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REVIEW ARTICLE

Recent Advances in Novel Recombinant RNAs for Studying Post-transcriptional Gene Regulation in Drug Metabolism and Disposition

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Abstract: Drug-metabolizing enzymes and transporters are major determinants of the absorption, disposition, metabolism, and excretion (ADME) of drugs, and changes in ADME gene expression or function may alter the pharmacokinetics/pharmacodynamics (PK/PD) and further influence drug safety and therapeutic outcomes. ADME gene functions are controlled by diverse factors, such as genetic polymorphism, transcriptional regulation, and co-administered medications. MicroRNAs (miRNAs) are a superfamily of regulatory small noncoding RNAs that are transcribed from the genome to regulate target gene expression at the post-transcriptional level. The roles of miRNAs in controlling ADME gene expression have been demonstrated, and such miRNAs may consequently influence cellular drug metabolism and disposition capacity. Several types of miRNA mimics and small interfering RNA (siRNA) reagents have been developed and widely used for ADME research. In this review article, we first provide a brief introduction to the mechanistic actions of miRNAs in post-transcriptional gene regulation of drug-metabolizing enzymes, transporters, and transcription factors. After summarizing conventional small RNA production methods, we highlight the latest advances in novel recombinant RNA technologies and applications of the resultant bioengineered RNA (BioRNA) agents to ADME studies. BioRNAs produced in living cells are not only powerful tools for general biological and biomedical research but also potential therapeutic agents amenable to clinical investigations.

Keywords: Drug-metabolizing enzyme, transporter, microRNA, siRNA, regulation, recombinant RNA.

1. INTRODUCTION

Determination of drug absorption, disposition, metabolism, and excretion (ADME) properties is essential in drug discovery and development to assess the druggability and predict efficacy, toxicity, and drug-drug interactions (DDIs) of a drug candidate. Knowledge of ADME or pharmacokinetics (PK) further guides the design of efficacious and safe dosing regimens for clinical practices under various conditions, such as applications to diverse populations. Changes in ADME may lead to an insufficient distribution of or overexposure to a drug in humans or result in unexpected levels of toxic metabolites, thereby causing toxicity or a reduced efficacy [1-3]. Therefore, understanding the key elements in the control of ADME/PK and relevant regulatory mechanisms could facilitate drug development and ensure the safe use of approved medications.

Drug-metabolizing enzymes highly expressed in human livers and other major ADME organs are responsible for the metabolism of drugs and other kinds of xenobiotics [3], including Phase I enzymes such as cytochrome P450 enzymes (CYPs or P450s) that catalyze the oxidations of drugs, and Phase II enzymes, such as UDP-glucuronosyltransferases (UGTs) and sulfotransferases (SULTs) that mediate important conjugation reactions. In addition to passive diffusion, drugs may be actively pumped into the cells by solute carrier (SLC) superfamily uptake transporters such as organic anion transporting polypeptides (OATPs) and organic cation transporters (OCTs), and are exported from the cells through efflux transporters mainly constituted by ATP-binding cassette (ABC) superfamily transporters such as P-glycoprotein (*ABCB1*/P-gp/MDR1), multidrug resistance-associated proteins (ABCCs/MRPs), and breast cancer-resistance protein (*ABCG2*/BCRP). Meanwhile, some metabolites are also transported or excreted by SLC or ABC transporters [4]. Overall, drug-metabolizing enzymes and transporters are determinants of ADME/PK which are inevitably linked to pharmacodynamic (PD) and safety of the drugs [5].

The expression, localization, and activities of drug-metabolizing enzymes and transporters are regulated by various factors, which thereby may affect the ADME/PK/PD profiles of drugs. One of the widely studied mechanisms is gene polymorphism, including single nucleotide polymorphism (SNP), copy number variation, and alternative splicing, through which the encoded protein with single or multiple amino acid changes, or variable length may cause changes in protein outcome, activity, and/or incorrect localization [5-8]. Another important mechanism underlying variations of ADME is the transcriptional regulation of ADME genes mediated by transcription factors (TFs), especially several nuclear receptors (NRs) such as pregnane X receptor (PXR or NR1I2) and constitutive androstane receptor (CAR or NR1I3). Upon ligand binding, the activated NR binds to target DNA and recruits other cofactors to activate target gene transcription [6, 9, 10]. On the other hand, the localization and function of enzymes and transporters could be regulated by post-translational modifications, such as phosphorylation, ubiquitination, glycosylation, and palmitoylation [11, 12]. Pathological and physiological conditions such as age, gender, pregnancy, and diseases in the liver, kidney, intestine, and other organs that are critical for ADME may also cause the alterations of those drug-metabolizing enzymes and transporters [7, 13]. Furthermore, exposure to drugs, environmental toxins, and diets that contain modulators of those proteins and/or inducers of liver or kidney injury or DDIs are known contributors to ADME/PK variations [14, 15]. Additionally, the contributions of epigenetic factors such as DNA methylation, histone acetylation, and noncoding RNAs (ncRNA) to ADME gene expression have been increasingly recognized in the past two decades [6, 16-21].

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MicroRNAs are a large family of regulatory ncRNAs approximately 22 nt in length that are derived from the genome [22]. The first miRNA, *lin-4*, was discovered in *Caenorhabditis elegans* (*C. elegans*) and identified to encompass complementary sequences to some repeated elements located in the 3′-untranslated region (UTR) of *lin-14* mRNA [23]. The base-paring and regulatory effects of *lin-4* RNA on LIN-14 protein levels were verified by another group almost at the same time [24]. In humans, more than 2,000 miRNAs have been identified and their functions in post-transcriptional gene regulation *via* targeting the miRNA-responsive elements (MREs), which are usually located within the 3'UTR of target transcripts accounting for more than two-thirds of human genes, have been well recognized. Subsequently, almost all cellular processes including drug metabolism and disposition have been revealed to be modulated by specific miRNAs [21, 22, 25-27]. Meanwhile, the concept of RNA interference (RNAi) was introduced, and synthetic double-stranded RNAs (dsRNAs) or short interfering RNAs (siRNAs; could be processed from dsRNAs) are effective to regulate target gene expression [28-30]. As such, RNAi has been recognized as a revolutionary approach and extensively used for the selective knockdown of a target gene. Indeed, exogenous siRNAs and endogenous miRNAs converge at the RNA-induced silencing complex (RISC) to achieve post-transcriptional regulation of target genes [31-33].

Extensive research has revealed that the majority of miRNAs post-transcriptionally regulate specific drug-metabolizing enzymes and transporters (Fig. **1**) by directly targeting the 3'UTR of their corresponding transcripts, while siRNAs are traditionally designed to act on the coding region (CDS) of target genes, and now increasingly on the 3'UTR (Fig. **2**). As the transcription of the ADME genes is controlled by NRs or other regulators, miRNAs could indirectly regulate ADME genes through targeting such factors [26]. Therefore, ADME/PK properties of drugs and other types of xenobiotics could be regulated by miRNAs through an interactive network consisting of miRNAs, ADME genes, nuclear receptors, and other forms of regulatory factors (Fig. **1**). Conversely, the biogenesis of miRNAs from miRNA-coding genes is also controlled by their own transcription factors or NRs that may be modulated by the drugs, leading to variations in drug exposure or ADME/PK [18, 34]. In this review, we first introduce the roles of miRNAs in the regulation of drug-metabolizing enzymes and transporters. After summarizing novel recombinant RNA technologies, we overview and discuss the applications of the recombinant or bioengineered RNA (BioRNA) agents to research on ADME/PK.

2. MICRORNAS IN POST-TRANSCRIPTIONAL REGULATION OF ADME GENES

2.1. MicroRNA Biogenesis and Mechanisms of miRNA/siRNA-Induced Gene Silencing

The canonical miRNA production process (Fig. **3**) begins with intranuclear, RNA polymerase II (Pol II)-mediated transcription of the miRNA-coding gene to produce a long, 5'-capped, 3'-polyadenylated stem-loop primary miRNA (pri-miRNA) transcript [35, 36]. Additionally, miRNAs encoded by the largest human miRNA gene cluster, C19MC, are transcribed in an RNA polymerase III-dependent manner [37-39]. The pri-miRNAs are then cropped into shorter miRNA precursors (pre-miRNA, ~70 nt) by the nuclear microprocessor complex which comprises the key enzyme Drosha, an RNase III family member, and the partner DGCR8/Pasha, a double-stranded RNA binding protein [40-42]. The pre-miRNAs are exported to the cytoplasm through the karyopherin Exportin-5 (Exp5) in a Ran guanosine triphosphate (Ran-GTP) dependent way [43]. The pre-miRNAs are subsequently cleaved into 19-25 nt miRNA duplexes with 2-nt 3' overhangs by another RNase III endonuclease, Dicer (Fig. **3**), along with the cofactors the human immunodeficiency virus transactivating response RNA-binding protein (TRBP), and protein kinase R-activating protein (PACT) within cytoplasm [38, 44-46].

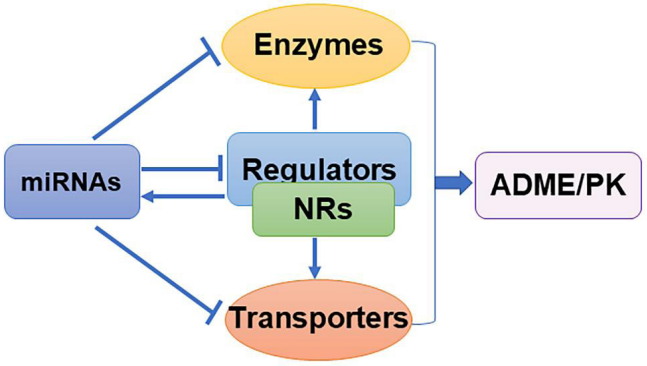

Fig. (1). Role of miRNAs in ADME/PK involving post-transcriptional gene regulation of metabolic enzymes, transporters, and their regulators. Genes encoding drug-metabolizing enzymes and transporters that are directly involved in the absorption, distribution, metabolism, and excretion of drugs are commonly referred to as ADME genes. MiRNAs have been shown to regulate ADME gene expression at the post-transcriptional level *via* direct targeting of ADME gene transcripts and/or acting on their regulators, such as nuclear receptors (NRs). Nuclear receptors typically bind to the DNA promoter regions of targeted ADME genes to achieve transcriptional gene regulation. Alteration of drug-metabolizing enzyme and transporter levels could result in changes in the ADME/PK properties of related drugs. *Vice versa*, the processing of miRNAs could be controlled by multiple regulators that may be affected by drugs or xenobiotics. (*A higher resolution / colour version of this figure is available in the electronic copy of the article*).

TRBP and/or PACT may not be necessary for the Dicer-catalyzed pre-miRNA cleavage, whereas TRBP is able to stabilize Dicer. Both TRBP and PACT play important roles in the recruitment of (AGO2), and stabilization of miRNAs, therefore they are essential for miRNA-mediated posttranscriptional gene regulation [44-46]. It should be noted that there are multiple branches in miRNA processing and the exact mechanism by which each factor participates in this process is still not fully disclosed [38, 47, 48].

The miRNA duplex is then loaded onto one member of Argonaute (AGO) proteins, AGO1-4, to form the pre-RISC, and then the complex releases the passenger strand to form the activated RISC [48-51]. The guide strand within the RISC binds to the target transcript *via* complementarity to suppress target protein expression *via* AGO-dominated mRNA degradation and/or protein translation inhibition [49, 51-56] (Fig. **2**). It has been recognized that miRNAs usually bind to the target transcript *via* partial complementarity, and consequently repress protein translation by interfering with the recruitment and functions of the factors involved in protein translation, whereas perfect or near-perfect base-pairing of the miRNA or siRNA with target transcript leads to mRNA degradation and translation inhibition [52, 57]. In mammals, all four AGOs are functional in protein translation inhibition, while only AGO2 is capable of being an endonuclease to slice the mRNAs [49, 53]. While some studies showed that widespread mRNA degradation was induced by miRNAs with imperfect miRNA-mRNA pairing in mammals, probably through the deadenylation, decapping, and finally 5'- to 3'- decay pathway [58-62], protein outcome is the hallmark of the miRNA-controlled gene regulation. By contrast, siRNAs are generally designed to perfectly match targeted sequences that are usually located within the CDS and sometimes the 3'UTR of the target genes (Fig. **2**).

2.2. MicroRNAs in the Regulation of Drug-metabolizing Enzymes

Enzymes that mediate the metabolism of drugs and some exogenous substances are classified into two groups, Phase I enzymes

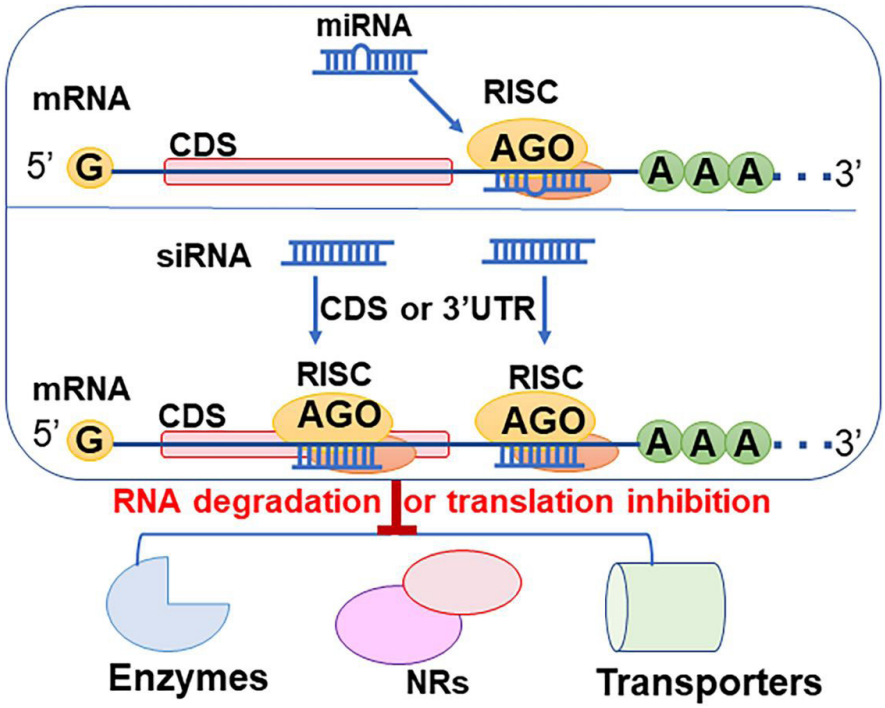

Fig. (2). Mechanisms of miRNA- and siRNA-mediated ADME gene regulation. The functional strand of the miRNA or siRNA duplex is loaded into an Argonaute protein (AGO) to form the RNA-induced silencing complex (RISC) and subsequently binds to target mRNA through complementary base pairing to exert RNA degradation and/or translation inhibition for the control of drug-metabolizing enzyme, transporter, and NR protein levels. MiRNAs usually bind to the 3'-untranslated region (3'UTR) of target transcript *via* imperfect complementarity, while siRNAs are often designed to target the coding region, and now increasingly 3'UTR, of target mRNA through perfect base pairing. (*A higher resolution / colour version of this figure is available in the electronic copy of the article*).

that catalyze the oxidation, reduction, and hydrolysis of the substances to introduce functional groups, and Phase II enzymes that are responsible for conjugation reactions to bring in small molecule moieties. While some drugs may be conjugated directly, drugs are usually subjected to sequential reactions catalyzed by Phase I and Phase II enzymes to form metabolites with higher polarity, through which the drugs are often deactivated, sometimes activated, and finally eliminated [63]. P450 superfamily enzymes that function as monooxygenases participate in more than 75% of Phase I biotransformations, whereas members of the UGT superfamily are the major enzymes involved in Phase II metabolism in humans, which together form the predominant components of the xenobiotic metabolizing system [21, 63, 64].

The human P450 superfamily is divided into 18 families that are comprised of 57 well-characterized members in total. It is predicted that almost all the P450 enzymes might be regulated by miRNAs, among which 17 isoforms were identified experimentally as targets for dozens of miRNAs [21, 64, 65]. Tsuchiya *et al*. validated CYP1B1 as a direct target of miR-27b [66], which is the first report on the miRNA-mediated post-transcriptional regulation of a drug-metabolizing enzyme. The direct association of miR-27b and CYP1B1 3'UTR was predicted by *in silico* analysis and verified by luciferase reporter gene assay through inhibition and re-introduction of miR-27b in human MCF-7 and Jurkat cells, and then the determination of CYP1B1 protein level and enzymic activity confirmed the downregulation of CYP1B1 by miR-27b, which together with mRNA level evaluation represent a classic methodology for the investigation of miRNA-mediated ADME gene regulation. The same group also revealed the inverse correlation of miR-27b and CYP1B1 in breast cancer tissues, which could be explained by the suppressive effect of miR-27b on CYP1B1 expression, indicating the significance of miRNA-mediated gene regulation in cancer. Meanwhile, Pan *et al*. showed that miR-27b acts as a suppressor of CYP3A4 [67], the most abundant human P450 isoform in both the liver and intestine. The authors first identified that miR-27b directly targets CYP3A4 3'UTR using two separate computational algorithms and luciferase reporter assays. Interestingly, levels of CYP3A4 protein, transcripts containing CDS or 3'UTR were all downregulated by miR-27b, among which 3'UTR-containing transcripts showed a lower level than the CDS, suggesting both translation inhibition and mRNA degradation mechanisms are involved in miR-27-controlled CYP3A4 regulation. Another direct target of miR-27b, vitamin D receptor (VDR/NR1I1), an NR known to transcriptionally control CYP3A4 expression, was further identified [67]. Likewise, retinoid X receptor alpha (RXRα; NR2B1), an NR that can control CYP3A4 expression was reported to be targeted by miR-27b [68]. The above studies demonstrate that CYP3A4 could be directly and indirectly regulated by miR-27b which might lead to an overt greater effect. In addition, another study revealed the regulation of some human P450s by specific miRNAs, such as CYP2C19 by miR-29a-3p [69], CYP2B6 by miR-25-3p [70], and CYP2E1 by miR-214-3p [71], in which an electrophoretic mobility shift assay was developed to evaluate the miRNA-MRE interactions. More miRNA-governed regulation of P450 enzymes can be found in some recent reviews [20, 21, 26, 72].

The human UGTs are a family of membrane proteins composed of 19 functional isoforms that are categorized into four subfamilies, with UGT1A as the most abundant subfamily [73]. All9 members of the UGT1A subfamily are encoded by a single gene and share the same 3′UTR. Papageorgiou *et al.* comprehensively screened the regulatory effects of a miRNA mimic library containing 2048 known human miRNAs on the UGT1A family using UGT1A 3'UTR luciferase reporter gene assays in HEK293 cells. MiR-103b, miR-141-3p, miR-200a-3p, miR-376b-3p, and miR-1286 were

shown to decrease the luciferase activity of reporter gene-carrying UGT1A 3'UTR by more than 30%, and corresponding MREs for individual miRNAs were also identified within the UGT1A 3'UTR. The functional study demonstrated that miR-21-3p, miR-103b, miR-141-3p, miR-200a-3p, and miR-376b-3p mimics inhibited the activity and mRNA levels of UGT1A1 and UGT1A6 in human colon cancer cell line LS180. Furthermore, miR-21-3p, miR-141-3p, and miR-200a-3p were shown to suppress the mRNA levels and metabolic activities of UGT1A1 in primary human hepatocytes [74]. In addition, miR-491-3p was found to strongly inhibit the UGT1A1 3'UTR luciferase reporter activity [74], which is consistent with an earlier study showing that miR-491-3p repressed the mRNA levels of UGT1A1, UGT1A3, UGT1A6, as well as drug-metabolizing activity of UGT1A1, by directly targeting UGT1A 3'UTR in HuH-7 cells [75]. Some studies also reported the regulatory effects of miRNAs on other Phase II enzymes, such as the suppression of human sulfotransferase 2A1 (SULT2A1) by hsa-miR-495-3p and hsa-miR-486-5p [76]. Findings on miRNA-controlled regulation of Phase II enzymes can be found in some reviews published recently [26, 77, 78].

2.3. MicroRNAs in the Regulation of Transporters

Drug transporters are crucial for the absorption, distribution, and excretion of drugs, other exogenous and endogenous substances, and their metabolites, and therefore play important roles in drug efficacy and toxicity. In some cases, transporter-based drug-drug interactions may occur and lead to variations in ADME/PK. Transporters that mediate the transmembrane movement of drugs between different body fluid compartments, cells, and tissues are classified as two major superfamilies, the ABC family which are ATP-dependent efflux transporters, and the SLC family which mainly function as uptake transporters without the assistance of ATP. Transporters located on epithelial cells in the intestine, liver, kidney, and the brain are of particular interest as they play critical roles in the translocation of the drugs across those epithelial cells, which may subsequently determine the PK/PD of relevant drugs [79]. In addition, the upregulation of efflux transporters such as MDR1, MRPs, and BCRP has been demonstrated to be one of the key mechanisms of multi-drug resistance (MDR), a major obstacle in cancer chemotherapy. As such, tremendous efforts are being made to investigate the regulatory mechanisms of those MDR proteins [79-82].

Many miRNAs have been shown to modulate ABCC/MRP protein outcomes through post-transcriptional gene regulation, such as ABCC1/MRP1 by miR-1291, miR-145, miR-1268a, miR-185-5p, miR-7, miR-210-3p, miR-133a and miR-326 [83-89], ABCC2/MRP2 by let-7c, miR-379, miR-297, miRNA-133a, miR-490-3p [90-94], ABCC3/MRP3 by miR-149, miR-192-5p, miR-181b-2-3p [95-97], and ABCC4/MRP4 is regulated by miR-124a (miR-124-3p) and miR-506 [98, 99]. However, alternative splicing and/or polyadenylation might occur during mRNA maturation, which might lead to variable lengths of mRNAs and/or 3'UTRs, and thus change the number or sequence of possible binding sites of miRNAs [100]. Bruhn *et al*. reported that the mRNAs of ABCC1, ABCC2, and ABCC3 all include multiple 3′UTR variants in human cancer cell lines and tissues [101]. Using luciferase reporter assay, they found that miR-379 could not suppress the activity of the shorter ABCC2 3'UTR variant lacking respective MRE [101]. A similar study showed that a truncated ABCG2 3'UTR was highly expressed in drug-resistant colorectal cancer cells [102]. As ABCG2 is a validated miR-519c target, and the truanted ABCG2 transcript lacking miR-519c MRE site escaped from miR-519c induced RNA degradation and/or translation inhibition, which could contribute to ABCG2 overexpression and MDR. Moreover, the same group revealed one more direct target of miR-519c, HuR, which is a binding protein of ABCG2 and could stabilize ABCG2 mRNA as well, suggesting that ABCG2 expression could be controlled by miR-519c through direct and indirect pathways [102-104].

The SLC transporters associated with the influx of drugs and xenobiotics mainly include the following subfamilies, OCTs (SLC22), organic anion transporters (OATs, SLC22), the organic cation/carnitine transporters (OCTNs, SLC22), OATPs (SLCO), and multidrug and toxin extrusion proteins (MATEs, SLC47A) [79]. There are increasing evidence showing that miRNA-mediated post-transcriptional regulation is one cause of interindividual variations of these proteins. Gene expression analysis with 26 human liver samples revealed that miR-24 levels are negatively associated with the levels of hepatocyte nuclear factor 4 alpha (HNF4α) mRNA as well as OATP2B1 mRNA and protein [105]. HNF4α is a liver-enriched nuclear receptor that controls hepatic OATP2B1 transcriptional expression, and it has been reported as a direct target of miR-24 [106]. In addition, the computational analysis predicted 2 MREs for miR-24 within OATP2B1. Thus, the authors investigated the direct effect of miR-24 on OATP2B1 using a luciferase reporter assay. The results demonstrated that miR-24 was able to suppress OATP2B1 expression by promoting mRNA degradation and translation inhibition [105]. Then the suppression of endogenous OATP2B1 and HNF4α expression by miR-24 was confirmed in HepaRG cells by using miR-24 precursor [105]. Interestingly, another group reported miR-24 mediated regulation of OATP2B1 using OATP2B1-overexpressing HEK293 cells and Caco-2 cells around the same time [107]. However, inhibition of miR-24 did not lead to OATP2B1 upregulation in Caco-2 cells. These studies indicate that miRNA-controlled post-transcriptional regulation may cause interindividual differences in tissue and/or disease-specific expression of drug transporters, offering new insights into causes of interindividual variations in ADME and the development of more effective drug treatment, especially for advanced cancer. There are also some recent reviews on the role of miRNAs in regulating drug transporters [6, 26, 108-110].

2.4. MicroRNAs in the Regulation of Transcription Factors in ADME

Many transcription factors especially nuclear receptors play vital roles in the transcriptional gene regulation of drug-metabolizing enzymes and transporters. Nuclear receptors, such as PXR, CAR, VDR, retinoid X receptor (RXR, NR2B1), and peroxisome proliferator-activated receptor α (PPARα or NR1C1)), typically bind with the corresponding exogenous or endogenous ligands to be activated and then form dimers with their respective partners. The NR dimers subsequently bind to target genes to activate transcription [6, 9, 111]. Contemporary studies on miRNA-mRNA association usually start with miRNA array, deep-sequencing, or bioinformatics analysis to predict the network of possibly involved miRNAs and genes. Thus, specific miRNA-mediated NR and ADME gene regulation may be investigated more extensively.

Global transcriptome analysis of liver tissues from donors with and without inflammation disclosed a miRNA-NR-P450 regulation network [112]. Luciferase reporter studies validated the putative MREs in the 3'UTRs of RXRα, CYP2C8, CYP2C9, CYP2C19, and CYP3A4 for miR-130b-3p, miR-452-5p, miR-155-5p, miR-155-5p/6807-5p, and miR-224-5p, respectively. Functional studies further showed that miR-155-5p remarkably repressed the activities of CYP1A2, 2C9, 2C19, 2D6 and 3A4, whereas miR-452-5p moderately suppressed the activities of CYP1A2, 2B6, 2C8 [112]. An earlier study by the same group revealed that miR-130-3p directly interacted with CYP2C9 and suppressed the metabolic activities of CYP2C9, as well as CYP1A2, CYP2B6, CYP2C8, CYP2C19, and CYP3A4, although precise mechanisms remain elusive [113]. As RXRα usually dimerizes with PXR, CAR, or PPARα to regulate P450 expression, the suppression of RXRα by miR-130b-3p [112] could be one reason for the broad effects of miR-130b-3p on multi-

ple P450s. Likewise, NR or other regulators could be involved in the influence of miR-155 on the expression and activities of multiple P450s, which warrants further investigation. These studies also suggest that miRNAs may coordinate with other factors to regulate ADME gene expression under some disease conditions such as inflammation. There are also several reviews covering miRNA regulation of NRs and regulatory factors in ADME [6, 26, 114].

3. CONVENTIONAL SMALL RNA REAGENTS AND PRODUCTION METHODS

Gain- and loss-of-function are common approaches to investigate the roles of miRNAs in the regulation of ADME gene expression and effects on drug metabolism and disposition. Corresponding miRNA agents and antisense oligos (antagomirs or inhibitors) have been developed and produced to imitate and inhibit the functions of miRNAs, respectively. In addition, siRNA agents may be produced to selectively inhibit or silence the ADME gene of interest. Some common, conventional, small RNA reagents are summarized herein, and production methods are discussed.

3.1. Short Hairpin (shRNA) Expression Plasmid and Virus

With the understanding of miRNA biogenesis (Fig. **3**), small RNA expression systems have been established using DNA plasmids or virus vectors (Fig. **3A**). In particular, the DNA sequence encoding either a pre-miRNA (or pre-miRNA like shRNA) or siRNA duplex connected by a small, 4- to 29-nt loop (shRNA) is cloned into target plasmid or virus vectors driven by an RNA pol III or II promoter, predominantly pol III promoter (*e.g*., H1 or U6). Early studies directly employed the natural pre-miRNA sequences, and target miRNAs were proved to be effectively expressed to regulate target genes [115, 116]. Further studies demonstrated that pre-miRNA-coding sequences with flanking sequences from the pri-miRNAs would notably improve the performance of target miRNAs, which has become a common way to design miRNA expressing-plasmids or virus vectors [117, 118]. The shRNA is usually designed as a simple stem-loop structure consisting of a 19- to 29-nt, perfectly matched duplex connected by a small loop [118, 119]. Furthermore, shRNAs are also designed by using natural pre-miRNA and/or pri-miRNA, through which the shRNAs maintain the structural characteristics of natural miRNAs, including the flanking sequences, loops, stems, and bulges of pre-miRNAs. Interestingly, it was reported that the siRNA duplex embedded in a natural miRNA expression scaffold showed comparable gene silencing effects with mitigated toxicity as simple stem-loop shRNA carrying the same siRNA sequence [117, 120, 121].

The shRNA-expressing plasmids are transfected into the target cells using transfection reagents, while the viruses could directly infect target cells after being packaged with host cells. The plasmid DNA and DNA reversely transcribed from virus RNA could be integrated into the genome of host cells (Fig. **3A**) to achieve expression of target miRNA or siRNA by following the miRNA biogenesis pathways (Fig. **3**). Plasmids are suitable for cell lines with high transfection efficiency, while virus infection is a viable transgenic method for cell types that are hard to be transfected, such as primary cells and suspension cells. While the use of the stable shRNA-expressing cell line is to the benefit of experiment reproducibility, avoiding multiple rounds of experiments, the establishment of stable cell lines is time-consuming, and unexpected changes might occur in the established cell models. It is also possible that the introduced exogenous genes could be lost after multiple passages. Therefore, it is necessary to frequently monitor the levels of target small RNA and its target genes. Because the long-lasting gene knockdown is sometimes not desired as it may lead to side effects, some inducible shRNA-encoding plasmids or virus systems have been developed accordingly [118, 122]. However, the plasmids and viruses are not "true" RNA agents, and the small RNA levels and regulatory effects highly depend on the transfection and integration efficacy as well as biogenesis in the host cells. Therefore, the actual levels or doses of target small RNAs are largely variable.

3.2. Synthetic RNA Agents

Synthetic RNA analogs (Fig. **3B**) produced by chemical synthesis or *in vitro* transcription are the most widely used RNAi agents. Phosphoramidite-based, solid-phase chemical synthesis has been developed as a common and automatic method for the production of target RNA agents that could also accommodate a variety of chemical modifications [123]. RNAs are synthesized by the repetitive addition of individual nucleosides on the solid support through the sequential deprotection, coupling, capping, and oxidation (if phosphite is the activated phosphorous ester) steps [123]. In addition, protective groups such as triisopropylsilyloxymethyl (TOM), bis(2-acetoxyethoxy) methyl orthoester (ACE) have been developed and introduced in the synthesis system to protect the 2'-hydroxy group of oligoribonucleotides. After desired number and order of nucleoside addition cycles, the reaction is terminated, followed by the RNA cleavage from the solid support and removal of base-protecting groups. The synthetic products may be purified by high-performance liquid chromatography (HPLC) to obtain ready-to-use RNAs with high purity. Furthermore, the internucleotide bonds can be modified in several forms, among which phosphoramidite is the most commonly used [123, 124]. To obtain better physicochemical and PK properties such as higher metabolic stability and longer half-life, various chemical modifications are also commonly introduced into the ribose or nucleobases of target RNA reagents.

Fire *et al*. first found that small RNAs synthesized biochemically with T3 and T7 polymerase could be used to achieve effective RNA interference [29]. Another study showed that siRNAs and shRNAs synthesized by T7 RNA polymerase were effective to silence target genes [125]. Both studies suggest that RNAi reagents produced by *in vitro* transcription serve as alternatives to chemically synthesized RNA materials. Indeed, *in vitro* transcription is a widely used enzymatic method for the production of single-stranded RNAs (ssRNAs). Transcription is carried out by using an RNA polymerase (*e.g*., T7 RNA polymerase) and a DNA template with a corresponding promoter. The yield of target RNAs relies mainly on the activity and reliability of the polymerase and subsequent purification steps. *In vivo* transcription represents a simple and versatile method as various types of RNA agents in variable lengths can be produced with ease. However, previous studies have shown that the *in vitro* transcription fidelity is template-dependent, and mutations or unexpected nucleotides at 3′ or 5′ ends may be introduced into the products [126, 127]. In addition, recombinant Dicer could be used to process single- or double-stranded RNAs, produced by chemical synthesis, *in vitro* transcription, or other means, to offer the desired siRNA or miRNA reagents.

With the advantage of incorporating specific chemical modifications, synthetic RNAs produced by *in vitro* transcription and chemical synthesis generally lack natural posttranscriptional modifications that are largely present on nucleobases [127-129]. In addition, artificial modifications introduced in the synthetic agents may increase the risk of side effects, such as immunogenicity. Synthetic RNA reagents are often delivered into cells (Fig. **3B**) by using various kinds of commercial transfection reagents to induce transient or short-term gene silencing that unlikely triggers any feedback or adaptive effects in the stably expressed systems. On the other hand, a sharp increase in cytosol miRNA/siRNA level within a short period of time might cause off-target effects, and appropriate controls should be included to properly interpret the observations. Similar to plasmid materials, the effectiveness of synthetic RNAs is also dependent upon the transfection efficiency or intracellular uptake [122, 127, 130].

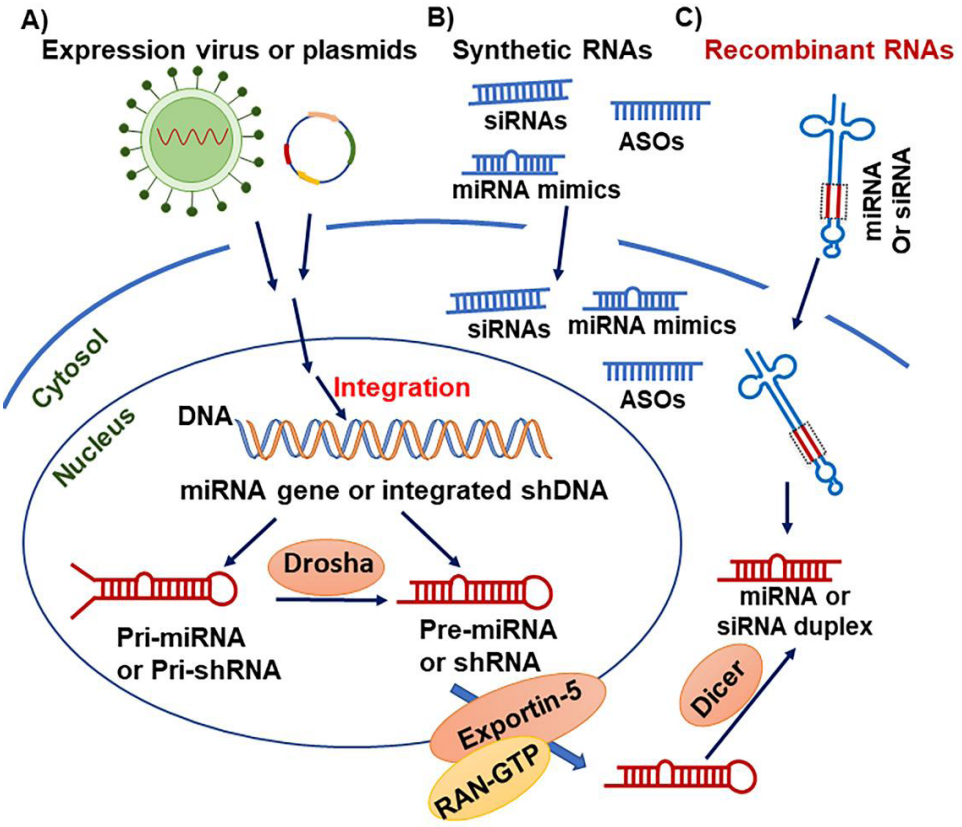

Fig. (3). MicroRNA biogenesis and common RNAi agents used in ADME research. Production of endogenous miRNAs is initiated by transcription of miRNA-encoding genes to produce primary miRNAs (pri-miRNA) that are subsequently sliced by nucleic RNase III enzyme Drosha to release precursor miRNAs (pre-miRNAs). Pre-miRNAs are then exported to the cytoplasm by Exportin-5 in a Ran guanosine triphosphate (Ran-GTP) dependent way. The cytoplasmic pre-miRNAs are further cleaved by another RNase III enzyme Dicer to produce miRNA duplexes. (**A**) MiRNA precursor or shRNA expressing plasmids or viruses are the conventional RNAi agents designed by mimicking the natural miRNA biogenesis pathways. After transfection or infection, desired small RNA-encoding genes are integrated into the genome of the host cells, and then the miRNAs or siRNAs are derived by following the natural miRNA biogenesis processes, except that some designs of short, stem-loop shRNAs bypass the pri-miRNA production step. (**B**) Small RNA analogs produced by chemical synthesis or *in vitro* transcription are the most widely used RNA reagents in basic and clinical studies. In addition to double-stranded miRNA mimics and siRNAs, another kind of synthetic RNA, single-stranded antisense oligos, namely antagomirs, are also used for the inhibition of miRNAs. (**C**) Recombinant or bioengineered RNA agents made in live cells using the tRNA fused pre-miRNA as a carrier are a novel class of RNAi agents, which, upon introduced into cells, are processed to target miRNA or siRNA duplex in a Dicer-dependent or independent manner. (*A higher resolution / colour version of this figure is available in the electronic copy of the article*).

4. NOVEL RNA BIOENGINEERING TECHNOLOGIES

Inspired by the development of recombinant protein production technologies and the applications of bioengineered proteins to structural and functional studies as well as protein drug development, considerable efforts have been made to create recombinant RNA or RNA bioengineering technologies (see reviews [33, 127, 129, 131]). To overcome the degradation by host RNases and achieve high-level expression of desired biologic RNA agents (Fig. **3C**) by live cell fermentation, two major approaches have been established for RNA bioengineering. One is to use specific RNAs with relatively compact and stable structures, such as 5S ribosomal RNA (rRNA) [132], transfer RNA (tRNA) [133, 134], hybrid tRNA/pre-miRNA [135, 136], and circular RNAs [137], as scaffolds or carriers to accommodate the payload RNAs. The other approach is to use host cells with deficient RNases [138, 139]. As the latter has a limited number of examples, this review is limited to the discussion of the RNA carrier approach.

4.1. Ribosomal RNA Scaffold

Ribosomal RNAs are key components of ribosomes, and rRNAs are the most abundant RNA species in eukaryotes (around 60% of total RNAs) and prokaryotes (over 90%). Bacterial rRNAs are comprised of three kinds of rRNAs 16S, 23S, and 5S [140]. Two consecutive studies from one research group showed that the so-called “tagged RNA molecules”, which were constructed by a 17-nucleotide sequence embedded into the 5S rRNA of *Vibrio proteolyticus* or *Pseudomonas putida*, could be stably expressed and accumulated to relatively high levels [141, 142]. Building on these findings, the 5S rRNA was developed as a carrier to accommodate multiple kinds of RNAs, including 13-nt or 50-nt random sequences, vascular endothelial growth factor (VEGF) aptamer, malachite green (MG) aptamer, all of which were highly expressed and showed some binding activities [132, 143, 144]. One optimal 5S rRNA-based recombinant RNA system is to insert the RNA sequence of interest (with DNAzyme-specific sequences) into stem II, thereby completely substituting the stem III - loop C region of 5S rRNA. The recombinant RNA is thought to retain the overall 5S rRNA structure and be recognized DNAzymes. *E. coli* ribosomal promoters P1 and P2 as well as T1 and T2 transcription terminators were used in those studies. After transformation and overexpression, target RNA was purified by using preparative polyacrylamide gel electrophoresis (PAGE) [144]. It was reported that several milligrams of pure RNAs could be produced from one gram of bacteria. While representing a potential way to produce RNAi agents, the

reliability of the rRNA scaffold and the application of resulting RNAs are less explored.

4.2. Transfer RNA Scaffold

Transfer RNAs are another class of abundant RNAs in cells, with simple and stable structures. The earliest reports of successful overexpression of tRNA species *in vivo* were published in the 1980s [145, 146]. Later, Ponchon *et al*. described the development and application of tRNA as a scaffold for the production of recombinant RNAs through bacterial fermentation [133, 134, 147]. Specifically, the RNA of interest is inserted into the tRNA by replacing the 3-nt anticodon region of the original tRNA. The resulting construct maintains the TΨC and D loops, as well as the cloverleaf structure of natural tRNA. It is speculated the recombinant RNA could be recognized as natural tRNA by the host cells, thereby circumventing the RNase-mediated slicing, and being accumulated to significant levels in bacterial cells. The expression vector of the recombinant RNA is composed of a lipoprotein (lpp) gene promotor or T7 promoter, the coding sequence of the recombinant RNA, followed by a ribosomal RNA operon transcription terminator, and an ampicillin resistance tag. The tRNA scaffold has been employed to express various kinds of target RNAs, for instance, an epsilon sequence of human hepatitis B virus (HBV), *Aquifex aeolicus* tmRNA domain, *E. coli* 16S ribosomal RNA decoding site, MG aptamer, Sephadex aptamer, hammerhead ribozymes, pre-miRNAs, and co-expressed RNA–protein complexes [133, 134, 148-152]. The recombinant RNAs could be purified by different methods, such as filtration, anion exchange, or affinity chromatography, to obtain up to tens of milligrams of pure RNAs. The desired RNAs could be released by RNase, ribozyme, or DNAzyme, or used directly for structural or functional studies. Although tRNA scaffold has emerged as a promising approach, the expression levels of recombinant RNAs have been revealed to largely depend upon the structures/sequences of the recombinant RNAs themselves [135, 148, 150].

4.3. Hybrid, tRNA Fused pre-miRNA (tRNA/pre-miRNA) Carrier

We initially intended to use the tRNA scaffold to produce pre-miRNAs for ADME and efficacy studies [148, 150], and unexpected results led to the establishment of a novel, robust and versatile tRNA/pre-miRNA carrier-based platform technology (Fig. **4**) [135, 136, 153]. After successfully constructing a set of pre-miRNA-expressing plasmids using a bacterial methionyl-tRNA scaffold, we surprisingly found that levels of recombinant tRNA/pre-miRNAs varied widely in bacteria, and most of them were not or minimally expressed and accumulated, and tRNA itself could not be overexpressed at all [135, 136]. Nevertheless, a few chimeras, such as tRNA/pre-miR-34a and tRNA/pre-miR-1291, were expressed at relatively high levels, which triggered us to directly utilize the high-expressing tRNA/pre-miR-34a or other pre-miRNA as a novel carrier to produce biologic RNAi molecules. Specifically, the small RNA of interest is incorporated into the tRNA/pre-miR-34a carrier by substituting the miR-34a duplex. Indeed, the tRNA/pre-miR-34a carrier provided a high-level expression of target miRNA and siRNA agents which accounted for > 10% of the total bacterial RNA. In addition, the tRNA/pre-miRNA carrier can accommodate many other forms of small single-stranded RNAs such as aptamers, although the overall success rate for all tested RNAs was around 30% [135, 136]. Thus, the tRNA/pre-miR-34a scaffold was further optimized by refining the pre-miR-34a sequence towards a more stable structure with fewer bulges. The optimized tRNA/pre-miR-34a carrier was superior to the first generation of tRNA/pre-miRNA carrier, offering much greater expression levels (> 30% of target RNA in total bacterial RNA) and a > 80% success rate for 42 designed recombinant RNAs [136]. Further identification of proper human tRNAs led to the establishment of third-generation, fully-humanized tRNA/pre-miRNA carriers that are more biocompatible with human cells for functional and therapeutic studies while retaining the expression levels and increasing success rate to about 100% [153].

The tRNA/pre-miRNA carrier is also characterized by its flexibility to accommodate almost all kinds of small RNAs with variable lengths. Besides replacing the miRNA duplex, the payload small RNAs could also be inserted at specific locations of the pre-miRNA, including the 5' or 3' end [127]. Following the construct design (Fig. **4**), the coding sequence of BioRNA is cloned into the target vector consisting of a proper promoter. After sequencing confirmation, the BioRNA-expressing plasmid is transformed into *E. coli* for fermentation production. Subsequently, total RNAs are extracted and separated by urea PAGE to validate the overexpression of target BioRNA, as indicated by the appearance of an extra strong band at the expected size compared to the wild-type bacteria (Fig. **4**). An anion-exchange fast protein liquid chromatography (FPLC) method has further developed and optimized to separate the BioRNA from the total RNAs with high yields [149, 153]. Fractions of target RNA are collected, combined, and concentrated to offer pure RNA. The quality of the final product is further validated by high-performance liquid chromatography (HPLC) analysis (Fig. **4**), and BioRNAs showing high purity (> 97%) are ready for further studies [154]. This tRNA/pre-miRNA-based RNA bioengineering technology has been proven as the most robust approach to achieve a consistent, high-yield, and large-scale production (tens of milligrams per liter of culture) of small RNA-loaded BioRNAs which are a novel class of true biologic RNAs for research and development [32, 33, 128, 129].

5. APPLICATIONS OF BIORNAS TO ADME RESEARCH

Mass spectrometry (MS)-based sequencing/mapping analyses of the BioRNAs revealed that such RNAs consist of a minimal number of natural post-transcriptional modifications [135, 148]. Deep sequencing studies showed that BioRNAs are readily processed to target miRNAs in a Dicer-dependent or independent manner after being introduced into human cells [136]. Functional studies further demonstrated that BioRNAs are active in regulating target gene expression and managing tumor progression and metastasis [136, 153, 155-157]. Herein, we focus on the discussion of the modulation of ADME gene expression by BioRNAs (Table **1**).

5.1. Recombinant RNAs for Drug Metabolism Studies

The first use of recombinant miRNA to modulate drug-metabolizing enzymes was published in 2014 [150]. It only involved a tRNA scaffold, and the tRNA fused pre-miR-27b was expressed at a low level (around 0.5 mg per liter of culture). After the purified tRNA/pre-miR-27b was introduced into human cells, mature miR-27b was released in a dose- and time-dependent manner. The functional study showed that BioRNA/miR-27b effectively suppressed both mRNA and protein levels of CYPA3A4 and the protein of VDR, and consequently inhibited the enzymatic activity of CYP3A4, as indicated by midazolam 1'-hydrolation metabolism. In addition, another recombinant miR-27b produced at a much higher yield by using the first generation of tRNA/pre-miR-34a carrier had similar regulatory effects on CYP3A4 and drug-metabolizing activity [158], demonstrating that BioRNAs are effective to modulate target P450 gene expression.

Previous studies have suggested that miR-34a directly targets RXRα and HNF4α, two NRs involved in P450 regulation, which might contribute to the observed negative correlation between miR-34a and CYP3A4 as well as CYP2C19 [106, 159, 160]. Therefore, BioRNA/miR-34a was utilized to determine the impact of miR-34a on the PK of P450 probe drugs in mouse models *in vivo* [161]. The results indicated that recombinant miR-34a slightly increased systemic exposure to midazolam, phenacetin, and dextromethorphan in

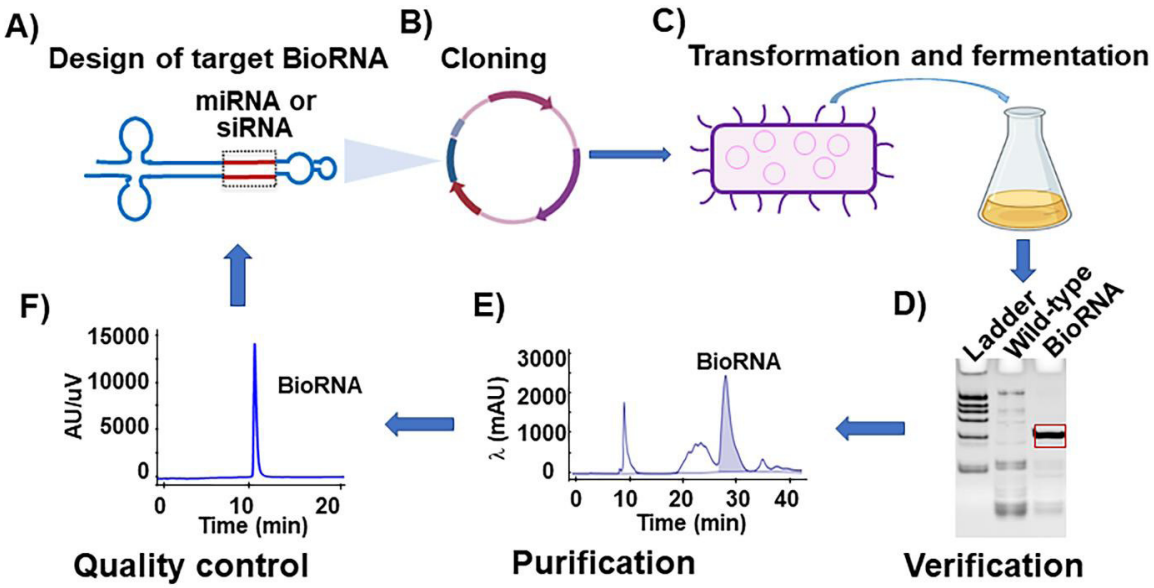

Fig. (4). Schematic illustration of the novel tRNA/pre-miRNA carrier-based RNA bioengineering platform technology. (**A**) Target recombinant RNA or bioengineered RNA (BioRNA) is designed using a tRNA/pre-miRNA carrier with payload miRNA or siRNA or other small RNA. (**B**) The coding sequence of the BioRNA is cloned into a target vector to offer the BioRNA-expressing plasmid. (**C**) The plasmids are transformed into *E. coli* to express the BioRNAs through overnight fermentation. (**D**) Overexpression of the target RNA is readily verified by urea polyacrylamide gel electrophoresis (PAGE) analysis. The strong extra band shown at the expected size in the BioRNA expressing sample, as compared to the wild-type *E. coli* total RNA sample, indicates the successful high-level expression of target BioRNA. (**E**) Target BioRNA is separated from the total bacterial RNA by using an anion-exchange fast protein liquid chromatography (FPLC) method. (**F**) The purity of the final BioRNA product is further quantified by high-performance liquid chromatography (HPLC) analysis. BioRNAs with high homogeneity (> 97%) can be used for further studies. (*A higher resolution / colour version of this figure is available in the electronic copy of the article*).

Table 1. Recombinant small RNA agents are shown to modulate ADME gene expression and alter DMPK.

Small RNA	ADME Gene	Effects	References
miR-27b-3p	VDR	Downregulates VDR mRNA and protein in LS180 cell line	[150, 158]
	CYP3A4	Downregulates CYP3A4 mRNA and protein expression and the subsequent metabolic activity in LS180 cell line	[150, 158]
miR-1291-5p	ABCC1	Downregulates MRP1/ABCC1 protein expression in PC cells and sensitizes the cells to doxorubicin treatment	[148]
	ASS1 (indirect)	Downregulates ASS1 protein expression in PC cells and suppresses arginine synthesis in ASS1 highly expressed PC cells	[163]
	GLUT1	Downregulates GLUT1 protein expression and suppresses glucose uptake and glycolysis in PC cells	[163]
miR-328-3p	ABCG2	Downregulates ABCG2 mRNA and protein expression and increases intracellular accumulation of mitoxantrone	[158]
	LAT1	Downregulates LAT1 protein expression in OS cells	[166]
	GLUT1	Downregulates GLUT1 protein expression and suppresses glucose uptake and glycolysis in OS cells	[166]
let-7c-5p	ABCC5 ABCC4	Downregulates MRP5/ABCC5 protein expression to increase the intracellular level of 5-Fu in HCC cells. Downregulate MRP4/ABCC4 protein level slightly.	[167]
miR-124-3p	ABCC4	Downregulates MRP4/ABCC5 protein expression in A549 cells	[136]
	MCT1	Downregulates MCT1 protein expression in OS cells	[153]
miR-34a-5p	CYPs	Showed minor or no effects on the PK of co-administered CYP substrate drugs in mice	[161]
NRF2-siRNA	NRF2	The siRNA effectively suppresses NRF mRNA and protein expression and subsequently decreases ABCC3/4 and ABCG2 mRNA levels to sensitize OS cells to chemotherapeutic drugs	[168]

mice [161]. This study exemplifies the utility of BioRNAs for *in vivo* studies on the potential influence of miRNAs on ADME gene expression and most importantly, the evaluation of possible interactions between therapeutic miRNAs or siRNAs and co-administered drugs.

5.2. Recombinant RNAs in the Modulation of Drug Transporters

There are also many reports on the application of BioRNAs to modulate drug transporter gene expression and functional consequences. MiR-1291-5p has been disclosed to target ABCC1, an important efflux transporter in MDR [89]. Following expression and purification, BioRNA/miR-1291 was utilized to investigate the role of miR-1291-5p in ABCC1 protein expression and drug resistance [148]. The results demonstrated that recombinant miR-1291-5p sensitized PANC-1 cells to doxorubicin *via* downregulating ABCC1 protein levels, supporting the role of miR-1291-5p in ADME and indicating the possible use of miRNA to overcome MDR. Furthermore, glucose transporter protein type 1 (GLUT1/SLC2A1) has been validated as a direct target for miR-1291-5p [162]. Our recent study verified the suppression of GLUT1 protein expression by BioRNA/miR-1291-5p in human carcinoma

cells and subsequently, changes in intracellular glucose uptake and glycolysis capacity [163]. Moreover, a rate-limiting enzyme in arginine synthesis, argininosuccinate synthase (ASS1), was shown to be downregulated by miR-1291-5p, despite the exact mechanism being unknown. By inhibiting GLUT1 and ASS1, the BioRNA/miR-1291 treatment sensitized pancreatic cancer (PC) cells to chemotherapy and arginine deprivation therapy [163]. These results from studies with BioRNA/miR-1291 indicate that miR-1291 may play an important role in the regulation of drug transport as well as nutrient metabolism and transport critical for cancer metabolism.

MiR-328-3p is another miRNA that has been shown to regulate several drug and nutrient transporters such as ABCG2 [164], GLUT1 [165], and large neutral amino acid transporter 1 (LAT1/SLC7A5) [166]. Li *et al*. verified the suppressive effect of miR-328-3p on ABCG2 expression by using BioRNA/miR-328-3p [158]. Comprehensive studies showed that BioRNA/miR-328-3p was selectively processed into mature miR-328-3p in breast cancer cells, resulting in a reduction of ABCG2 protein levels. This led to a greater intracellular accumulation of mitoxantrone, thereby enhancing the sensitivity of cancer cells to mitoxantrone [158]. Furthermore, a most recent study using BioRNA/miR-328 verified two direct targets for miR-328-3p, GLUT1 and LAT1 [166]. In silico analyses predicted MREs for miR-328-3p in the 3'UTR of *SLC2A1* and *SLC7A5*, and the subsequent luciferase reporter studies supported the interactions between miR-328-3p with 3'UTRs of *SLC2A1* and *SLC7A5*. Interestingly, while BioRNA/miR-328-3p reduced LAT1 protein levels in cells, it did not change the overall amino acid profiles that may be controlled by multiple factors. By contrast, the repression of GLUT1 protein levels by BioRNA/miR-328-3p resulted in lower cellular glucose uptake and glycolysis [166]. As a result, combination treatment with BioRNA/miR-328 and cisplatin or doxorubicin exhibited synergistic anti-proliferative activities against osteosarcoma (OS) cells. In addition, BioRNA/let-7c-5p was used to effectively repress the protein levels of ABCC5 and ABCC4, which contributed to its synergistic effects with 5-FU in the inhibition of cancer cell viability [167], and the use of BioRNA/miR-124-3p also validated the roles of miR-124-3p in the regulation of ABCC4 and MCT1 [136, 153].

5.3. Recombinant RNAs for the Modulation of Regulators

Besides miRNA agents, many siRNAs have been produced by using the tRNA/pre-miRNA carriers for the selective silencing of target genes [135, 168]. The nuclear factor erythroid 2-related factor 2 (NRF2) is a TF involved in the regulation of some efflux transporters and oxidative enzymes. Thus, activation/overexpression of NRF2 is also recognized as a tumor protective mechanism. A recombinant siRNA targeting NFR2, namely BioRNA/NRF2-siRNA, was thus designed and produced by using the tRNA/pre-miR-34a carrier [168]. NRF2 mRNA and protein were both suppressed significantly by BioRNA/NRF-siRNA, leading to the attenuation of NRF2-activated oxidative enzymes and the elevation of reactive oxygen species. Moreover, BioRNA/NRF-siRNA increased the sensitivity of osteosarcoma cells to chemotherapeutic drugs, doxorubicin, cisplatin, and sorafenib, which could be explained, at least partially, by the downregulation of NRF2-controlled efflux transporters, ABCC3, ABCC4, and ABCG2 [168]. These findings support the utility of BioRNAs in studying post-transcriptional gene regulation in ADME and offer insights into developing new therapeutic strategies including the use of bioengineered RNA molecules.

CONCLUSION

To sum up, the miRNA-controlled post-transcriptional gene regulation has been revealed as another important mechanism underlying broad interindividual variations of drug-metabolizing enzyme and transporter expression and activity under different physiological, pathological, and environmental conditions. The miRNAs are an addition to transcription factors and nuclear receptors as well as other epigenetic factors to form a complex network that coordinates the regulation of ADME gene expression. Alterations of protein outcomes of drug-metabolizing enzymes and/or transporters by regulatory miRNAs may change the ADME of relevant drugs and thus influence drug efficacy or safety. Nevertheless, as most studies are based on *in vitro* models focusing on specific pathways, more *in vivo* studies and disease-relevant conditions with comprehensive analyses of the overall ADME/PK properties are highly warranted to increase the depth of current knowledge on the roles of miRNAs in the regulation of ADME/PK. Moreover, additional efforts are expected to bridge the gap between mechanistic studies and experimental therapeutics, such as drug efficacy and safety, to advance the understanding of the clinical importance of miRNAs. With the disclosure of miRNA functions in modulating MDR and nutrient transporter protein outcomes, opportunities arise to develop new and more effective therapeutic strategies. On the other hand, dysregulated miRNAs might serve as biomarkers for the evaluation of ADME/PK or drug efficacy and toxicity.

Groundbreaking advances have been made in the production of RNA agents for ADME research. Synthetic RNAs and plasmid or virus-based vectors are two major forms of conventional materials being used for studying miRNA functions and RNAi research. However, the former are RNA analogs synthesized *in vitro* with extensive chemical modifications that may exhibit distinct biological activities and safety profiles, and the latter are DNA-based reagents that rely on efficient transcription in host cells. It is also unknown how chemical modifications would influence the unwinding of miRNA or siRNA duplex, stability of the passenger or sense strand, and selectivity, efficacy, and off-target effects of the guide or antisense strand. In addition, many previous reports did not even specify the functional strand of the studied miRNA, and almost all studies did not disclose the exact chemical modifications of the RNAi agents used. Thus, recombinant RNA technologies have been developed to produce true biologic RNA agents to better recapitulate the properties and functions of natural RNAs. Among them, the tRNA/pre-miRNA carrier-based technology is the most reliable to successfully produce target BioRNAs consisting of payload small RNAs. Studies have demonstrated a precise release of payload miRNAs, either 5p or 3p, from the pre-miR-34a carrier, confirming the predominant, functional strand of interest. Research with unparalleled BioRNAs has further advanced the understanding of miRNA pathways and their significance in ADME. Such BioRNAs produced in live cells have emerged as a novel class of biologic RNA molecules for studying miRNA regulatory mechanisms, gene functions, and new therapeutic approaches.

LIST OF ABBREVIATIONS

AGO	=	Argonaute
DDIs	=	Drug-Drug Interactions
dsRNAs	=	Double-Stranded RNAs
miRNAs	=	MicroRNAs
ncRNA	=	Noncoding RNAs
NRs	=	Nuclear Receptors
OATPs	=	Organic Anion Transporting Polypeptides
OCTs	=	Organic Cation Transporters
PK/PD	=	Pharmacokinetics/Pharmacodynamics
siRNA	=	Small Interfering RNA
UGTs	=	UDP-Glucuronosyltransferases
UTR	=	Untranslated Region

CONSENT FOR PUBLICATION

Not applicable.

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CONFLICT OF INTEREST

The authors declare no conflict of interest financial or otherwise.

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