg/mL bovine collagen type I, and 0.01 mg/mL BSA. THLE-2 and THLE-3 cells were then cultured in BEGM, which was generated by supplementing BEBM with SingleQuot reagents according to the manufacturer's instructions (Lonza Group AG), as well as 5 ng/mL epidermal growth factor and 70 ng/mL *O*-phosphorylethanolamine. All culture media were supplemented with 10% FBS, and all cell lines, verified to be free of mycoplasma contamination, were cultured in a humidified incubator (37°C, 5% CO₂). Cell counting for all experiments was performed using the Countess II FL system (Thermo Fisher Scientific; Waltham, MA).

Protein isolation and Western Blot analysis.

All cells used to compare the basal levels of proteins of interest were cultured in specified vessels, not treated with any agent, and harvested at 80% confluence. Human Huh7, HepG2, Hep3B, and THLE-2 cells used in experiments assessing the modulation of ABC transporter and LIN28B protein levels were seeded into six-well plates (500,000 cells/well) and transfected with 15 nM of biologic let-7-5p or control RNA, or Lipofectamine vehicle only for 72 h. Cells were then lysed in RIPA buffer containing protease inhibitor cocktail and remained on ice for 30 min before thorough mixing and centrifugation. Supernatant was collected for each sample, and protein concentration was quantified using the Pierce BCA protein assay kit. Protein samples (30 µg) were loaded into individual lanes of a 10% TGX Stain-Free SDS-PAGE gel, electrophoretically separated, and imaged with the Chemidoc MP system (Bio-Rad) for total protein determination before being transferred to PVDF membrane using the Trans-Blot Turbo Transfer System (Bio-Rad). Membranes were treated with 5% blotting-grade blocker for 2 h (25°C) to minimize background

signal and then incubated overnight (4°C) with primary antibodies against c-MYC (1:1,000; Cell Signaling Technology Cat# 5605), PD-L1 (1:1,000; Cell Signaling Technology Cat# 86744), CDK6 (1:1,000; Santa Cruz Biotechnology; Cat# sc-177), LIN28B (1:1,000; Cell Signaling Technology; Cat# 11965), Cat # 3978), ABCC1/MRP1 (1:2,500; Proteintech; Cat# 67228-1-Ig), ABCC2/MRP2 (1:500; Santa Cruz Biotechnology; Cat# sc-518048), ABCC3/MRP3 (1:1,000; Cell Signaling Technology; Cat# 39909), ABCC4/MRP4 (1:1,000; Abcam; Cat# ab15602), ABCC5/MRP5 (1:200; Santa Cruz Biotechnology; Cat# sc-376965), or β -actin (1:1,000; Sigma-Aldrich; Cat# A5441). Membranes were then incubated in one of the following HRP-linked secondary antibodies for 2 h (25°C): anti-mouse IgG (1:3,000; Cell Signaling Technology; Cat# 7076), anti-rabbit IgG (1:10,000; Jackson ImmunoResearch; Cat# 111-035-003); anti-rat IgG (1:3,000; Cell Signaling Technology; Cat# 7077). Membranes were then briefly incubated in a 1:1 mixture of Clarity Western Enhanced Chemiluminescence Substrates, and protein images were obtained with the Chemidoc MP system. Image Lab software (Bio-Rad) was used to quantify target protein band intensity, which was normalized to total protein and β -actin levels within the corresponding sample, with control RNA set as 1.0. All BioRNA/let-7-5p treatments were performed in biological triplicate (N = 3), with individual protein lysate samples were pooled for Western blot analyses that were conducted technically at least twice.

RNA extraction and stem-loop reverse transcription, quantitative real-time PCR (RT-qPCR) analyses

Human Huh7, HepG2, Hep3B, and THLE-2 cells were seeded into twelve-well plates (250,000 cells/well) and transfected with 15 nM of BioRNA/let-7-5p or control RNA, or Lipofectamine

vehicle for 48 h in biological triplicate (N = 3/group). Direct-zol RNA miniPrep kit (Zymo Research) was used to isolate total RNA from treated cells, and RNA concentration was determined using a Tecan Spark microplate reader. Specific stem-loop RT and qPCR primers were designed for individual let-7-5p isoforms as detailed (**Supplemental Table S1**). Total RNA (300 ng) was reverse-transcribed to cDNA using NxGen M-MuLV reverse transcriptase and corresponding stem-loop RT primers. Quantitative PCR analyses utilized sequence-specific forward primers for each let-7-5p isoform and its corresponding vehicle and RNA controls. U6 small RNA levels were separately assessed for each sample using U6-specific primers (**Supplemental Table S1**). Real time analyses were prepared using the iTaq Universal SYBR Green Supermix according to manufacturer instructions and performed on a CFX96 Touch Real-Time PCR System (Bio-Rad). For each sample, levels of let-7-5p isoforms were normalized to their respective U6 small RNA levels, and the formula $2^{-\Delta\Delta CT}$ was used to calculate fold change of individual let-7-5p isoforms. Each biological sample was analyzed with two technical replicates.

Cell growth and viability assays

Human Huh7, Hep3B, and HepG2 cells were seeded into 96-well plates at a density of 7,500 cells/well and transfected with BioRNA/let-7-5p or control RNA (15 nM), or Lipofectamine vehicle for 72 h (N = 3/group). An Incucyte Live-Cell Imaging and Analysis system (Sartorius AG; Göttingen, Germany) was used to determine cell confluency values for every sample at 4 h intervals, allowing construction of cell growth curves. Absolute cell confluency values were fit to the Malthusian exponential growth equation, $Y = Y_0 * \exp/(k * x)$, using GraphPad Prism (San Diego, CA). Kinetic parameters were estimated for each treatment, including the starting

confluency (Y_0), rate constant (k), and doubling time ($\ln(2) / k$). Cells were removed from the incubator after 72 h and the CellTiter-Glo 2.0 Cell Viability Assay Kit was used to further validate cell viability.

Statistical analysis

All values are expressed as mean $\pm$ standard deviation (SD). Statistical analysis was performed using one- or two-way ANOVA with Bonferroni or Tukey *post-hoc* tests, where appropriate (GraphPad Prism; San Diego, CA). Differences between groups were considered significant when the probability value was less than 0.05 ($P < 0.05$).

Results

Identification of proper liver cell lines for the study of let-7-5p controlled target gene expression.

To identify suitable cell models to investigate functional differences of let-7-5p isoforms, the basal protein levels of several known and predicted let-7-5p targets were compared across seven liver cell lines without any treatments. Overall, liver cells showed variable basal protein levels of cancer-related (c-MYC, CDK6, LIN28B, PD-L1) and ABC transporter (ABCC1-5/MRP1-5) targets (**Figure 1**). Human HCC cells (Huh7, HepG2, Hep3B) showed basal expression of several validated let-7-5p targets important in cancer (c-MYC, CDK6, LIN28B) but did not show expression of human PD-L1 protein (**Figure 1A**). Notably, basal expression of LIN28B protein was highly variable across human HCC cells, with Huh7 exhibiting basal LIN28B levels that were dramatically higher than HepG2 and Hep3B. Densitometric analysis of LIN28B revealed relative intensity values across human HCC cells, with HepG2 and Hep3B exhibiting 38% and 27% of the present Huh7 LIN28B signal respectively, normalized to corresponding total protein (**Figure 1A**). Conversely, Huh7 showed the lowest basal protein levels of both c-MYC and CDK6 across human HCC cells. Huh7, HepG2, and Hep3B also demonstrated the highest basal protein expression of several transporter targets (ABCC2/MRP2, ABCC3/MRP3, ABCC5/MRP5). Basal transporter levels were comparable overall, with the lowest ABCC2/MRP2 levels detected in Hep3B cells. Observed basal ABCC1/MRP1 protein levels were comparably high across human HCC cells and transformed healthy human liver epithelial cells (THLE-2 and THLE-3), with the highest basal levels detected in HepG2 and THLE-2 and the lowest observed in Huh7 (**Figure 2B**). Uniquely,

high PD-L1 protein levels were detected in THLE-2 and THLE-3 cells with minimal or no basal protein expression of other targets (**Figure 1**). Both murine liver carcinoma cell lines (Hepa1-6 and AML12) demonstrated high basal protein expression of c-MYC and ABCC4/MRP4, with Hepa1-6 also exhibiting some basal expression of ABCC3/MRP3. Basal expression of ABCC4/MRP4 protein was detected in all cell lines but was the most highly expressed in AML12 cells (**Figure 1**). The basal protein expression of known let-7-5p target ABCC5/MRP5 was detected across all tested cell lines but showed the highest expression in human HCC cells (Huh7, HepG2, Hep3B) (**Figure 1B**)

BioRNA/let-7-5p agents are processed to target let-7-5p isoforms in human HCC cells.

Human HCC cells (Huh7, Hep3B, HepG2) with high basal protein levels of let-7-5p targets (LIN28B and ABCC5/MRP5) were thus selected for further investigation. To assess whether BioRNA/let-7-5p agents were effectively processed to their respective -5p isoforms, stem-loop RT-qPCR analyses were employed to measure individual let-7-5p miRNA levels following transfection with BioRNA/let-7-5p for 48 h. Levels of each let-7-5p miRNA were measured by individual stem-loop RT-qPCR analyses with sequence-specific primers (**Supplemental Table S1**). The results showed that BioRNA/let-7-5p treatments resulted in higher levels of corresponding let-7-5p isoforms in Huh7, HepG2, and Hep3B cells, with some consistent differences observed across individual cell lines and isoforms (**Figure 2**). Across all three human HCC cells, 17 out of 21 BioRNA/let-7-5p treatments exhibited 100-fold or greater change of corresponding let-7-5p miRNA, with many treatments also far exceeding this value. All four RT-qPCR analyses that fell short of this 100-fold change threshold were observed in Hep3B cells,

which consistently showed overall lower fold change values compared to equivalent experiments in Huh7 and HepG2 cells. In Huh7 cells, let-7c, -d, and -g-5p all demonstrated higher than 10,000-fold change following treatment, with let-7c-5p being the most highly detected (**Figure 2**). In HepG2 cells, let-7d-5p showed the highest fold change near 10,000-fold, let-7g-5p showed the second highest near 1,500-fold, and remaining isoforms exhibited increases between 100- and 1,000-fold (**Figure 2B**). While overall findings in Hep3B cells showed comparatively lower fold changes, the comparative trend between individual isoforms was consistent with Huh7 cells, with the highest increase observed for let-7c, -7d, and let-7g-5p (over 500-1,000 fold) treatments ((Figure 2A)A, C). These findings demonstrate that let-7-5p isoforms are successfully released from BioRNA/let-7-5p agents and accumulated to varying degrees in human HCC cells, with let-7d-5p consistently exhibiting one of the highest fold increases across all cell lines.

Comparison of the effectiveness of individual let-7-5p isoforms to downregulate protein levels of ABCC5 and LIN28B in human HCC cells.

To determine the effectiveness of individual BioRNA/let-7-5p isoforms in modulating protein levels of known let-7-5p targets ABCC5/MRP5 and LIN28B, immunoblot analyses were performed in Huh7, HepG2, and Hep3B cells transfected with BioRNA/let-7-5p or control RNA. Across all cells, multiple BioRNA/let-7-5p treatments successfully downregulated ABCC5/MRP5 protein levels when compared to control RNA (**Figure 3A**). In Huh7 cells, let-7a, -c, and -d-5p treatments showed the largest decreases in ABCC5/MRP5 protein levels (34%, 24%, and 23% respectively), as compared to control RNA (**Figure 3A**). In HepG2 cells, let-7c, -d, and -g-5p treatments showed the strongest downregulation of ABCC5/MRP5 protein levels (57%, 65%, and

50% respectively) while let-7a-5p exhibited a modest suppression of ABCC5/MRP5 protein comparable to Huh7 (36%) (**Figure 3A**). Similar results were observed in Hep3B cells, where let-7c, -d, and -g-5p treatments strongly reduced ABCC5/MRP5 protein levels (46%, 50%, and 62% respectively) compared to control RNA, with let-7a-5p treatment showing only minor suppression of ABCC5/MRP5 protein levels (17%), distinct results in Huh7 and HepG2. Similarly, BioRNA/let-7g-5p treatment was among the most effective to downregulate ABCC5/MRP5 in HepG2 and Hep3B cells but notably showed minimal effects in Huh7 cells (**Figure 3A**). On the other hand, there was no or minimal reduction of ABCC2/MRP2 or ABCC4/MRP4 protein levels in HepG2, Hep3B, or Huh7 cells by any of the seven major let-7-5p isoforms examined in this study (**Supplemental Figure S1**).

Multiple BioRNA/let-7-5p isoforms also proved effective to downregulate LIN28B protein levels in human HCC cells (**Figure 3B**). Out of all tested isoforms, BioRNA/let-7d-5p consistently showed the strongest effects to reduce LIN28B protein levels, by about 61%, 77%, and 76% in Huh7, HepG2, and Hep3B cells respectively, as compared to control RNA (**Figure 3B**). While BioRNA/let-7d-5p was consistently the most effective, all treatments successfully downregulated LIN28B to variable degrees. Consistent with observed results for ABCC5/MRP5, BioRNA/let-7b-5p was the least effective treatment to reduce LIN28B protein levels but still showed decreases of 22%, 13%, and 31% in Huh7, HepG2, and Hep3B cells, respectively (**Figure 3B**). All other isoforms reduced LIN28B protein levels by about 33%, 35%, and 31% in Huh7, HepG2, and Hep3B cells, respectively. Collectively, these results demonstrate that several biologic let-7-5p isoforms effectively downregulate protein levels of ABCC5/MRP5 and LIN28B across different human HCC cells, with some variability. Additionally, our findings illustrate consistent differences

in the efficacy of individual let-7-5p isoforms to modulate protein levels of these targets, with let-7c and -d-5p proving the most consistently effective to suppress ABCC5/MRP5 in three distinct human HCC cells. Similarly, BioRNA/let-7d-5p was the most effective treatment to downregulate LIN28B protein, with a high degree of consistency across various cell lines (**Figure 3**).

Additionally, interactions were illustrated between individual let-7-5p isoforms and MREs within the 3'UTR of ABCC5/MRP5 and LIN28B mRNA transcripts (**Figure 3**). Each let-7-5p isoform shares a conserved seed sequence on its 5' end, mediating complementary 8mer interactions at each MRE within ABCC5/MRP5 and LIN28B mRNA transcripts (**Figure 3**). While all let-7-5p isoforms share complementary 8mer sites at the illustrated MREs, each isoform may differ in their number of additional complementary nucleobase pairings and G-U wobble base pairings upstream of the conserved seed sequence³³⁸. Notable differences are also present adjacent to the conserved seed sequence, with let-7d-5p possessing a unique adenosine as the first nucleobase on its 5' end (**Figure 3**). Individual let-7-5p isoforms were ordered at each MRE according to their TargetScan Context++ scores, with the lowest scores representing the most favorable miRNA/mRNA interaction. Context++ scores are calculated as the sum of 14 distinct features that characterize the MRE and its surrounding context, accounting for factors such as seed match identity (e.g. 8mer, 7mer-m8), supplementary non-seed base pairing, local AU abundance at the mRNA target, nucleotide identity at positions 1 and 8 of the miRNA, and thermodynamic stability of seed-pairing³³⁸. Overall, greater degrees of increase in let-7-5p isoforms (**Figure 2**) and their complementarities with target transcripts (**Figure 3**) forecast the strength of reduction of targeted protein levels (e.g., LIN28B by let-7d-5p) (**Figure 3**).

BioRNA/let-7-5p agents are processed to respective let-7-5p isoforms and variably regulate ABCC5/MRP5 protein levels in THLE-2 cells.

Human liver epithelial cells (THLE-2) were further utilized to assess the efficiency of BioRNA/let-7-5p processing and regulation of ABCC5/MRP5 protein levels. Stem-loop RT-qPCR analyses demonstrated selective processing of BioRNA/let-7-5p agents to their corresponding let-7-5p isoforms, with five out of seven isoforms exhibiting greater than 200-fold change (**Figure 4A**). Interestingly, observed fold change values for several isoforms in THLE-2 (most notably let-7a, -b, -e-5p) were of greater magnitude than those observed in Hep3B cells, but generally less than those observed in Huh7 and HepG2 cells. However, stem-loop RT-qPCR analyses in THLE-2 cells showed a comparative trend in the fold change of individual let-7-5p isoforms that was distinct from observations in human HCC cells. Unlike Huh7, HepG2, and Hep3B cells which consistently exhibited large increases in detected let-7c, d, and g-5p levels, THLE-2 cells demonstrated comparatively modest increases of these isoforms (**Figure 4A**). Additionally, THLE-2 cells showed an increase of let-7e-5p species greater than 500-fold, exceeding the fold-change values observed for this isoform in Huh7 and Hep3B cells.

The comparative effectiveness of let-7-5p isoforms to regulate ABCC5/MRP5 protein levels in THLE-2 were investigated following transfection with individual BioRNA/let-7-5p agents for 72 h. Consistent with the results obtained from multiple human HCC cells, let-7-5p isoforms showed variable degrees of efficacy to suppress ABCC5/MRP5 protein levels in THLE-2, with let-7c-5p (41%), let-7d-5p (51%), and let-7g-5p (48%) showing greater extents of downregulation as compared to control RNA (**Figure 4B**).

Bioengineered let-7-5p isoforms inhibit the growth of human HCC cells to variable degrees.

Cell growth and viability experiments were carried out to investigate the efficacy of biologic let-7-5p isoforms to inhibit the viability of human HCC cells (Huh7, HepG2, Hep3B). Following transfection with BioRNA/let-7-5p agents, cell confluency values were measured over 72 h and used to assess cell growth over time for each treatment. Data fitted well to the Malthusian exponential growth equation, $Y = Y_0 * \exp/(k * x)$, and relevant kinetic parameters were estimated (**Figure 5**). The results showed that multiple biologic let-7-5p agents effectively inhibited viability of human HCC cells, with key differences in their comparative effectiveness observed across different HCC cells (**Figure 5**). In Huh7 and HepG2 cells, all BioRNA/let-7-5p treatments exhibited increased doubling time ($\ln(2) / k$) and decreased rate constant (k) values compared to control RNA (**Figure 5A-B**). For Huh7 cells, let-7e, -f, -g-5p treatments resulted in the largest changes in these parameters while let-7a, -b, and -c-5p treatments showed the largest changes in HepG2 cells (**Figure 5A-B**). Results in Hep3B cells showed interesting differences, where only let-7a, -c, and -e-5p treatments exhibited increased doubling time compared to control RNA among them let-7c-5p was the most effective isoform (**Figure 5C**). It is noted that Hep3B and Huh7 cells treated with control RNA also exhibited large differences in doubling time when compared to vehicle control, whereas no difference was observed between control RNA and vehicle treatments in HepG2 cells (**Figure 5C**).

At the 72 h endpoint, cell viability values were also measured with CellTiter-Glo 2.0 Assay (**Figure 6**). Across all three cell lines and individual treatments, 72 h endpoint confluence values were

generally consistent with results obtained from CellTiter-Glo cell viability assay and Incucyte Live-Cell Imaging and Analysis method (**Figure 6**). Cell viability analyses showed differences between treatment groups that mirror changes to doubling time ($\ln(2) / k$) and rate constant (k) as described above. Consistent with changes to estimated parameters, BioRNA/let-7e, -f, and -g-5p treatments exhibited the strongest effects against Huh7 cell viability (**Figures 5-6**). Some small differences were observed in HepG2 cells, with BioRNA/let-7d and -e-5p treatments exhibiting stronger inhibition of endpoint viability compared to BioRNA/let-7b-5p that showed larger change in estimated parameters (**Figures 5-6**). Endpoint cell viability results in Hep3B cells also reflect the observed changes in estimated kinetic parameters, with BioRNA/let-7c-5p demonstrating the strongest inhibition of Hep3B cell viability compared to control RNA (**Figures 5-6**). Overall, growth curve and endpoint cell viability analyses showed multiple let-7-5p isoforms are effective to inhibit the viability and growth of different human HCC cells, with some key differences between isoforms and across cell lines.

Discussion

Multiple efflux transporters confer resistance to standard HCC therapies, with several studies supporting the role of efflux transporters such as ABCC2/MRP2, ABCC4/MRP4, and ABCC5/MRP5 in mediating resistance to drugs like sorafenib, fluorouracil (5-FU), and oxaliplatin^{167,332,339}. Therapeutic resistance may occur through direct efflux of administered drug as well as indirect factors related to endobiotic transport. Although ABCC5/MRP5 does not readily transport sorafenib, it is strongly induced in sorafenib-resistant HCC cells and provides protection against sorafenib-induced ferroptosis by promoting the expression of solute carrier SLC7A11³³². In HCC and many other types of cancers, the let-7 family are among the most consistently downregulated species of miRNAs, with repressed let-7 miRNA levels associated with increased expression of genes implicated in MDR (ABCC1-5/MRP1-5)^{317,329,340-342}. While several studies have demonstrated the potential of let-7-5p to regulate ABC transporters (ABCC2, ABCC4, ABCC5) and re-sensitize multiple cancers to therapy, the present study found minimal effects of let-7-5p isoforms on the protein outcomes of ABCC2/MRP2 or ABCC4/MRP4 in HCC cells *in vitro*, while exhibiting functional difference in regulating ABCC5 protein levels.

HCC progression and persistent MDR phenotype resulting from low let-7-5p expression is due in part to RNA-binding protein LIN28B, which is overexpressed in HCC and actively represses maturation of endogenous let-7 miRNA species through specific interaction with their primary (pri-) and precursor (pre-) miRNAs, preventing proper processing by the Drosha microprocessor and Dicer complexes respectively³⁴³⁻³⁴⁵. LIN28B, an established target of let-7-5p³⁴⁶⁻³⁴⁸, also promotes 3' polyuridination of pre-let-7 through terminal uridylyl transferases, suppressing let-7-

5p levels and therefore promoting overexpression of ABCC5/MRP5. MiRNA let-7-5p can also regulate expression of LIN28B through PTGR, a well-established feedback loop also supported by this study that interrogated the comparative efficacy of individual let-7-5p isoforms to repress protein levels of validated targets LIN28B and ABCC5 in HCC cells.

BioRNA/let-7d-5p treatment showed the strongest and most consistent downregulation of LIN28B protein levels across three human HCC cell lines. The superior efficacy of let-7d-5p to regulate LIN28B is directly correlated with its highest degree of target complementarity outside of the seed sequence for both MREs within the LIN28B 3'UTR. Illustrated alignments confirm let-7d-5p is the only isoform to form a stable C-G non-seed pair in both LIN28B MREs, distinct from the U-G wobble base pair present at this position for all other let-7-5p isoforms. This interaction specific to let-7d-5p fosters a higher degree of target complementarity outside the seed region ³⁴⁹ and provides a clear explanation for its superior regulatory strength observed in this study. Let-7d-5p also possesses a unique adenosine at its 5' end, which does not prevent 8mer interaction with MRE's in LIN28B or ABCC5/MRP5 transcripts, but does influence its natural biogenesis ³⁵⁰ and might also impact how well our BioRNA/let-7-5p agents are processed to target let-7-5p isoforms. Despite BioRNA/let-7d-5p being the most effective isoform to reduce LIN28B protein levels, it was not the most effective to inhibit HCC cell viability. This indicates that while the degree of LIN28B repression is important for HCC viability, let-7 miRNA exert anti-cancer effects through a broader network of targets that may differ between individual cell lines, emphasizing the importance of paired phenotypic assessments as performed in this study.

Central to present study was the evaluation of BioRNA/let-7-5p agents to repress protein levels of protective efflux transporter ABCC5/MRP5. In particular, let-7c, -7d, and 7g-5p were identified as more effective suppressors of ABCC5/MRP5 protein levels across distinct HCC cell backgrounds. These results further demonstrate the potential of BioRNA/let-7-5p for use in rational combination therapy and provide strong mechanistic rationale for co-administration of let-7-5p with existing drugs subject to efflux by ABCC5/MRP5, such as 5-FU¹⁶⁷. Considering the effective downregulation of LIN28B in Huh7 by BioRNA/let-7g-5p, the observed lack of regulation of ABCC5/MRP5 by this isoform is likely to be target specific but not necessarily correlated with let-7-5p MRE complementarity. Let-7g-5p can form the largest number of complementary nucleobase pairings outside of the seed sequence with the MRE within ABCC5. Most notably, let-7g-5p contains a cytosine near its 3' end where all other isoforms have uracil, allowing formation of a complementary C-G pairing where all other isoforms have a U-G wobble base pair, supporting its high degree of regulatory strength against ABCC5 observed in HepG2 and Hep3B cells. These results demonstrate a separation between the factors driving anticancer effects of BioRNA/let-7-5p agents from those governing regulation of MDR-related targets such as ABCC5/MRP5, dependent on the cellular microenvironment.

Overall, Huh7 cells exhibited a lower magnitude of ABCC5/MRP5 suppression than HepG2 and Hep3B cells despite showing the highest fold change of let-7-5p miRNAs. Potential factors explaining this lower PTGR magnitude specific to ABCC5/MRP5 in Huh7 is the variable basal expression of individual let-7-5p isoforms and high basal expression of IGF2BP1, an RNA binding protein with established influence on MDR that may stabilize mRNA transcripts of efflux transporters like ABCC5/MRP5 or limit accessibility to let-7 MREs within the 3'UTR^{351,352}. The

role of IGF2BP1 in protecting ABCC5/MRP5 mRNA was evident in a recent study utilizing a specific small molecule inhibitor of IGF2BP1 which suppressed ABCC5/MRP5 gene expression³⁵². Besides the variable basal levels of individual let-7-5p isoforms leading to distinct degrees of increase in specific let-7-5p isoforms upon BioRNA treatments, cell lines may also differ in their basal levels of protein targets or endogenous factors that dampen let-7-5p regulatory outcomes. In this study, ABCC5/MRP5 was found to be expressed at consistent levels across human HCC cells, while LIN28B expression was noticeably higher in Huh7 cells. Variable basal expression of let-7-5p miRNA, their corresponding protein targets, and related endogenous factors like IGF2BP1 underly the high heterogeneity observed in HCC, providing context to the cell line specific differences in let-7-5p treatment outcomes observed in this study. Additionally, the transfection efficiencies differ a lot among these HCC cell lines which likely contributes to the variability of particular effects.

Much like the unique sub-populations that comprise microenvironments of a heterogenous tumor, individual HCC cell lines possess distinct cellular morphologies and genetic backgrounds that foster variable energy metabolism and modulate responses to exposome or stress, such as xenobiotics and hypoxia. Further, the exosomes or extracellular vesicles released by HCC cells to facilitate cell-cell communications and microenvironments may differ much in their active components, including exosomal miRNAs³⁵³, highlighting a potential underlying factor that may contribute to variable let-7-5p effectiveness across individual HCC cell lines observed in this study. Under hypoxic conditions, hypoxia-inducible factor-1 alpha signaling largely affects the size, quantity, and composition of exosomes³⁵⁴ in which the upregulation of exosomal miR-155-5p has been revealed to promote angiogenesis in endothelial cells³⁵⁵. On the other hand, exosomal

oncogenic miR-155-5p derived from hypoxic tumor-associated macrophages promotes the progression of renal cell carcinoma³⁵⁶. Rather, it is noteworthy that exosomes represent a novel system for the delivery of therapeutic RNA molecules, including miRNAs³⁵⁷.

To robustly assess the effect of BioRNA/let-7-5p treatments on HCC cell viability, two methods were used in this study. The Incucyte system offers real-time label-free imaging and analysis of cell confluence over time, allowing us to capture subtle changes in growth kinetics (rate constant and doubling time) not captured by assays at single time point. Meanwhile, the live-cell imaging technology was complemented with biochemical CellTiter-Glo 2.0 assay that is based on measuring cellular ATP levels that reflects changes in cell proliferation and cellular energy state^{358,359}. By utilizing both imaging and biochemical information to assess HCC cell viability, the disadvantages of each approach are minimized to conduct more complete and unbiased assessment of treatment effects. Consistent with our recent findings³⁶⁰, orthogonal imaging and biochemical assays agreed well with one another, revealing functional differences among individual let-7-5p isoforms in modulating cell viability. This rigorous dual-pronged approach showing high concordance between orthogonal analyses supports the validity of our findings as genuine pharmacological effects of BioRNA/let-7-5p molecules.

While present study interrogates the comparative effects of seven major human let-7-5p isoforms in the regulation of target gene expression and HCC cell viability by using unique BioRNA/let-7-5p agents, these findings are limited to particular *in vitro* cell line models. The translational potential warrants further investigations with more clinically relevant *in vivo* or *ex vivo* models. Indeed, many association or correlation studies reported previously^{317,361-365} have demonstrated

the value of using human specimens for identifying specific miRNAs and defining their significance in the regulation of particular drug-metabolizing enzymes or ABC transporters, as well as developing new miRNA based therapeutics. On the other hands, our previous studies have extended some *in vitro* observations to *in vivo* studies to reveal the translational potential of miRNA replacement therapy in disease animal models^{83,160,360,366-370}, in which pharmacological synergism between an onco-suppressive miRNA with co-administered drug may be attributable to interactions occurring at both pharmacodynamic and pharmacokinetic levels¹⁷¹. Moreover, the present study is also limited to the use of gain-of-function approach, which is appropriate when examining the miRNAs (e.g., let-7-5p) downregulated and their targets of interest (e.g., ABCC5 and LIN28B) upregulated in the model systems (e.g., HCC cells) and aiming at developing miRNA replacement therapy. Further studies with loss-of-function approaches would undoubtedly advance the understanding of role of let-7-5p isoforms in PTGR under physiological condition or particular circumstances. Nevertheless, in contrast to previous research that commonly investigates a single let-7-5p isoform in one study, this comparative study identifies the most promising let-7-5p isoforms against HCC cell viability as well as regulating ABCC5 and LIN28B expression should inform the direction of future *in vivo* investigation of let-7 mono- and combination therapies.

In conclusion, this study established functional differences of seven major let-7-5p isoforms in the regulation of target gene expression and HCC cell viability *in vitro* whose effectiveness are dependent on the isoform's sequence and abundance as well as interactions with specific targets and cellular microenvironment. Among them, let-7c-5p and let-7d-5p were revealed as effective regulators of efflux transporter ABCC5, indicating their potential for combination therapy as chemo-sensitizing agents to improve the treatment of HCC. Effects of BioRNA/let-7-5p isoforms

against HCC cell viability were notably variable and not necessarily proportional to their regulatory strengths on a single oncogene such as LIN28B. These findings highlight possible approaches to combat MDR in HCC and demonstrate the value of this novel class of miRNA molecules to investigate PTGR of ADME genes that are produced by innovative RNA bioengineering platform technology.

Tables

Supplemental Table S1. Sequences of hsa-let-7-5p isoforms and primers for stem-loop RT real-time qPCR analyses. Complementary interactions between hsa-let-7-5p isoforms and corresponding stem-loop RT primers are underlined and color coded. Highlighted are corresponding hsa-let-7-5p segments and the isoform-specific qPCR primers. RT, reverse transcription; F, forward; R, reverse.

	miRNA sequence (5' to 3')
hsa-let-7a-5p	<u>UGAGGUAGUAGGUUGU</u> AUAGUU
hsa-let-7b-5p	<u>UGAGGUAGUAGGUUGU</u> GUGGUU
hsa-let-7c-5p	<u>UGAGGUAGUAGGUUGU</u> AUGGUU
hsa-let-7d-5p	<u>AGAGGUAGUAGGUUGC</u> AUAGUU
hsa-let-7e-5p	<u>UGAGGUAGGAGGUUGU</u> AUAGUU
hsa-let-7f-5p	<u>UGAGGUAGUAGAUUGU</u> AUAGUU
hsa-let-7g-5p	<u>UGAGGUAGUAGUUUGU</u> ACAGUU
	Stem-loop RT primer sequence (5' to 3')
hsa-let-7a, d, e, f-5p	GTCGTATCCAGTGCAGGGTCCGAGGTATTCGCACTGGATACGACA <u>AACTAT</u>
hsa-let-7b-5p	GTCGTATCCAGTGCAGGGTCCGAGGTATTCGCACTGGATACGACA <u>AACCAC</u>
hsa-let-7c-5p	GTCGTATCCAGTGCAGGGTCCGAGGTATTCGCACTGGATACGACA <u>AACCAT</u>
hsa-let-7g-5p	GTCGTATCCAGTGCAGGGTCCGAGGTATTCGCACTGGATACGACA <u>AACTGT</u>
	Real-time qPCR sequence (5' to 3')
hsa-let-7a-5p F	CGCGC <u>TGAGGTAGTAGGTTGT</u>
hsa-let-7b-5p F	CGCGCA <u>TGAGGTAGTAGGTTGT</u>
hsa-let-7c-5p F	CGCGC <u>TGAGGTAGTAGGTTGT</u>
hsa-let-7d-5p F	CGCGCA <u>AGAGGTAGTAGGTTGC</u>
hsa-let-7e-5p F	CGCGCA <u>TGAGGTAGGAGGTTGT</u>
hsa-let-7f-5p F	GCCGCG <u>TGAGGTAGTAGATTGT</u>
hsa-let-7g-5p F	GCCGCG <u>TGAGGTAGTAGTTTGT</u>
Universal R	GTGCAGGGTCCGAGGT
U6 F	CTCGCTTCGGCAGCACA
U6 R	AACGCTTCACGAATTTGCGT

Figures and Legends

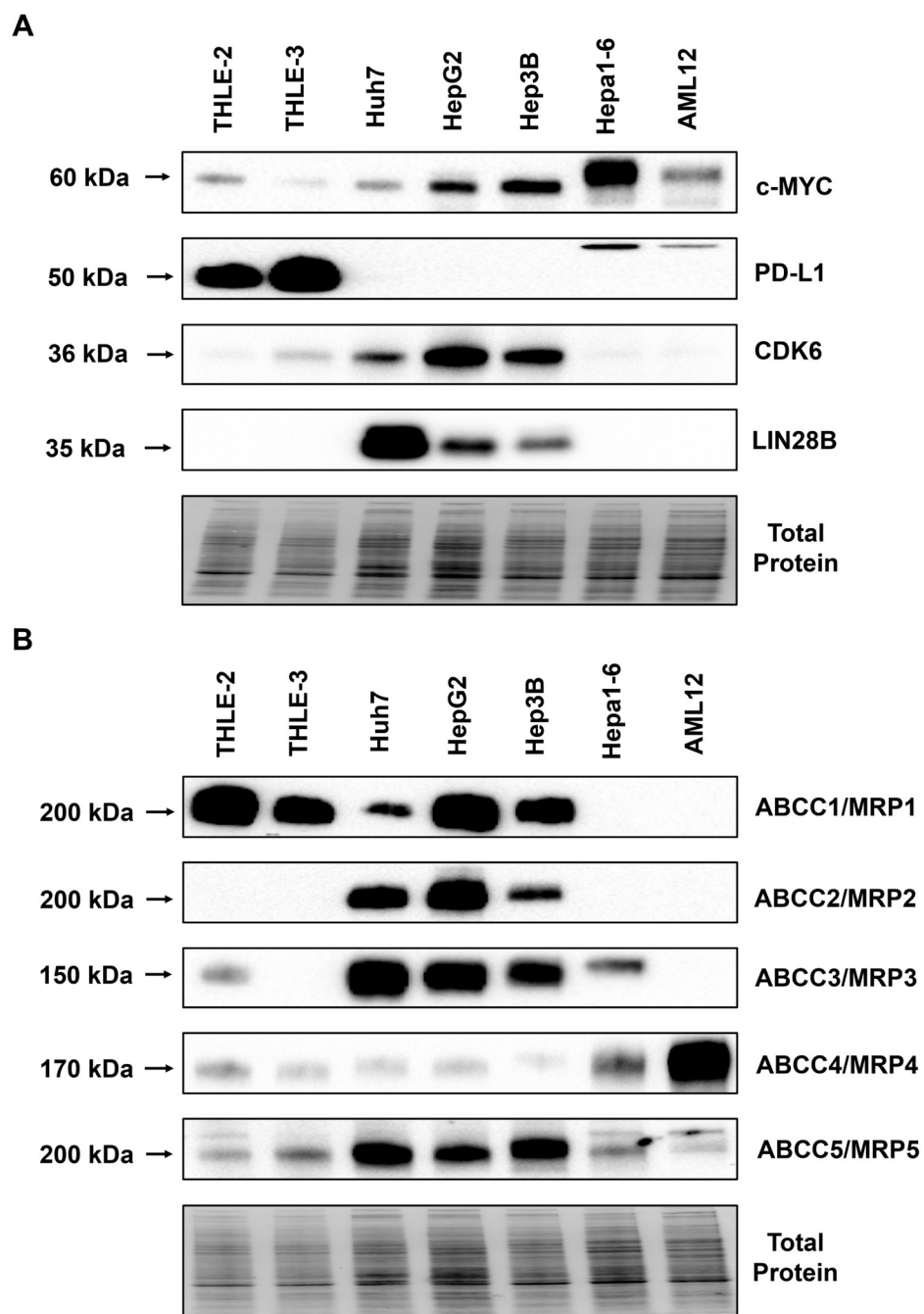


Figure 1. Comparison of basal levels of several proteins in a panel of liver cell lines, including human (Huh7, HepG2, and Hep3B) and murine (Hepa1-6 and AML12) hepatocellular carcinoma cells as well as two derived from healthy human liver epithelium (THLE-2 and THLE-3), without any treatments. (A) c-MYC, PD-L1, CDK6, and LIN28B are known let-7-5p targets important in cancer. (B) Common multidrug resistance transporters ABCC1-5 (MRP1-5) in which ABCC5 is a direct target of let-7-5p. Western blot analyses were conducted with selective antibodies.

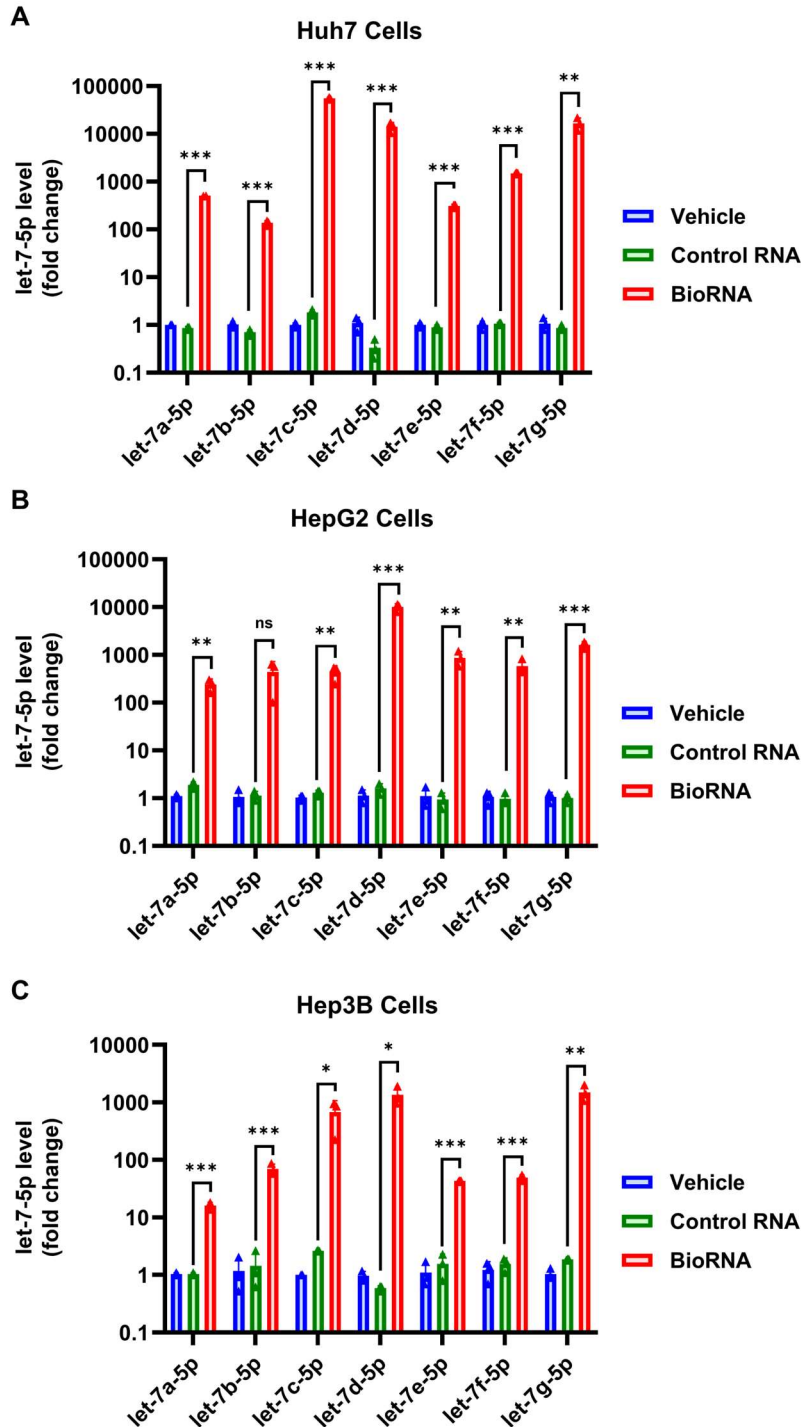


Figure 2. BioRNA^{Gly}/let-7-5p agents are selectively processed to their respective let-7-5p isoforms in (A) Huh7, (B) HepG2, and (C) Hep3B cells. Selective stem-loop RT real-time qPCR methods were used to measure individual let-7-5p isoforms, and their levels were normalized to U6 levels in corresponding samples. ***P < 0.001; **P < 0.01; *P < 0.05; and ns, not significant (One-way ANOVA with Bonferroni *post hoc* tests). All values are mean $\pm$ SD (N = 3 biological replicates per treatment group).

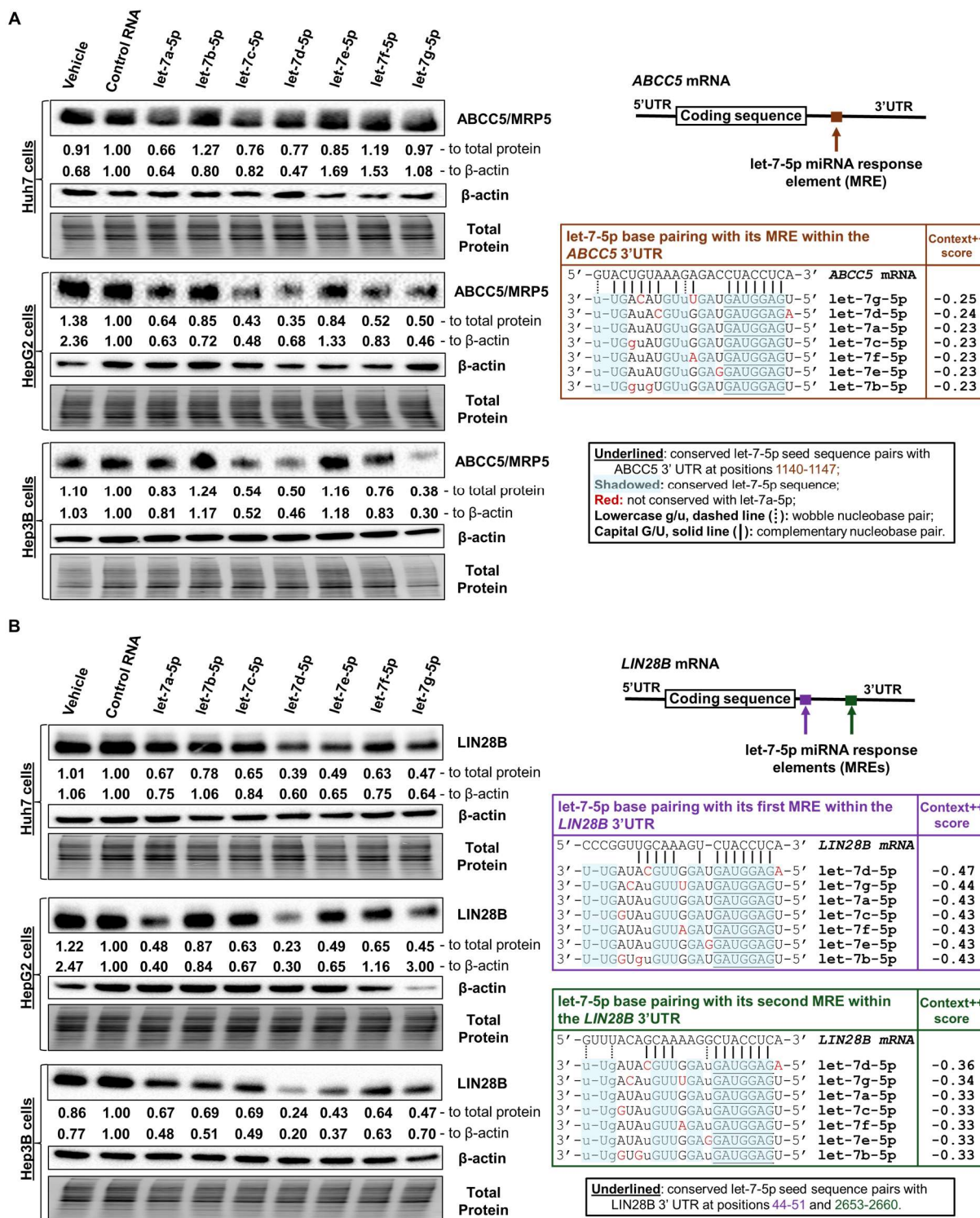
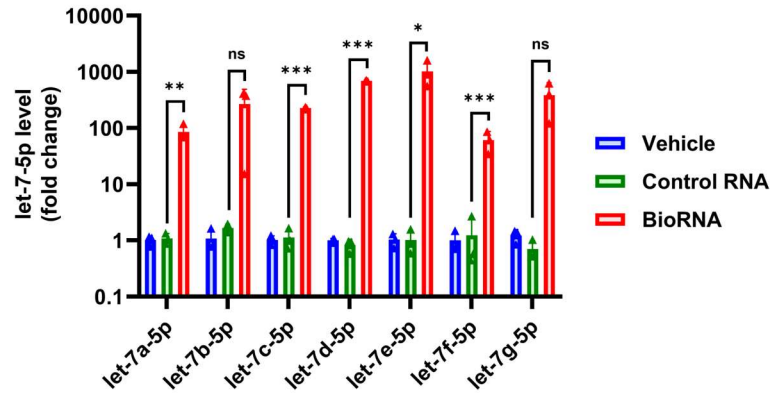


Figure 3. Comparison of the effectiveness of various let-7-5p isoforms to downregulate protein levels of (A) ABCC5/MRP5 and (B) LIN28B in multiple human liver cells. Huh7, HepG2, and Hep3B cells were treated with 15 nM of bioengineered let-7-5p or control RNA, or vehicle for 72 h, and Western blot analyses were performed to determine protein levels. Shown are representative blots. Protein band densities were normalized to corresponding total protein or β -actin levels, with control RNA group set as 1.0. Interactions of individual let-7-5p isoforms with respective MREs within (A) ABCC5/MRP5 and (B) LIN28B 3'UTR have been illustrated. Individual let-7-5p isoforms are listed according to the Context scores (TargetScan).

A THLE-2 Cells



B THLE-2 Cells

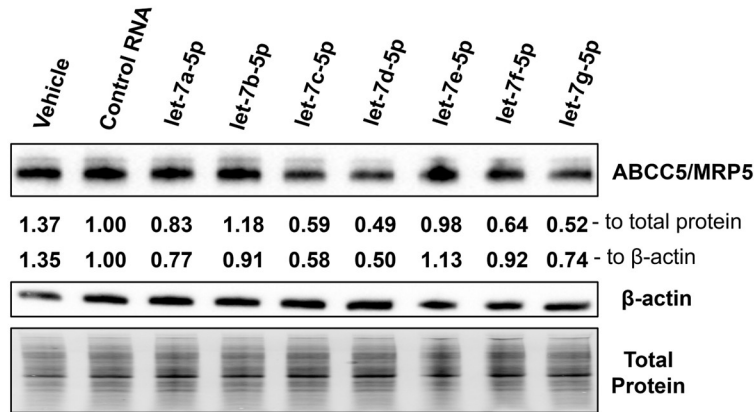


Figure 4. Release of let-7-5p isoforms from BioRNA^{Gly}/let-7-5p molecules and their effectiveness to suppress ABCC5/MRP5 protein levels in THLE-2 cells. (A) Levels of let-7-5p isoforms were measured by selective stem-loop RT real-time qPCR methods and normalized to respective U6 levels. ***P < 0.001; **P < 0.01; *P < 0.05; and ns, not significant (One-way ANOVA with Bonferroni *post hoc* tests). Values are mean ± SD (N = 3 biological replicates per treatment group). (B) Western blot analyses were performed to determine ABCC5/MRP5 protein levels in THLE-2 cells after 72 h transfection with 15 nM of RNA or vehicle. Shown are representative blots. Protein band densities were normalized to corresponding total protein levels or β-actin, with control RNA group set as 1.0.

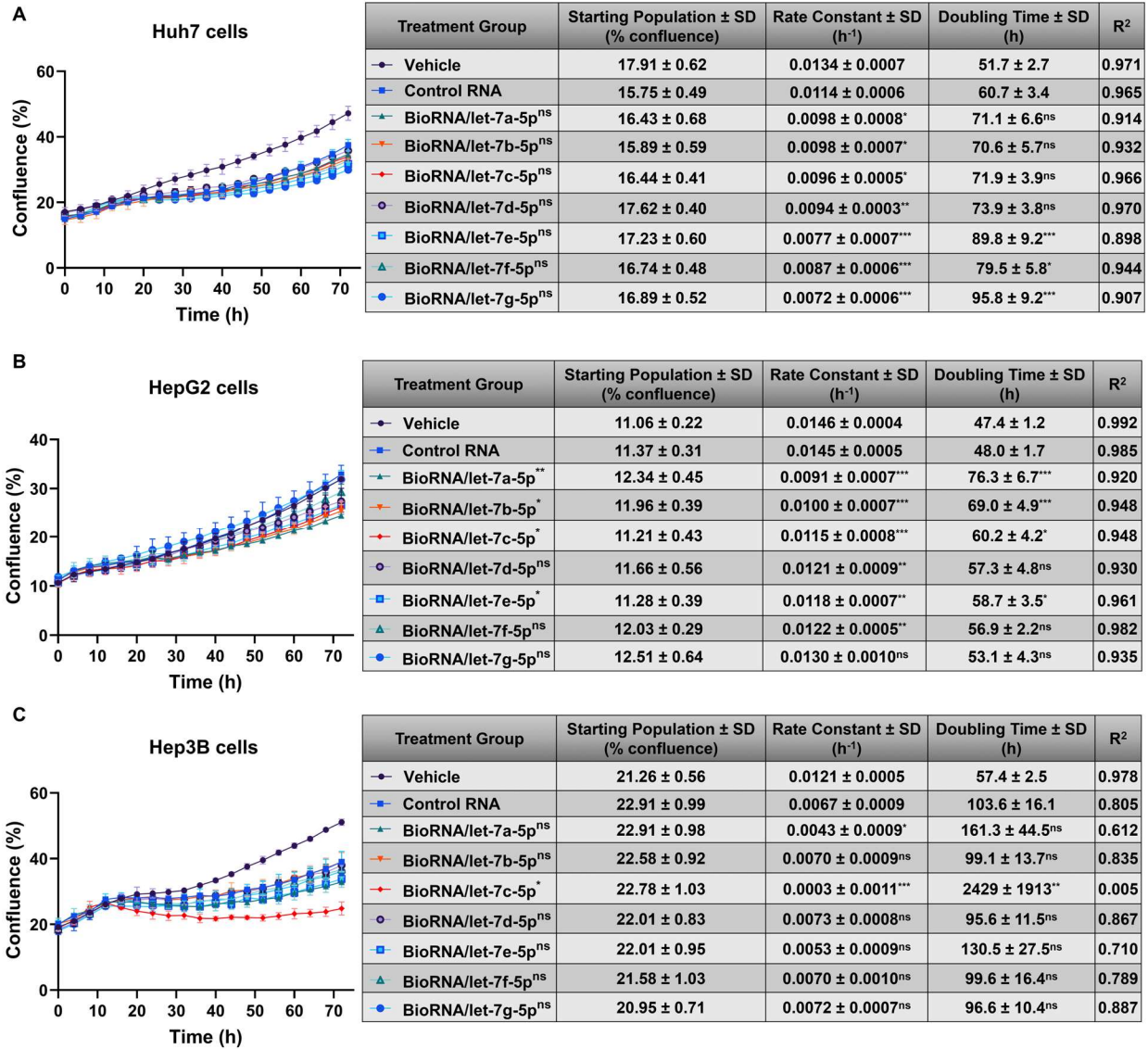


Figure 5. Efficacy of individual let-7-5p isoforms to inhibit (A) Huh7, (B) HepG2, and (C) Hep3B cell viability over time. Cell confluency was monitored for 72 h with an Incucyte Live-Cell Imaging and Analysis System, after the treatment with 15 nM of biologic let-7-5p or control RNA or vehicle. Cell confluency values were measured at 4 h intervals and used to construct cell confluency growth curves for each treatment. * $P < 0.05$; and ns, not significant; let-7c-5p compared to control RNA (Two-way ANOVA with Tukey *post hoc* tests). Data were fit to the exponential (Malthusian) growth equation, $Y = Y_0 * \exp/(k * x)$, and kinetic parameters were estimated, including the starting confluency (Y_0), rate constant (k), and doubling time ($\ln(2)/k$). *** $P < 0.001$; ** $P < 0.01$; * $P < 0.05$; and ns, not significant; individual let-7-5p isoforms compared to control RNA (One-way ANOVA with Bonferroni *post hoc* tests). All values are mean $\pm$ SD (N = 3 biological replicates per treatment group).

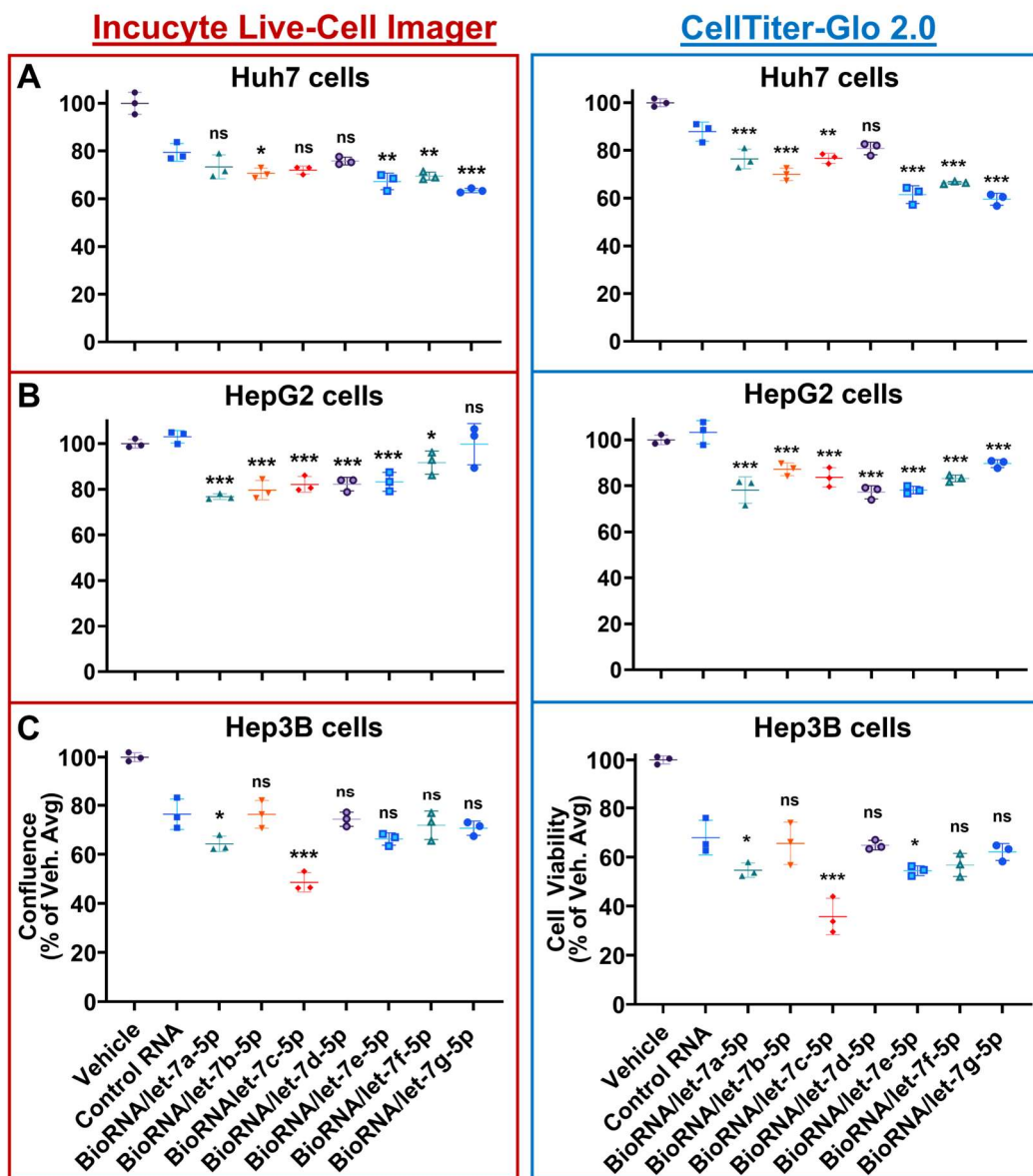
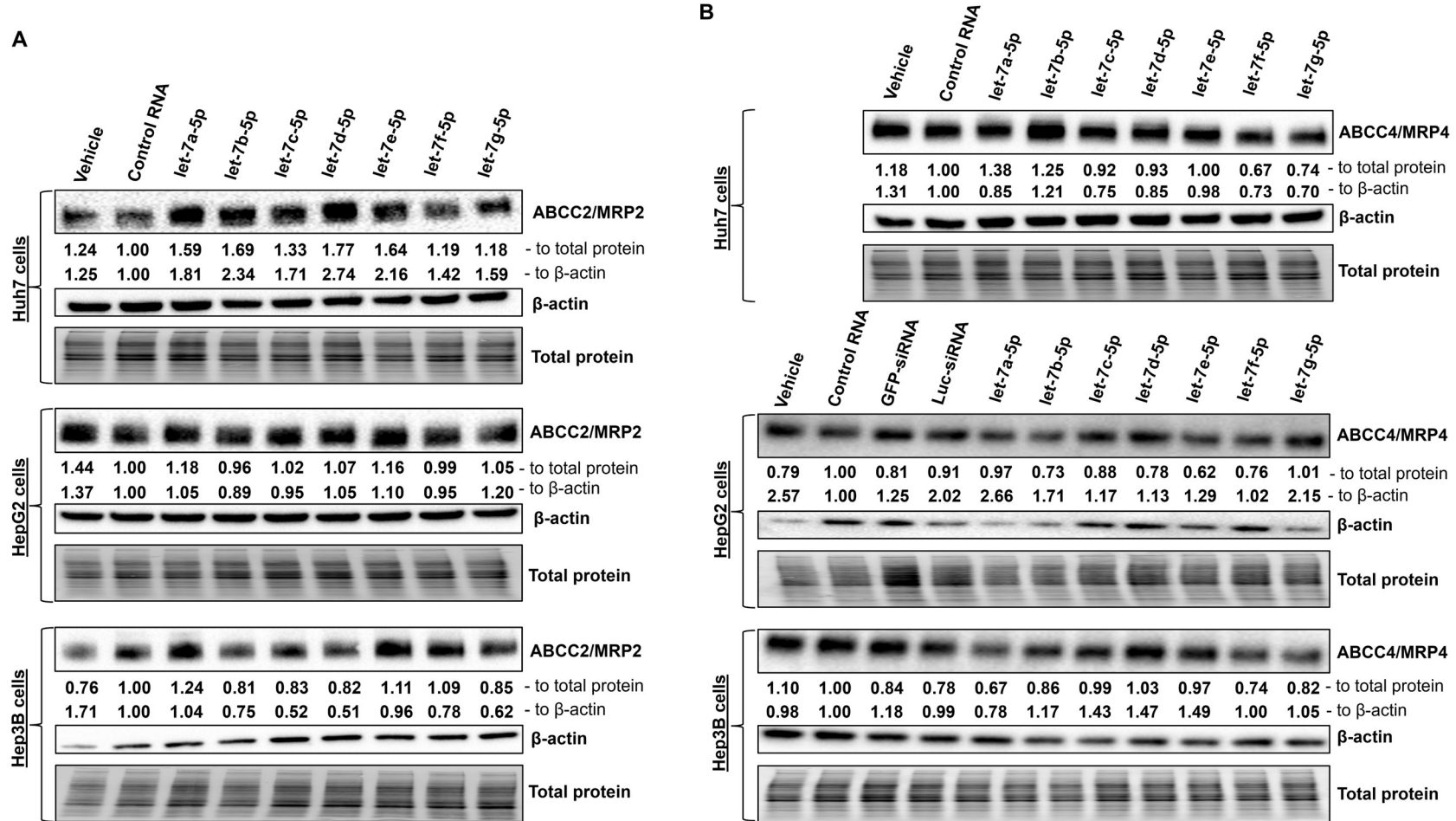


Figure 6. Comparison of Incucyte Live-Cell Imaging method (left) and CellTiter Assay (right) to determine BioRNA/let-7-5p efficacy to inhibit (A) Huh7, (B) HepG2, and (C) Hep3B cell viability, after 72-h treatment with 15 nM of RNA. Vehicle treatments were included as additional controls. ***P < 0.001; **P < 0.01; *P < 0.05; and ns, not significant; individual BioRNA/let-7-5p isoforms compared to control RNA (One-way ANOVA with Bonferroni *post hoc* tests). All values are mean $\pm$ SD and represented as % of vehicle control (N = 3 biological replicates per treatment group)



Supplemental Figure S1. BioRNA/let-7-5p isoforms exhibit minimal to no regulation of (A) ABCC2/MRP2 and (B) ABCC4/MRP4 protein levels in human HCC cells. Huh7, HepG2, and Hep3B cells were treated with 15 nM of bioengineered let-7-5p or control RNA, or vehicle for 72 h, and Western blot analyses were performed to determine protein levels. Protein band densities were normalized to corresponding total protein or β-actin levels, with control RNA group set as 1.0.

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