Rapid production of novel pre-microRNA agent hsa-mir-27b in *Escherichia coli* using recombinant RNA technology for functional studies in mammalian cells

Mei-Mei Li, Wei-Peng Wang, Wen-Juan Wu, Min Huang and Ai-Ming Yu

Department of Biochemistry & Molecular Medicine, UC Davis Medical Center, Sacramento, CA, United States (M.-M.L., W.-P.W., W.-J.W., Q.C., A.-M.Y.); Laboratory of Drug Metabolism and Pharmacokinetics, School of Pharmaceutical Sciences, Sun Yat-Sen University, Guangzhou, Guangdong, China (M.-M.L., M.H.); Center of Drug Metabolism and Pharmacokinetics, College of Pharmaceutical Sciences, Soochow University, Suzhou, Jiangsu, China (W.-P.W.)
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Address correspondence to: Prof. Ai-Ming Yu, Department of Biochemistry & Molecular Medicine, UC Davis Medical Center, 2700 Stockton Blvd., Suite 2132, Sacramento, CA 95817. Tel: +1 916-734-1566; Fax: +1 916-734-4418; Email: aimyu@ucdavis.edu.

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Abbreviations: miR or miRNA, microRNA; miR-27b, microRNA-27b; hsa-mir-27b, Homo sapiens pre-microRNA-27b; ncRNA, noncoding RNA; E. coli, Escherichia coli; CYP, cytochrome P450; CYP3A4, cytochrome P450 3A4; VDR/NR1I1, vitamin D receptor; PXR/NR1I2, pregnane X receptor; RXRα/NR2B1, retinoid X receptor alpha; FPLC, fast protein liquid chromatography; LC, liquid chromatography; MS, mass spectroscopy; 1α-VD3, 1-alpha-hydroxycholecalciferol; MDZ, midazolam; 1’-HO-MDZ, 1’-hydroxymidazolam; HAR, harmine.
Abstract

Noncoding microRNAs (miRNAs or miRs) have been revealed as critical epigenetic factors in the regulation of various cellular processes including drug metabolism and disposition. However, research on miRNA functions is limited to the use of synthetic RNA and recombinant DNA agents. Herein, we show that novel pre-miRNA-27b (mir-27b) agents could be biosynthesized in Escherichia coli using recombinant RNA technology, and recombinant tRNA/mir-27b chimera was readily purified to a high degree of homogeneity (> 95%) using anion-exchange fast protein liquid chromatography. The tRNA-fusion mir-27b was revealed to be processed to mature miRNA miR-27b in human carcinoma LS-180 cells in a dose and time dependent manner. Moreover, recombinant tRNA/mir-27b agents were biologically active in reducing the mRNA and protein expression levels of cytochrome P450 3A4 (CYP3A4) which consequently led to a lower midazolam 1’-hydroxylase activity. These findings demonstrate that pre-miRNA agents may be produced by recombinant RNA technology for functional studies.
MicroRNAs (miRNAs or miRs) are a large family of small non-coding RNAs (ncRNAs) that govern target gene expression in cells via translation inhibition or mRNA deadenylation/degradation mechanisms. Our recent study have showed that miR-27b regulates the protein and mRNA expression of cytochrome P450 3A4 (CYP3A4) (Pan et al., 2009a), the most abundant CYP enzyme expressed in human liver and intestine. Change in CYP3A4 expression, which is transcriptionally controlled by a number of nuclear receptors, such as vitamin D receptor (VDR/NR1I1) (Schmiedlin-Ren et al., 2001; Thummel et al., 2001), pregnane X receptor (PXR/NR1I2) (Lehmann et al., 1998; Xie et al., 2000) and retinoid X receptor alpha (RXRa/NR2B1) (Wang et al., 2008), may lead to large variability in drug metabolism (Wang and LeCluyse, 2003; Thummel and Lin, 2014). MiR-148a was also found to influence CYP3A4 expression via the targeting of PXR (Takagi et al., 2008). Further studies suggest that other miRNAs, such as miR-34a and miR-577, could be involved in the modulation of CYP3A4 expression (Vuppalanchi et al., 2013; Lamba et al., 2014; Oda et al., 2014; Wei et al., 2014). In addition, other CYP enzymes (e.g., CYP1B1) and drug transporters (e.g., P-glycoprotein, breast cancer resistance protein, and multidrug resistance-associated proteins) may be regulated by miRNAs, such as miR-27b, miR-124, miR-519c and miR-1291 (Tsuchiya et al., 2006; To et al., 2008; Zhu et al., 2008; Pan et al., 2009b; Haenisch et al., 2011; Li et al., 2011; Pan et al., 2013; Rieger et al., 2013; Shukla et al., 2013; Xie et al., 2013; Markova and Kroetz, 2014). These findings support the concept that miRNAs are critical epigenetic factors in the modulation of drug metabolism and disposition that might consequently alter drug efficacy (Yu, 2007; Yu and Pan, 2012; Ingelman-Sundberg et al., 2013; Yokoi and Nakajima, 2013).
Currently, viral or non-viral vector-based miRNA expression systems are heavily used for *in vitro* and *in vivo* studies on miRNA functions (Liu and Berkhout, 2011). These agents are literally recombinant DNA materials, and this approach is generally less efficient because it relies on the host cells or organisms to transcribe the DNA to miRNA precursors. RNA agents such as the miRNA mimics and precursors as well as antisense antagonirs produced by chemical synthesis are another major class of materials used in miRNA research (Ling et al., 2013). These synthetic RNA agents all consist of unnatural modifications and it is unknown how chemical modifications may alter the biological activity of miRNAs despite that the mimics exhibit a longer half-life. *In vitro* transcription (Beckert and Masquida, 2011) may produce RNA agents in variable lengths, whereas a large scale production requires more but inexpensive RNA polymerases. Very recently, tRNA (Ponchon and Dardel, 2007; Ponchon et al., 2009; Nelissen et al., 2012) and rRNA (Liu et al., 2010) have been used as scaffolds for successful production of recombinant RNAs in *Escherichia coli* (*E. coli*) for structural analyses. This recombinant RNA technology may also offer a novel means to biosynthesize RNA agents for functional studies.

In the present study, we showed that novel pre-miRNA hsa-mir-27b agents were successfully expressed in *E. coli* using recombinant RNA technology. Recombinant tRNA/mir-27b chimeras were isolated by anion-exchange Fast Protein Liquid Chromatography (FPLC). In addition, the purified tRNA/mir-27b agents were found to be processed to mature miR-27b in human carcinoma LS-180 cells, which consequently reduced CYP3A4 protein expression and resulted in a lower midazolam 1'-hydroxylase activity. These findings may offer novel clues for the development of natural pre-miRNA agents for functional studies in drug metabolism.
Materials and Methods

Chemicals and Materials. Midazolam (MDZ) and its metabolite 1’-hydroxymidazolam (1’-HO-MDZ) were purchased from Cambridge Isotope Laboratories (Tewksbury, MA) and Cayman Chemical Company (Ann Arbor, MI), respectively. 1-Alpha-hydroxycholecalciferol (1α-VD3) was bought from EMD Millipore (Billerica, MA). Primers were synthesized by Integrated DNA Technologies (Coralville, IA). Restriction enzymes including SalI and AatII as well as the T4 DNA ligase were purchased from New England Biolabs (Ipswich, MA). All other chemicals and organic solvents were purchased from Sigma-Aldrich (St. Louis, MO) or Thermo Fisher Scientific Inc. (Waltham, MA).

Prediction of RNA Secondary Structure. The secondary structures of pre-miRNAs, tRNA and chimeric RNAs (Figure 1A) were predicted using the CentroidFold (http://www.ncrna.org/centroidfold) (Sato et al., 2009), Centroidhomfold (http://www.ncrna.org/centroidhomfold) (Hamada et al., 2011), and RNAstructure (http://rna.urmc.rochester.edu/RNAstructureWeb/Servers/Predict1/Predict1.html) (Reuter and Mathews, 2010).

Construction of Plasmids. To express the pre-miR-27b agents using tRNA scaffold (Figure 1A), the DNA fragments encoding 123-nt and 149-nt hsa-mir-27b were first amplified from human genomic DNA by PCR using the primers 5’- ACG CGT CGA CCC AGC GAT GAC CTC TCT AAC -3’ (forward) and 5’- CAT CGA CGT CCT TAA CTG TCC CCA TCT CAC C -3’ (reverse), and 5’- ACG CGT CGA CCG TCC CTT TAT TTA TGC CCA GC -3’ (forward) and
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5’- CAT CGA CGT CCG GCT CCA ACT TAA CTG TCC -3’ (reverse), respectively. The amplicon was cloned into the vector pBSMrnaSeph (kindly provided by Dr. Luc Ponchon, Université Paris Descartes, France) (Ponchon and Dardel, 2007; Ponchon et al., 2009) (Figure 1B) after being linearized by restriction endonucleases SalI and AatII. All hsa-mir-27b inserts were confirmed by Sanger sequencing analysis.

**Expression of Recombinant ncRNAs.** Recombinant ncRNA chimera and control tRNAs were expressed using HST08 *E. coli* strain (Clontech, Mountain View, CA), as described (Ponchon and Dardel, 2007; Ponchon et al., 2009). Total RNAs were isolated from *E. coli* using the Tris-HCl-saturated phenol extraction method, quantitated using NanoDrop (Thermo Scientific), and analyzed by denaturing urea (6 M) polyacrylamide (8%) gel electrophoresis (PAGE) to assess the expression of recombinant ncRNAs.

**Purification of Recombinant ncRNAs.** Recombinant ncRNAs were purified using a NGC QUEST 10PLUS CHROM FPLC System (Bio-Rad, Hercules, CA) consisting of two dual-piston pumps, a MULTI UV/Vis detector and a BioFrac fraction collector. For the isolation of tRNA/mir-27b, a UNO Q1 anion-exchange column (Bio-Rad) was equilibrated with five column volumes (at a flow rate 1.0 mL/min for 5 min) of Buffer A (10 mM sodium phosphate, pH = 7.0). The total RNAs (~ 1.0 mg) was then loaded onto the column and separated using a gradient elution method at the same flow rate 1.0 mL/min, 10 mL of 100% Buffer A, 5 mL of 0-50% Buffer B (Buffer A consisting of 1 M sodium chloride), 5 mL of 50% Buffer B, and 30 mL of 50-80% Buffer B, followed by 1 mL of 80-100% Buffer B, 2 mL of 100% Buffer B, 2 mL of 0-100% Buffer A, and 5 mL of 100% Buffer A. For the purification of tRNA/MSA, a UNO Q6
anion-exchange column (Bio-Rad) was equilibrated with five column volumes (at a flow rate 6.0 mL/min for 5 min) of Buffer A. After total RNAs (~ 5.0 mg) was loaded onto the column, and tRNA/MSA was separated using a gradient elution method at the same flow rate 6.0 mL/min, 6 mL of 100% Buffer A, 6 mL of 0-50% Buffer B, 48 mL of 50% Buffer B, and 60 mL of 50-60% Buffer B, followed by 6 mL of 60-100% Buffer B, 12 mL of 100% Buffer B, 6 mL of 0-100% Buffer A, and 12 mL of 100% Buffer A. FPLC traces were monitored at 260 nm using the UV/Vis detector. After analyzed on a denaturing PAGE gel, the fractions containing pure chimeric RNAs were pooled. Recombinant ncRNAs were precipitated with ethanol, reconstituted with nuclease-free water, and then desalted and concentrated with Amicon ultra-0.5 mL centrifugal filters (30 KD; EMD Millipore, Billerica, MA). The purity of ncRNAs was further validated using PAGE and quantified using NanoDrop before other experiments.

**Cell Culture and Transfection.** Human colon carcinoma LS-180 cells were purchased from ATCC (Manassas, VA) and cultured in Eagle's Minimum Essential Medium (ATCC) supplemented with 10% fetal bovine serum (Life Technologies, Carlsbad, CA) at 37°C in 5% carbon dioxide. Cells were transfected with purified recombinant ncRNAs using Lipofectamine 2000 (Life Technologies).

**Reverse Transcription Quantitative Real-Time PCR (RT-qPCR).** Regular RT-qPCR analysis of mRNA levels and stem-loop RT-qPCR analysis of miRNA levels were conducted using the methods described previously (Pan et al., 2009a; Li et al., 2011), except that total RNAs were isolated from LS-180 cells using ZR RNA MiniPrep kit (Zymo Research, Irvine, CA), cDNA was synthesized from total RNA with iScript Reverse Transcription Supermix (Bio-Rad), and
qPCR was conducted using SsoAdvanced SYBR Green Supermix (Bio-Rad) on a CFX96 Touch real-time PCR system (Bio-Rad). The same primers (Pan et al., 2009a; Li et al., 2011) were used for the analysis of mature miR-27b, U74, CYP3A4, VDR and GAPDH levels. Primers 5’- ACC TCT CTA ACA AGG TGC AG GCT TAG -3’ (forward) and 5’- CAC CTT CTC TTC AGG TGC AGA AC -3’ (forward) were used for the analysis of pre-miRNA mir-27b. U74 was used as internal control for miR-27b, and GAPDH was utilized for other analytes. The relative expression was calculated using the formula $2^{-\Delta C_T}$, where $\Delta C_T$ was the difference in $C_T$ value between the analyte and internal standard, and then normalized to the control treatment.

**Immunoblot Analysis.** LS-180 cell were treated with 2.5 µM 1α-VD3 or vehicle for 96 h, and then transfected with 50 nM tRNA/mir-27b or tRNA/MSA for 72 h. Cell lysates were prepared for immunoblot analysis using RIPA lysis buffer (Rockland Immunochemicals, Gilbertsville, PA) consisting of complete protease inhibitor cocktail (Roche Diagnostics, Mannheim, Germany), while total RNAs were isolated for qPCR analysis as described above. Protein concentrations were determined using the BCA Protein Assay Kit (Pierce, Rockford, IL). Whole-cell proteins (50 µg/lane) were separated on 10% sodium dodecyl sulfate-polyacrylamide gels (SDS-PAGE) and electrophoretically transferred onto PVDF membranes (Bio-Rad, Hercules, CA). After incubated with selective antibody against CYP3A4 (BD Biosciences, San Jose, CA), VDR or GAPDH (Santa Cruz Biotechnology, Santa Cruz, CA) and subsequently with a horseradish peroxidase rabbit anti-mouse IgG (BD Bioscience) or a peroxidase-conjugated goat anti-rabbit IgG (Jackson ImmunoResearch, West Grove, PA), proteins were visualized with an enhanced chemiluminescence detection system (Bio-Rad) and images were acquired by ChemiDoc MP Imaging System (Bio-Rad).
Midazolam 1’-Hydroxylation Activity Assay. Cellular MDZ metabolism was conducted as reported (Schmiedlin-Ren et al., 2001). Briefly, LS-180 cells were first treated with 2.5 µM 1α-VD3 for 96 h and then transfected with 50 nM tRNA/mir-27b or control tRNA/MSA. At 72 h post-transfection, 5 µM MDZ was administered to the media and cells were maintained at 37 °C. Two hundred µL of media were collected at different time points. After adding 200 µL of media containing 100 nM harmine (HAR; internal standard), analytes were extracted with 3 mL ethyl acetate. The extracts were evaporated and reconstituted for LC-MS/MS quantitation of MDZ and 1’-HO-MDZ using a AB SCIEX 4000 QTRAP System (AB SCIEX LLC, Framingham, MA) coupled with a Prominence LC-20AD HPLC system (Shimadzu, Columbia, MD), as we described previously (Granvil et al., 2003; Felmlee et al., 2008). All data were collected and processed using the Analyst software.

Statistical Analysis. All values were mean ± standard deviation (SD). Depending on the number of groups and variances, data were compared with unpaired Student’s t-test, or two-way ANOVA (GraphPad Prism, San Diego, CA). Difference was considered statistically significant if the probability was less than 0.05 (P < 0.05).
Results and Discussion

To better maintain the hairpin structure of hsa-mir-27b (97 nt in length) and evaluate the impact of length on the expression of recombinant ncRNAs, we extended the 5’ and 3’ flanking sequences of hsa-mir-27b to 123 nt and 149 nt. The secondary structures of resultant tRNA/mir-27b-123nt (227 nt in total) and tRNA/mir-27b-149nt (253 nt) chimeras as well as the tRNA scaffold and hsa-mir-27b were thus predicted using different algorithms (CentroidFold, Centroidhomfold and RNAstructure). All predictions consistently showed a conservation of the stem-loop structure of hsa-mir-27b within chimeric ncRNAs (Figure 1A), suggesting that chimeric tRNA/mir-27b would be accessible by cellular endoribonucleases such as Drosha and Dicer for the production of mature miR-27b. Therefore, their corresponding DNA segments were inserted into the pBSMmirSeph vector (Figure 1B) linearized by endonucleases SalI and AatII, and all clones were confirmed by Sanger sequencing analyses before the expression of recombinant ncRNAs.

To assess if recombinant tRNA/mir-27b was expressed, total RNAs were isolated from bacteria at 16 h post-transformation with hsa-mir-27b expression plasmid and subjected to RNA electrophoretic mobility assay. A successful expression of chimeric tRNA/mir-27b and tRNA/MSA was indicated by the appearance of new RNA bands ~200 nt and ~100 nt in length, respectively, in E. coli transformed with ncRNA expression plasmids, as compared to untreated bacteria (Figure 1C). It is noteworthy that the electrophoretic mobility of chimeric tRNA/pre-miRNA and tRNA/MSA is greater than that indicated by the single-stranded RNA markers. This is likely due to the presence of “double-stranded” stem structure in these ncRNAs (Figure 1A).
While tRNA/MSA was consistently expressed at a high level (~ 10 mg/L culture) (Ponchon and Dardel, 2007; Ponchon et al., 2009), the expression levels of tRNA/mir-27b were relatively low (< 500 μg/L culture) and thus the size of hsa-mir-27b showed minimal effect on tRNA/mir-27b expression (Figure 1C). This is also different from the tRNA/mir-1291 that was usually expressed at over 10 mg/L culture, whereas the levels were sharply decreased with the increase of mir-1291 length (unpublished data). Indeed, the lower expression of recombinant tRNA/mir-27b was associated with the occurrence of other RNA fragments (e.g., ~ 150 nt, Figure 2A), suggesting that tRNA/mir-27b might be susceptible to bacterial RNases. Therefore, further study is warranted to improve the expression level of recombinant tRNA/mir-27b chimera.

An anion-exchange FPLC method was thus developed for the purification of recombinant ncRNAs. Elution with low to high concentrations of sodium chloride solution successfully separated the recombinant tRNA/mir-27b (eluted at 31.2 min; Figure 1D) from other small RNAs, tRNAs and 5S rRNA using the Q1 column. Likewise, tRNA/MSA (18.5 min; data not shown) was nicely separated from other RNAs using the Q6 column. The purified tRNA/mir-27b (Figure 1E) and tRNA/MSA (data not shown) indeed showed a high degree of homogeneity (> 95%). A higher yield was also achieved for tRNA/MSA using the anion-exchange FPLC method (i.e., ~0.5 mg of tRNA/MSA from 5 mg of total RNAs obtained from 200 mL culture; 10% recombinant RNA/total RNAs) than that using affinity chromatography (unpublished data). In contrast, around 100 μg of tRNA/mir-27b was generally purified from 5 mg of total RNAs obtained from 200 mL culture, equivalent to a 2% yield of recombinant RNA/total RNAs, which is mainly due to the low expression level of tRNA/mir-27b (Figure 1C). Indeed, the FPLC purification yields of other high-level expressing pre-miRNAs including mir-1291 were all...
comparable (unpublished data), suggesting that the anion-exchange FPLC described in this study is a reliable method for the purification of recombinant ncRNAs.

To delineate whether chimeric tRNA/mir-27b can be processed into mature miR-27b, human colon carcinoma LS-180 cells were transfected with the purified recombinant tRNA/mir-27b and control tRNA/MSA, which is an excellent cell model system for studying CYP3A4 regulation (Schmiedlin-Ren et al., 2001; Thummel et al., 2001) and comprised of necessary machinery for miR-27b biogenesis (Pan et al., 2009a). Selective stem-loop RT and regular qPCR assays were employed to quantify mature miRNA miR-27b and pre-miRNA mir-27b, respectively (Pan et al., 2009a; Li et al., 2011) (Figure 2). The selectivity of stem-loop RT-qPCR for the analysis of mature miR-27b was supported by a low C_T value (< 25) for the cDNA prepared from total RNAs of tRNA/mir-27b-transfected cells versus much higher C_T values for the same amount of cDNAs prepared from the purified tRNA/mir-27b (> 30) and total RNAs of untreated or tRNA/MSA-transfected cells (> 28), as well as the decrease of mir-27b levels versus increase of miR-27b over time in the cells after transfection with tRNA/mir-27b (Figure 2C and 2D). The data showed a sharp increase in hsa-mir-27b levels in the cells transfected with tRNA/mir-27b (Figure 2A and 2C), indicating a successful delivery of recombinant tRNA/mir-27b into the cells. Such an over 100-fold increase of hsa-mir-27b levels persisted till 72 h post-transfection (Figure 2C), highlighting the stability of tRNA/mir-27b within the cells. Consequently, the levels of mature miR-27b were increased in a dose (Figure 2B) and time (Figure 2D) dependent manner in LS-180 cells transfected with tRNA/mir-27b. In addition, the levels of mature miR-27b formed from the same dose of tRNA/mir-27b-123nt and tRNA/mir-27b-149nt (Figure 2B) at the same time points (Figure 2D) were not significantly different. Together, these results support the
production of mature miR-27b from recombinant tRNA/mir-27b, despite that it remains unknown how exactly the chimeric tRNA/mir-27b are processed in the cells and what ribonucleases are involved in the processes.

Our previous studies have demonstrated that the well-conserved miR-27b can regulate CYP3A4 expression in human carcinoma cells through targeting of the 3-untranslated regions of CYP3A4 and VDR/NR1I1 (Pan et al., 2009a). Therefore, we evaluated whether recombinant tRNA/mir-27b is effective in the modulation of CYP3A4 expression in LS-180 cells. RT-qPCR analyses (Figure 3A) using gene specific primers showed that CYP3A4 mRNA levels were reduced around 10-fold by both tRNA/mir-27b-123nt and tRNA/mir-27b-149nt in LS-180 cells, whereas VDR mRNA levels were not altered. Immunoblot analyses (Figure 3B) using selective antibodies revealed that CYP3A4 and VDR protein levels were suppressed 30-50% and ~20%, respectively, in LS-180 cells after transfection with chimeric tRNA/mir-27b. These results indicate that recombinant tRNA/mir-27b is biologically active in the inhibition of CYP3A4 expression in LS-180 cells after being processed to mature miR-27b (Figure 2), which may involve posttranscriptional and transcriptional regulatory mechanisms (Pan et al., 2009a; Yu, 2009). Furthermore, we investigated the consequent effect of altered CYP3A4 expression on cellular drug metabolism capacity. MDZ, a widely used CYP3A4 probe drug that is not transported by P-glycoprotein or other transporters, was employed to assess cellular CYP3A4 enzymatic activity. MDZ and 1’-HO-MDZ, the major metabolite produced by CYP3A4 were quantified by specific and sensitive LC-MS/MS method. Our data (Figure 3C) revealed that the [MDZ]/[1’-HO-MDZ] metabolic ratios were increased over 5-fold in LS-180 cells at various time points after transfection with either tRNA/mir-27b-123nt or tRNA/mir-149nt, indicated a
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reduction of CYP3A4 enzymatic activity. Together, these results show that recombinant mir-27b chimeras are functional in the modulation of CYP3A4 protein outcome and consequently influence cellular drug metabolism capacity.

In summary, this study demonstrates a successful production of novel, biologically-active mir-27b agents using the tRNA scaffold. Recombinant tRNA/mir-27b chimeras are readily purified to a high degree of homogeneity using an anion-exchange FPLC method developed in the present study, despite that their expression levels are relatively lower than that of control tRNA/MSA and other pre-miRNAs (unpublished data). Our data show that the mir-27b chimeras are processed to mature miR-27b in human carcinoma LS-180 cells in a dose and time dependent manner. In addition, our results indicate that recombinant mir-27b suppresses CYP3A4 and VDR protein expression and results in a lower cellular drug metabolism capacity. These findings suggest that pre-miRNAs can be produced using tRNA based recombinant RNA technology, which represent novel ncRNA agents for studying miRNA functions in the control of drug metabolism and disposition.
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Authorship Contributions

Participated in research design: Yu, Li, Wang, Wu and Huang.

Conducted experiments: Li, Wang and Wu.

Contributed to new reagents or analytical tools: Yu, Li, Wang and Wu.

Performed data analysis: Li, Wang, Wu, Huang and Yu.

Wrote or contributed to the writing of the manuscript: Yu, Li, Wang, Wu, and Huang.
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References


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Footnotes

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Send reprint requests to: Dr. Ai-Ming Yu, Department of Biochemistry & Molecular Medicine, UC Davis School of Medicine, 2700 Stockton Boulevard, Sacramento, CA 95817, USA. Phone: 916-734-1566; Fax: 916-734-4418; E-mail: aimyu@ucdavis.edu.
Figure Legends

**Figure 1. Design and production of recombinant tRNA-fusion pre-miR-27b agents.** (A) Secondary structures of tRNA/MSA, hsa-mir-27b, and chimeric tRNA/mir-27b-123nt and tRNA/mir-149nt were predicted by CentroidFold. Consistent results were obtained using Centroidhomfold and RNAstructure. (B) The target hsa-mir-27b inserts encoding mir-27b-123nt and mir-27b-149nt were ligated into the pBSMrnaSeph vector linearized by endonucleases Sal I and Aat II to produce the recombinant pre-miRNA expression plasmids (pBSMmirSeph). (C) The tRNA/mir-27b-123nt and -149nt chimeras were successfully expressed in *E. coli*. The arrows indicate the recombinant ncRNA bands at expected sizes. Total RNAs (1 μg per lane) were analyzed by denaturing polyacrylamide (8%) gel electrophoresis. Untreated bacteria (blank) served as control. (D) FPLC traces during the purification of chimeric tRNA/mir-27b-149nt. Total RNAs were separated using anion-exchange FPLC and monitored at 260 nm. (E) Denaturing polyacrylamide (8%) gel electrophoresis indicated that fraction #6 consisted of high-purity (> 95%) recombinant tRNA/mir-27b-149nt. Similar results were obtained for tRNA/mir-27b-123nt (data not shown).

**Figure 2. Recombinant pre-miRNA mir-27b chimera is processed to mature miRNA miR-27b in human LS-180 cells.** The levels of mir-27b (A) and miR-27b (B) were increased in a dose dependent manner in LS-180 cells at 24 h post-transfection with the purified tRNA/mir-27b (3 and 10 nM), as determined by selective qPCR analysis. In addition, mir-27b (C) and miR-27b (D) levels varied in LS-180 cells at 24, 48, 72 and 96 h post-transfection with 10 nM chimeric tRNA/mir-27b. Cells treated with the same doses of tRNA/MSA or vehicles were used as
controls. GAPDH and U74 were utilized as internal control for the quantification of mir-27b and miR-27b, respectively. Values are mean ± SD of triplicate treatments with separate cultures and analyzed using two-way ANOVA. *P < 0.01, compared to the corresponding control treatment (vehicle or tRNA/MSA) at the same dose or time point. #P < 0.01, compared to the data obtained from the same tRNA/mir-27b agent at a lower dose (3 nM) or early time point (24 h).

**Figure 3. Recombinant pre-mir-27b is effective in the modulation of CYP3A4 expression.**  
(A) qPCR analyses revealed that recombinant tRNA/mir-27b significantly (*P < 0.001, one-way ANOVA) reduced CYP3A4 mRNA expression levels in LS-180 cells, compared to the tRNA scaffold. GAPDH was used as an internal control. (B) Western blot analyses showed that LS-180 cells transfected with tRNA/mir-27b had lower CYP3A4 protein expression levels than the cells transfected with control tRNA. GAPDH was used as a loading control. (C) CYP3A4 enzymatic activity, as measured by [MDZ]/[1'-HO-MDZ] metabolic ratio at various time points after the exposure to MDZ (at 72 h post-transfection), was significantly (*P < 0.001, two-way ANOVA) lower in LS-180 cells treated with tRNA/mir-27b than the tRNA/MSA control. MDZ and metabolite concentrations were determined by the LC-MS/MS method. Values are mean ± SD of triplicate treatments with separate cultures. *P < 0.05, compared to the control tRNA/MSA treatment.
Fig. 2

(A) Relative mir-27b expression (fold) at different doses of recombinant ncRNA (nM).

(B) Relative miR-27b expression (fold) at different doses of recombinant ncRNA (nM).

(C) Relative mir-27b expression (fold) at different time points after transfection (h).

(D) Relative miR-27b expression (fold) at different time points after transfection (h).

Legend:
- Vehicle
- tRNA/MSA
- tRNA/mir-27b-123nt
- tRNA/mir-27b-149nt

Symbols:
- * indicates significant difference compared to vehicle
- # indicates significant difference compared to tRNA/MSA

Fig. 3

(A) Relative CYP3A4 mRNA expression (%)

(B) Western Blot for CYP3A4, VDR, and GAPDH

(C) MDZ/[1H]-HO-MDZ levels after MDZ treatment (0, 30, 120, 240 min)