Supplementary Information accompanying

FolamiRs: Ligand-targeted, vehicle-free microRNA replacement therapy

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List of Supplementary Materials

Supplemental Materials and Methods

Materials

Amino acids for peptide synthesis were purchased from Aapptec, USA. *N*-Hydroxybenzotriazole (HOBt) and benzotriazol-1-yloxytris(pyrrolidino)phosphonium hexafluorophosphate) (PyBOP) were obtained from Sigma-Aldrich and Chem-Impex Int. (Chicago, IL) respectively. Solid phase peptide synthesis (SPPS) was performed using a standard peptide synthesis apparatus (Chemglass, Vineland, NJ) by following standard Fmoc-solid phase peptide synthesis procedure*(54)*. Unless otherwise specified, all other chemicals were purchased from Sigma-Aldrich (St. Louis, MO). All folate and folate-NIR dye conjugates were purified by preparative reverse phase (RP)-HPLC (Agilent) and LC/MS analyses were obtained using an Agilent mass spectrometer coupled with a UV diode array detector.

Synthesis of Folate-EDA conjugate:

In a peptide synthesis vessel ethylenediamine, polymer-bound (200-400 mesh)-resin (1.000 g, 0.17 mmol, 1. eq.) was loaded and swollen with dichloromethane (3 mL) followed by dimethylformamide (3 mL) for 1 h. Fmoc-Glu-OtBu solution (0.1808 g, 0.425 mmol, 2.5. eq.) was added to the vessel in DMF, *N,N*-Diisopropylethylamine (DIPEA-i-Pr_2Net, 0.2202 g, 1.7 mmol, 10. eq.) and (Benzotriazol-**1**-yloxy) tripyrrolidinophosphonium hexafluorophosphate (PyBOP, 0. 2212 g, 0.43 mmol, 2.5. eq.). Argon was bubbled for 4h, the coupling solution was drained, and the resin was washed with DMF (3x10 mL) and isopropanol (i-PrOH) (3x10 mL). Kaiser tests were performed to assess reaction completion. Fmoc deprotection was carried out using 20% piperidine in DMF (3x10 mL), before each amino acid coupling. The above sequence was repeated to complete the reaction with TFA-Pteroic-acid. The resin was washed with 50% ammonium hydroxide in DMF 3x10 mL (5 min) to cleave the trifluoro-acetyl protecting group on pteroic acid and washed with i-PrOH (3x10 mL) followed by DMF (3x10 mL). The resin was dried under argon for 30 min. Folate-EDA peptide was cleaved from the resin using a cleavage mixture consisting of 95% CF_3COOH, 2.5% H_2O and 2.5% triisopropylsilane. Ten mL of the cleavage mixture was added and argon was bubbled for 1.5 h. The cleavage mixture was drained into a clean flask. The resin was washed an additional 3 times with cleavage mixture. The combined mixture was concentrated under reduced pressure to a smaller volume (~ 5 mL) and precipitated by adding cold ethyl ether. The precipitate was collected by centrifugation, washed with ethyl ether (3 times) and dried under high vacuum. Crude reaction mixture was purified by RP-HPLC, (mobile phase A = 10 mM ammonium acetate, pH = 7; organic phase B = acetonitrile; method: 0% B to 30% B in 35 minutes at 13 mL/min) and furnished folate-EDA at 65% yield. LC-MS (A = 10 mM ammonium bicarbonate, pH = 7; organic phase B = acetonitrile; method: 0% B to 30% B in 12 minutes) R_T = 3.26 min ($M+H^+$ = 484.0).

Synthesis of Folate-DBCO conjugate:

To a stirred solution of Folate-EDA (0.0100 g, 0.0206 mmol, 1 eq.) and NHS-DBCO (0.0091 g, 0.0227 mmol, 1.1 eq.) in DMSO, DIPEA (0.0039 g, 0.0309 mmol, 1.5 eq.) was added dropwise. The reaction mixture continued with stirring at room temp. Progress of the reaction was monitored by LC-MS. After complete conversion of Folate-EDA the crude reaction mixture was purified by RP-HPLC (mobile phase A = 10mM ammonium acetate, pH = 7; organic phase B = acetonitrile; method: 0% B to 50% B in 35 minutes at 13 mL/min), and furnished Folate-DBCO at 85% yield. LC-MS (A = 10 mM ammonium bicarbonate, pH = 7; organic phase B = acetonitrile; method: 0% B to 50% B in 12 minutes) R_T = 4.62 min ($M+H^+$ = 771.0).

Synthesis of Folate-SS-DBCO conjugate:

To a stirred solution of Folate-EDA (0.0100 g, 0.0206 mmol, 1 eq.) and NHS-SS-DBCO (0.0128 g, 0.0227 mmol, 1.1 eq.) in DMSO, DIPEA (0.0039 g, 0.0309 mmol, 1.5 eq.) was added dropwise. The reaction mixture continued with stirring at room temp. Progress of the reaction was monitored by LC-MS. After complete conversion of Folate-EDA the crude reaction mixture was purified by RP-HPLC (mobile phase A = 10 mM ammonium acetate, pH = 7; organic phase B = acetonitrile; method: 0% B to 50% B in

35 minutes at 13mL/min), and furnished Folate-SS-DBCO at 82% yield. LC-MS (A = 10 mM ammonium bicarbonate, pH = 7; organic phase B = acetonitrile; method: 0% B to 50% B in 12 minutes) R_T = 5.4 min ($M+H^+$ = 934.0).

Synthesis of Folate-Cys (Folate-SH) conjugate:
In a peptide synthesis vessel H-Cys(Trt)-2-Cl-Trt-resin (100-200 mesh, 0.200 g, 0.088 mmol, 1 eq.) was loaded and swollen with dichloromethane (3 mL) followed by dimethylformamide (3 mL) for 1 h. To the vessel was then introduced the Fmoc-Orn(Boc)-OH solution (0.080 g, 0.176 mmol, 2.0 eq.) in DMF, i-Pr_2NEt (0.0684 g, 0.528 mmol, 6.0 eq.), and PyBOP (0.1832 g, 0.35 mmol, 4.0 eq.). Argon was bubbled for 4h, the coupling solution was drained, and the resin was washed with DMF (3x10 mL) and i-PrOH (3x10 mL). Kaiser tests were performed to assess reaction completion. Fmoc deprotection was carried out using 20% piperidine in DMF (3x10 mL), before each amino acid coupling. The above sequence was repeated to complete the reaction with Fmoc-Glu-OtBu (0.0749 g, 0.176 mmol, 2.0 eq.) and TFA-Pteroic-acid (0.0359 g, 0.088 mmol, 1.0 eq.) coupling steps. The resin was washed with 2% hydrazine in DMF 3x10 mL (5 min) to cleave the trifluoro-acetyl protecting group on pteroic acid and washed with i-PrOH (3x10 mL) followed by DMF (3x10 mL). The resin was dried under argon for 30 min. Folate-Cys peptide was cleaved from the resin using a cleavage mixture consisting of 92.5% CF_3COOH, 2.5% H_2O, 2.5% ethanedithiol and 2.5% triisopropylsilane. Ten mLs of the cleavage mixture was introduced and argon was bubbled for 1.5 h. The cleavage mixture was drained into a clean flask. The resin was washed an additional 3 times with cleavage mixture. The combined mixture was concentrated under reduced pressure to a smaller volume (~ 5 mL) and precipitated in ethyl ether. The precipitate was collected by centrifugation, washed 3 times with ethyl ether and dried under high vacuum. Crude reaction mixture was purified by RP-HPLC, (mobile phase A = 10 mM ammonium acetate, pH = 7; organic phase B = acetonitrile; method: 0% B to 30% B in 35 minutes at 13 mL/min) and furnished Folate-Cys at 72% yield. LC-MS (A = 10 mM ammonium bicarbonate, pH = 7; organic phase B = acetonitrile; method: 0% B to 30% B in 12 minutes) R_T = 3.73 min ($M+H^+$ = 659.0).

Synthesis of Folate-NIR dye conjugate:
To a stirred solution of Folate-Cys (0.010 g, 0.015 mmol, 1 eq.) and Maleimide-NIR Dye (0.019 g, 0.017 mmol, 1.1 eq.) in DMSO, DIPEA (0.0029 g, 0.0228 mmol, 1.5 eq.) was added dropwise. The reaction mixture continued with stirring at room temp. Progress of the reaction was monitored by LC-MS. After complete conversion of Folate-Cys the crude reaction mixture was purified by RP-HPLC, (mobile phase A = 10 mM ammonium acetate, pH = 7; organic phase B = acetonitrile; method: 0% B to 30% B in 35 minutes at 13 mL/min) and furnished Folate-NIR at 85% yield. LC-MS (A = 10 mM ammonium bicarbonate, pH = 7; organic phase B = acetonitrile; method: 0% B to 30% B in 12 minutes) R_T = 3.30 min ($M+H^+$ = 1179.0)

Synthesis of Folate-DBCO-NIR dye conjugate:
To a stirred solution of Folate-NIR dye (0.0050 g, 0.0027 mmol, 1 eq.) and NHS-DBCO (0.0016 g, 0.0041 mmol, 1.5 eq.) in DMSO, DIPEA (0.0054 g, 0.0041 mmol, 1.5 eq.) was added dropwise. The reaction mixture continued with stirring at room temp. Progress of the reaction was monitored by LC-MS. After complete conversion of Folate-NIR dye the crude reaction mixture was purified by RP-HPLC, (mobile phase A = 10 mM ammonium acetate, pH = 7; organic phase B = acetonitrile; method: 0% B to 50% B in 35 minutes at 13 mL/min) and furnished Folate-DBCO-NIR at 86% yield. LC-MS (A = 10 mM ammonium bicarbonate, pH = 7; organic phase B = acetonitrile; method: 0% B to 50% B in 12 minutes) R_T = 4.75 min ($M/2+H^+$ = 1043.0).

Synthesis of Folate-SS-DBCO-NIR conjugate:
To a stirred solution of Folate-NIR dye (0.0050 g, 0.0027 mmol, 1 eq.) and NHS-SS-DBCO (0.0023 g, 0.0041 mmol, 1.5 eq.) in DMSO, DIPEA (0.0054 g, 0.0041 mmol, 1.5 eq.) was added dropwise. The reaction mixture continued with stirring at room temp. Progress of the reaction was monitored by LC-MS.

After complete conversion of Folate-NIR dye the crude reaction mixture was subjected to purification by R-HPLC, (mobile phase A = 10 mM ammonium acetate, pH = 7; organic phase B = acetonitrile; method: 0% B to 50% B in 35 minutes at 13 mL/min) and furnished Folate-SS-DBCO-NIR at 84% yield. LC-MS (A = 10 mM ammonium bicarbonate, pH = 7; organic phase B = acetonitrile; method: 0% B to 50% B in 12 minutes) R_T = 4.991 min ($M/2+H^+$ = 1125.0).

Preparation of Folate-miRNAs (FolamiRs):
MiRNA duplexes were constructed using two RNA oligonucleotides: denoted as miR-34a-5p guide strand and miR-34a-3p passenger strand (Integrated DNA Technologies). The miR-34a-3p passenger strand comprises a 20nt RNA oligo double modified with an azide linker on the 5′ end and 2′-O-methyl RNA bases on the 3′ end. The miR-34a-5p guide strand comprises a 22 nt RNA oligo with minimal modifications on the 3′ with 2′-O-methyl RNA bases. A scrambled miRNA (Negative control) synthesized with the same modifications was used to form a control duplex. A bi-orthogonal click reaction was performed between Folate-DBCO and azide modified antisense miR-34a (or scramble) as shown in **Supplemental Fig 1.** For experiments with siLuc2 the oligos with the following sequences were used: sense strand: 5′ GGACGAGGACGAGCACUUCUU 3′ and antisense strand: 5′ GAAGUGCUCGUCCUCGUCCUU 3′. Click reaction*(55)* was performed at a 1:10 molar ratio (azide oligo: Folate DBCO) at room temperature in water for eight hours and then cooled to 4 °C for four hours. Unconjugated folate was removed from the reaction using Oligo Clean and Concentrator (Zymo Research) per manufacturer instructions. Conjugation was verified using 15% tris-base, acetic acid, ethylenediaminetetraacetic acid (TAE) native PAGE and MALDI spectral analysis. For folate-NIR compound conjugation an additional verification was done using Licor Odyssey CLX (Licor).

After conjugation, the miR-34a-5p guide strand was annealed to the folate-passenger strand and NIR-folate-passanger strand conjugates. Briefly, folate-miR-34a-3p and miR-34a-5p were mixed in an equal molar ratio (1:1, final concentration 5 μM each) in annealing buffer: 10 mM Tris buffer pH 7 (Sigma), supplemented with 50 mM NaCl (Sigma), and 1 mM EDTA (Sigma), and incubated at 95 °C for five minutes and then ramp cooled to room temperature over a period of one hour and then stored at -80 °C.

Stability assay in serum:
The duplex RNA oligonucleotides and the FolamiR conjugates were incubated in 50% fetal bovine serum (Sigma) in water at 37 °C for the indicated times. RNA samples were collected and analyzed using 15% TAE polyacrylamide gel electrophoresis (PAGE).

Flow cytometry:
FR positive MDA-MB-231 cells and FR negative A549 cells grown as described previously were detached by trypsinization and washed twice in ice-cold phosphate buffered saline (PBS; pH 7.4) and resuspended to a density of 1×10^7 cells/mL in serum free medium. Cell viability was determined by trypan blue exclusion and cells were only used if the viability of cells was >80%. Next, flow cytometric analyses were performed following stanadard protocols. Briefly, $1x10^6$ cells were incubated with PE anti-FOLR1 antibody (Cat. 908303, Biolegend) or matched isotype antibody (Cat. 400213, Biolegend) as a control and analyzed by flow cytometric analysis using LSR Fortessa flow cytometer (BD Biosciences, San Jose, CA, USA). Data was analyzed using FlowJo software v10 (Tree Star, Inc, OR, USA). Functionality of the FR was confirmed firstly by incubating MDA-MB-231 and A549 cells with FolamiR-34a-NIR (50nM) followed by flow cytometric analyses as described above, and secondly by microscopy analysis of cells incubated with folate-fluorescein isothiocyanate (FITC)*(56)* at a final concentration of 50 nM. Cells were evaluated at different time points using an Olympus IX73 microscope equipped with a 1.25X objective, Olympus DP80 camera, and CellSens 1.11.

In vitro FolamiR delivery:
MDA-MB-231 cells (HTB-26) and A549 non-small cell lung cancer cells (CCL-185), both mycoplasma free as determined by testing for mycoplasma contamination via MycoAlert Mycoplasma Detection Kit (Lonza), were grown in RPMI 1640 medium, no folic acid (Life Technologies) supplemented with 10% fetal bovine serum (Sigma), 100 U mL^{-1} penicillin and 100 µg mL^{-1} streptomycin (Hyclone, GE Healthcare Life Sciences) and maintained at 37 °C in 5% CO2. Authentication of MDA-MB-231 cells was performed using Short Tandem Repeat (STR) profiling (American Type Culture Collection – ATCC, USA). A549 cells were purchased directly from ATCC, and thus their authentication has been confirmed.

For luciferase reporter experiments, a miR-34a sensor plasmid was generated by inserting the antisense sequence to miR-34a into the 3′ untranslated region of Renilla luciferase in the vector (psiCHECK, Promega). MiR-34a specific silencing was confirmed in MDA-MB-231 cells by transiently transfecting a miR-34a sensor or a mutated miR-34a sensor. MiR-34a sensor expressing cells were transfected with a miR-34a mimic using Lipofectamine RNAimax (Life Technologies) to confirm silencing mediated by exogenous miRNA. To generate stable clones, MDA-MB-231 cells were seeded in six-well plates at a density of $1x10^6$ cells/well and were transfected with 2 ug of miR-34a sensor plasmid using Lipofectamine 2000 (Life Technologies). Stable clones were selected using Hygromycin B (500µg/mL; Hyclone, GE Healthcare Life Sciences) as a selection marker. Single clones were evaluated for Renilla expression and the clone with the highest Renilla expression was selected.

MB-231 sensor cells were seeded into 96-well plates containing FolamiR-34a, Fol-SS-34a or FolamiR-NC (negative control) in folic acid and serum free RPMI medium for a final concentration of 50 nM. Untreated and unconjugated duplex miRNA were included as controls. Renilla luciferase values were obtained 24, 48, 72, 96 and 120 h post incubation using the Renilla Glo Luciferase kit (Promega) following the manufacturer instructions. Renilla levels were normalized to FolamiR-NC for each time point. Experiments were performed three times with technical triplicates for each condition.

FR dependent response:
FR positive MDA-MB-231 cells and FR negative A549 cells were transfected with 500 ng of a miR-34a sensor plasmid using Lipofectamine 2000 (Life Technologies). After 24 hours 4000 cells/well were seeded into 96-well plates containing FolamiR-34a or FolamiR-NC (negative control) in folic acid and serum free RPMI medium for a final concentration of 50 nM or 100 nM. Cells transfected with Lipofectamine RNAimax (Life Technologies) were used as a control to monitor miR-34a Renilla sensor response to miR-34a mimic. Untreated and unconjugated duplex miRNAs were included as controls. Renilla luciferase values were obtained 96 hours post incubation using the Renilla Glo Luciferase kit (Promega) following the manufacturer instructions. Renilla levels of FolamiR-34a treated cells were normalized to FolamiR-NC for each time point and unconjugated duplex miRNA treated cells were normalized to untreated. Experiments were performed three times with technical triplicates for each condition.

In vitro FR binding competition assay:
In vitro FR binding competition assays were performed as described previously *(57, 58)*. Briefly, FR positive MDA-MB-231 cells and FR negative A549 cells grown as described previously were detached by trypsinization and washed twice in ice-cold phosphate buffered saline (PBS; pH 7.4) and resuspended to a density of 1×10^7 cells/mL in serum free medium. Next, 100 µL of the cell suspension was incubated with FolamiR-34a-NIR to a final concentration of 50nM in the absence or presence of 1 to 100 fold molar excess of folate glucosamine conjugate. Cells were incubated at 4ºC for 20 minutes and washed twice with ice cold PBS and analyzed for displacement of FolamiR-34a-NIR binding by flow cytometric

analysis using LSR Fortessa flow cytometer (BD Biosciences, San Jose, CA, USA). Data was analyzed using FlowJo software v10 (Tree Star, Inc, Ashland, Ore).

Functional competition was verified using MDA-MB-231 sensor cells incubated with FolamiR-34a or FolamiR-NC to a final concentration of 50nM in the absence or presence of 1 to 100 fold molar excess of folate glucosamine conjugate. Untreated and unconjugated duplex miRNA were included as controls. Renilla luciferase values were obtained 96 hours post incubation using the Renilla Glo Luciferase kit (Promega) following the manufacturer instructions. Renilla levels of FolamiR-34a treated cells were normalized to FolamiR-NC for each time point and unconjugated duplex miRNA treated cells were normalized to untreated. Experiments were performed three times with technical triplicates for each condition.

Cell proliferation assays:
A Sulforhodamine B (SRB, Sigma) assay was used as a proxy for cell proliferation in 96-well plates*(59)*. Briefly, following FolamiR treatment cells were fixed with 10% tricholoroacetic acid in complete media and stained for 1 hour with 0.4% (wt/vol) SRB in 1% acetic acid. Unbound dye was removed by four washes with 1% acetic acid. Finally, protein-bound dye was extracted with 10 mm unbuffered Tris base and absorbance at 510 nm was obtained using a GloMax Multi+ spectrophotometer (Promega). Absorbance values (proxy for cell mass) were normalized to that of cells cultured in the presence of FolamiR-NC for each time point.

Flank tumor establishment:
For single-dose studies, subcutaneous tumors were induced in female Nu/Nu (NU-Foxn1^{nu}; Charles River) congenic mice (6 weeks, n = 5) by subcutaneous injection of 5 X 10^6 MDA-MB-231 sensor cells suspended in 200 μL of Matrigel (Corning). For longitudinal studies, parental MDA-MB-231 cells were used. Due to the observation that rodents present high plasma and tissue levels of 5-methyl-tetrahydrofolate, the naturally occurring form of folate, (around 10-fold higher than in humans)*(60)* mice were maintained on folic acid deficient diet (Envigo, TD.95247) two weeks prior tumor implantation and during the experiment series. A folate-deficient diet has shown to reduce folate levels to physiological levels seen in humans*(58)*. To determine tumor growth, individual tumors were measured using a vernier caliper and tumor volume was calculated by: tumor volume (mm^3) = width × ($length^2$) x 2^{-1}. Animals were excluded if tumors had not reached a volume of 150 cm^3 by the time of treatment. For single dose experiments, animals were injected intravenously (i.v.) with 5 nmol of FolamiRs after acquisition of luminescent and florescence signals (day 0, Fig. 1a). For multiple dosing experiments, animals were randomized into experimental arms by minimizing the differences in their mean tumor size. When tumor volume reached ~200 mm^3 animals were treated with i.v. injections of the indicated molar concentration of FolamiR every three days. All experimental protocols were approved by the Purdue Animal Care and Use Committee and were in compliance with NIH guidelines for animal use.

Bioluminescent and infrared imaging:
RediJect Coelenterazine h Bioluminescent Substrate (PerkinElmer) was administered per the manufacturer's protocol for *in vivo* monitoring of tumor bioluminescence using IVIS Lumina II (Caliper) or Spectral AMI (Spectral Instruments). Luminescent values were acquired at multiple points after injection of substrate starting at 20 minutes and only maximal mean radiance values were reported. Infrared imaging was conducted using IVIS Lumina II (Caliper) at 745 nm excitation and ICG emission filters. Non-invasive longitudinal monitoring of tumor luminescence and fluorescence was conducted by whole-animal imaging performed at the following time points: 0, 24, 48, and 72 h (n = 3 animals per experimental group). Gross organ images were acquired using the 800 nm channel in the Licor Odyssey CLX (Licor).

Serum cytokines:
Serum samples from multiple dosing experiments (24 hours after last injection) were used to test for IL-6, and tumor necrosis factor (TNFα) concentrations using the mouse specific cytokine Multi-Analyte ELISArray Kit (Qiagen) according to manufacturer's instructions. Briefly, serum samples were thawed on ice and cleared from debris by centrifugation at 1000 x *g* at 4 °C for 10 min before the analysis. All samples or standards were added to a 96-well plate together with assay buffer. Plates were shaken gently and incubated for 2 h at room temperature. Supernatant was removed and wells were washed. A detection antibody was added and the plates were incubated for 1 h at room temperature. Plates were rinsed and incubated with Avidin-HRP solution for 30 min at room temperature. The wells were washed and development solution was added to acquire data using a GloMax plate reader (Promega). Absorbance values were acquired at 450 nm and 570 nm. The 570 nm readings were subtracted from the 450 nm readings. Cytokine standard curves were used to calculate the cytokine concentrations in serum samples (pg/mL). The limits of detection were as follows: IL-6 58.8 pg/mL, and TNFα 30.5 pg/mL. LPS treated Nu/Nu mice (NU-Foxn1^{nu}; Charles River) were used as positive controls. Mice received an intra-peritoneal injection of 500 ng kg^{-1} lipopolysaccharides (LPS, L6529, Sigma) and serum was collected two hours post injection.

Maximum Tolerated Dose (MTD) study:
Balb/c mice (8 weeks of age) were administered one intravenous injection of 33.3, 10 or 1 nmol of FolamiR-34a. Animals were observed post administration for 2 weeks. The mice were observed for changes in body weight and clinical observations (rapid weight loss, diarrhea, rough hair coat, hunched posture, lethargy, labor breathing, neurological signs, etc.). The mice were allowed *ad libitum* feed and water. A necropsy was performed at the end of the study. Whole blood, serum, and organ tissue were collected for further analysis.

Induction of tumor formation in Kras;p53 mice:
Induction of tumor formation in *Kras*$^{LSL\text{-}G12D/+}$; *Trp53*$^{flx/flx}$ (FVB.129 background) double mutant mice (6 to 10 weeks old) was performed based on the method of DuPage et al*(45)*. Briefly, lung specific transgene activation was achieved via intratracheal delivery of Adenoviral particles (10^6 PFU) encoding for Cre recombinase. Tumors were allowed to preform for eight weeks prior experiments.

Tumor progression monitoring using Magnetic Resonance Imaging (MRI):
MRI scans of induced and non-induced animals (scans of healthy tissue) were obtained using a 7.0 Tesla Bruker Biospec 70/30 USR Scanner (Billerica, MA) and a 40 mm mouse volume coil at the Purdue MRI Facility. Animals were anesthetized using a 2.5 % v/v isoflurane in O_2 for 5 minutes and then moved to the heated animal bed where anesthesia was set to 2%. Respiration rate was monitored *via* pressure sensor. A low-resolution multiplane scout scan was obtained using the following parameters: TR=4s, TE= 1.5 ms, FOV= 30 x 30 mm^2, slice thickness= 1 mm, data matrix= 256 x 256, 7 slices per plane (axial, coronal and sagittal), approximate time of scan per mice= 1 minute. The scout scan was used to align the spine of the mouse to collect high-resolution images of the lungs using the following parameters: TR=4s, TE= 1.5 ms, FOV= 30 x 30 mm^2, slice thickness= 0.5 mm, data matrix= 256 x 256, 30 slices per plane (axial and coronal), approximate time of scan per mice= 5 minutes. Quantification of tumor burden was conducted following the manual segmentation protocol described by Krupnick et al*(61)*. This analysis of tumor burden by MRI takes advantage of the vast difference in MR image intensities between tumor tissue (bright) and normal lung tissue (dark) and uses the average lung image intensity as a proxy for tumor burden*(62)*. Briefly, lung MR images are manually segmented using ImageJ 2.0.0 and the average lung image intensity normalized to that of the liver within the same animal is calculated. To determine tumor progression within an animal the average lung image intensity is then normalized to the first day of treatment. Furthermore, tumor and whole lung volumes per animal were calculated using three-dimensional reconstruction using ITK-Snap and Paraview 5.2 software (Kitware, NY, USA)*(63)*.

Tumor/whole lung ratios were obtained at the indicated times showing the percentage of lung volume occupied by tumors.

Folate uptake studies in Kras;p53 mice:
For folate uptake studies, 5 nmoles of OTL38 (kindly provided by On Target Laboratories, LLC, West Lafayette, IN), a fluorescent imaging conjugate composed of folate tethered to a fluorescent near infrared (NIR) dye currently in clinical trials (Clinical trial identifier: NCT02769533)*(32, 47, 48)*, were delivered systemically through the tail vein into healthy and tumor bearing mice (n=3; 8 weeks post transgene activation). Twenty-four hours after the injection animals were sacrificed and perfused with saline. Whole organ images were acquired using the 800 nm channel in the Licor Odyssey CLX (Licor). Lungs were fixed in 10% buffered formalin and paraffin embedded according to standard procedures. Sections were stained by hematoxylin and eosin (H&E) and evaluated using an Olympus IX73 microscope equipped with a 1.25X objective, Olympus DP80 camera, and CellSens 1.11. Tumor burden was calculated using ImageJ 2.0.0, which represents the tumor area relative to the total lung area obtained from three independent sections for each animal. Unstained mounted sections were evaluated in the 800 nm channel in the Licor Odyssey CLX (Licor) and using a Nikon TiS microscope equipped with a 20X objective, an ICG band pass filter (Ex: 780-800; Ex: 810-860; Semrock, Brightline), a xenon/mercury light source (Nikon, Japan), Photometrics QuantEM EMCCD camera, and NIS-Elements (Nikon, Japan).

FolamiR treatment in Kras;p53 mice:
For multiple dosing experiments with FolamiR-34a, tumor bearing animals (8 weeks) were randomized into experimental arms by minimizing the differences in their MRI measured tumor burden. Animals were treated with i.v. injections of 1 nmol FolamiR every three days (10 injections total) and tumor progression was monitored using a 7.0 Tesla Bruker Biospec 70/30 USR Scanner (Billerica, MA) as described above every week for four weeks. Twenty-four hours after the final injection animals were sacrificed and perfused with saline. Lungs were harvested, fixed in 10% buffered formalin and paraffin embedded according to standard procedures. Sections were stained by hematoxylin and eosin (H&E) and evaluated as described earlier. Mice were maintained on a folic acid deficient diet (Envigo, TD.95247) starting at six weeks after tumor induction and during the experiment series. All experimental protocols were approved by the Purdue Animal Care and Use Committee and were in compliance with NIH guidelines for animal use.

In vivo blocking of FR:
Subcutaneous tumors were induced in female Nu/Nu (NU-Foxn1^{nu}; Charles River) congenic mice (6 weeks, n = 3) following injection of 5 X 10^6 FR positive MDA-MB-231 sensor cells (right side) and FR human A549 cells (left side) suspended in 200 µL of Matrigel (Corning). Tumors were allowed to form and mice bearing A549 and MDA-MB-231 tumors of similar size were included in the experiment. For the *Kras;p53* mouse model, tumors were allowed to form for eight weeks after transgene activation (n=3). Tumor formation was monitored using MRI. Competition studies were performed in mice (n = 3) by co-administration, via the tail vein, of 5 nmoles of FolamiR-34a-NIR for the xenograft model or 5 nmoles of OTL38 for the *Kras;p53* mouse model in the presence or absence of 500 nmoles (≥100-fold molar excess) of folic acid glucosamine. Folic acid glucosamine conjugate was used because of its increased solubility at low pH compared folic acid and to prevent precipitation in the kidneys *(64)*. *In vivo* whole animal imaging and *ex vivo* tissue distribution studies were performed as described above.

RNA isolation and miRNA expression analyses using quantitative PCR (qPCR):
MDA-MB-231 derived tumors from Nu/Nu mice (NU-Foxn1^{nu}; Charles River) and lung tumors from *Kras*$^{LSL\text{-}G12D/+}$; *Trp53*$^{flx/flx}$ mice were collected in RNA Later (Life Technologies) and stored at -80 °C. Tumor tissues (50mg) were placed in 2 mL collection tubes containing 700 µL QIAzol lysis reagent (Qiagen) and 1.4 mm ceramic beads. Samples were disrupted using a bead mill (Fisher Scientific) at 4 m

s^{-1} for 3 minutes. Total RNA was extracted using RNeasy Mini Kit (Qiagen) according to the manufacturer instructions. Next, cDNA was generated using miScript II RT Kit (Qiagen) and miScript HiFlex Buffer using 1 µg of total RNA. For miR-34a standard generation, miR-34a mimic (Life Technologies) was used for cDNA synthesis. qRT-PCR was performed with miRNA primer assays (Qiagen). The reactions were processed using a QuantStudio 6 Flex Real-time PCR machine (Life Technologies) using miScript SYBR Green PCR Kit (Qiagen) under the following cycling steps: 15 min at 95 °C; 40 cycles at 95 °C for 15 s, 55 °C for 30 s, 70 °C for 30 s; melting curve from 95 °C to 60 °C at 1.6 °C s^{-1}. Three technical repeats for each biological replicate (at least 3) were carried out. MiR-34a copy number was determined using a standard curve covering 1 x 10^8 copies to 1 x 10^3 copies.

Supplementary Figures

Supplemental Figure 1: Folate ligand synthesis

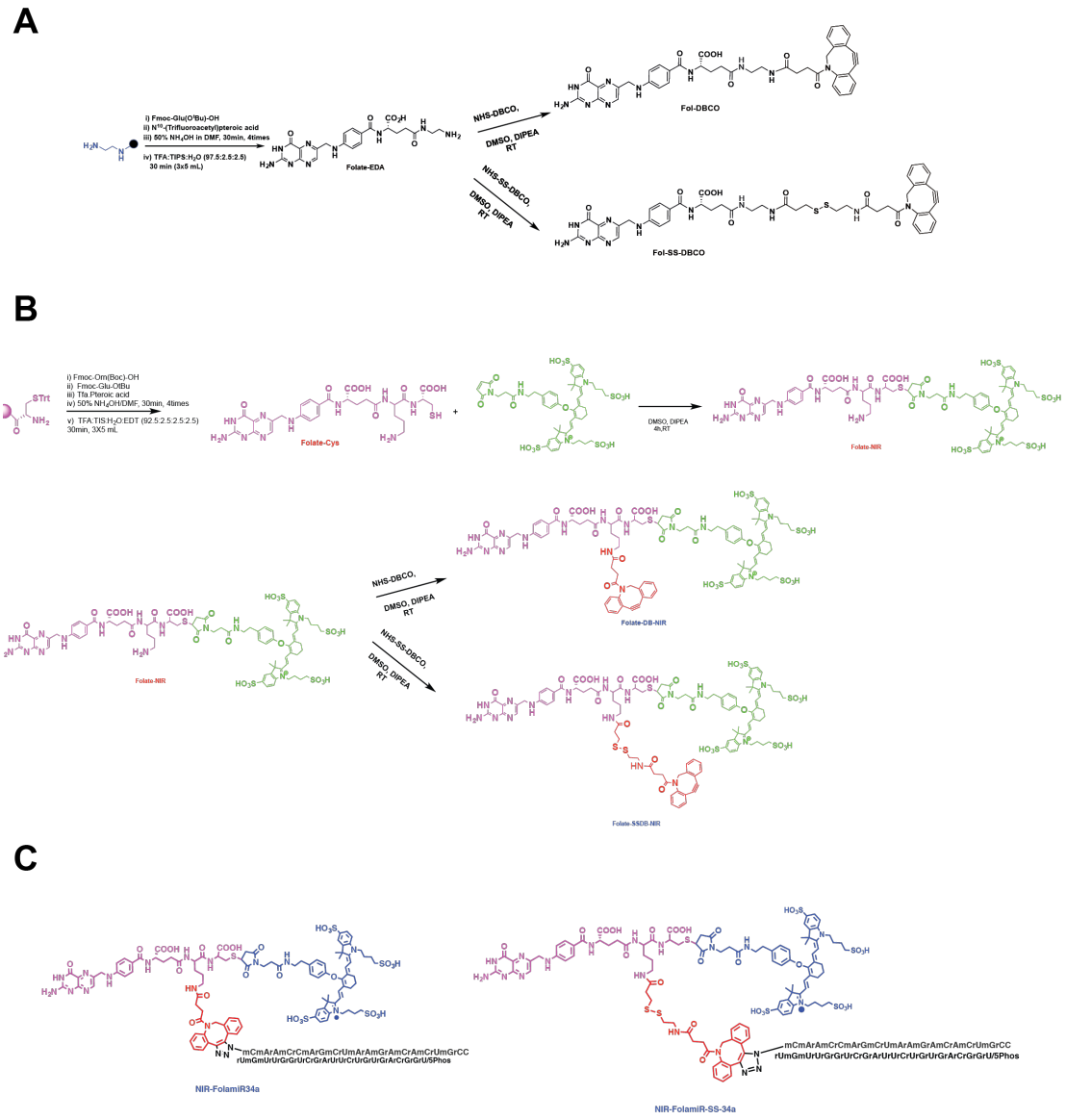
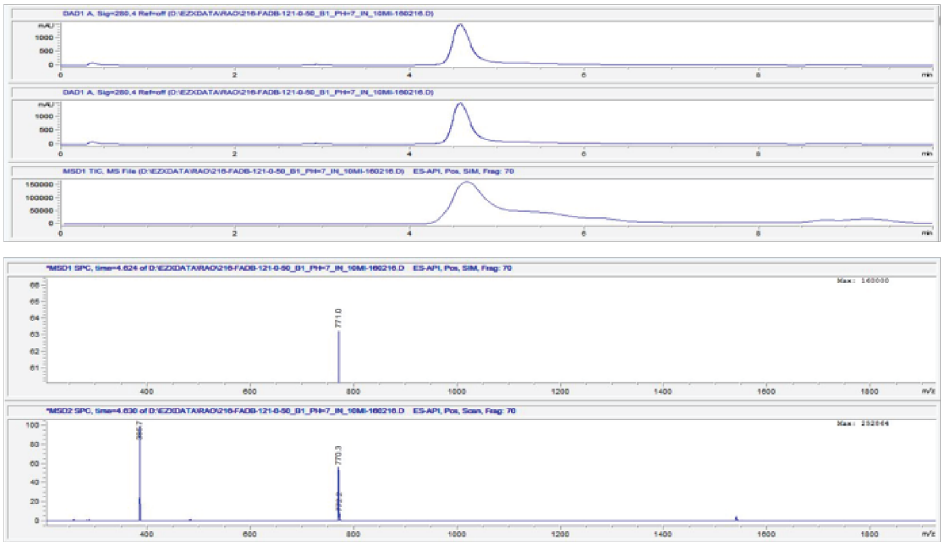
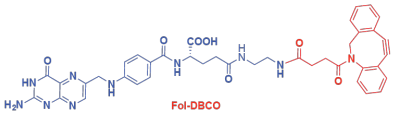


Figure S1. General synthesis procedure of folate conjugates. A) Synthesis of folate-DBCO and folate-SS-DBCO used for conjugating miRNAs via bio-orthogonal click reaction. B) Synthesis of folate-DBCO-NIR and folate-SS-DBCO-NIR. C) Structure of FolamiR34a-NIR and FolamiR-SS-34a-NIR conjugates.

Supplemental Figure 2: Verification of folate-DBCO conjugate synthesis

A



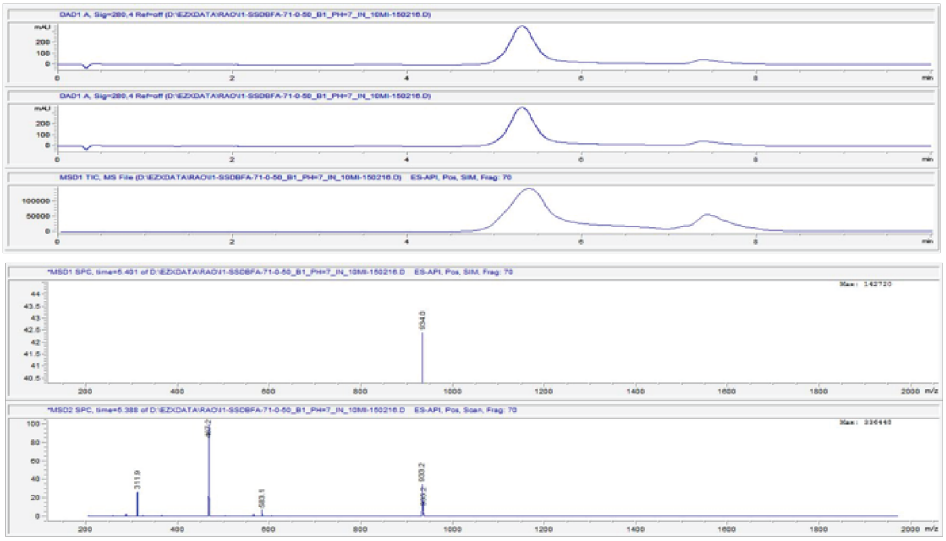


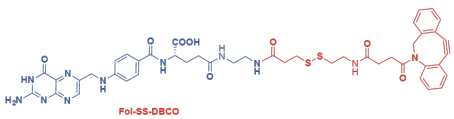
Chemical Formula: $C_{40}H_{38}N_{10}O_7$

Exact Mass: 770.29

Molecular Weight: 770.81

B





Chemical Formula: $C_{45}H_{47}N_{11}O_8S_2$

Exact Mass: 933.31

Molecular Weight: 934.06

Figure S2. LC-MS spectra of folate conjugates. A) LC-MS of Folate-DBCO conjugate. B) LC-MS of Folate-SS-DBCO conjugate.

Supplemental Figure 3: Verification of folate-miRNA conjugation

A

	miR-34a-3p		
Folate ligand	**1**	**2**	**3**
Folate		+	
Folate-SS			+

B

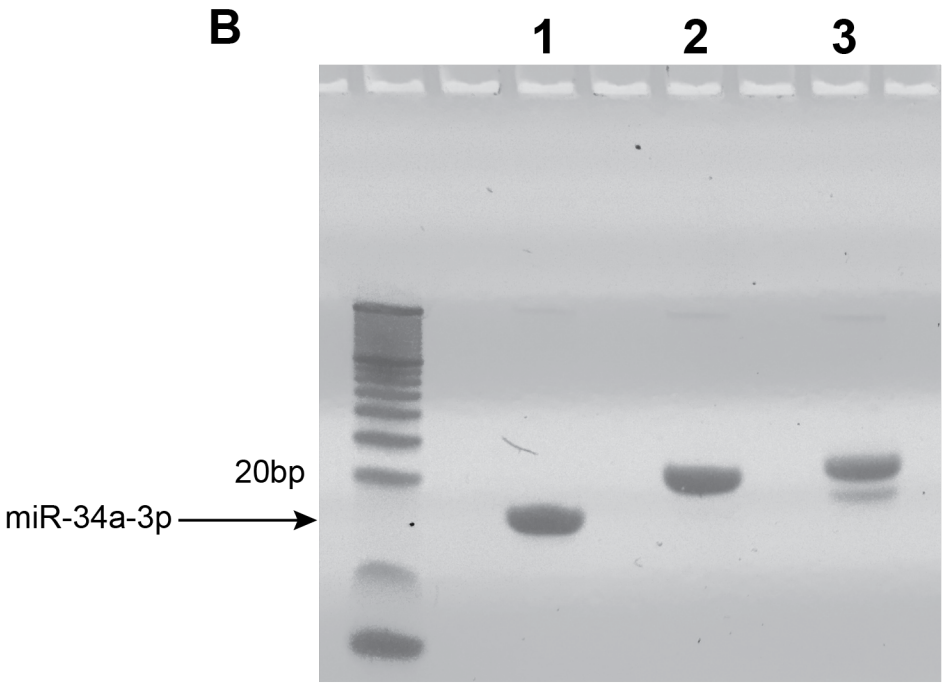
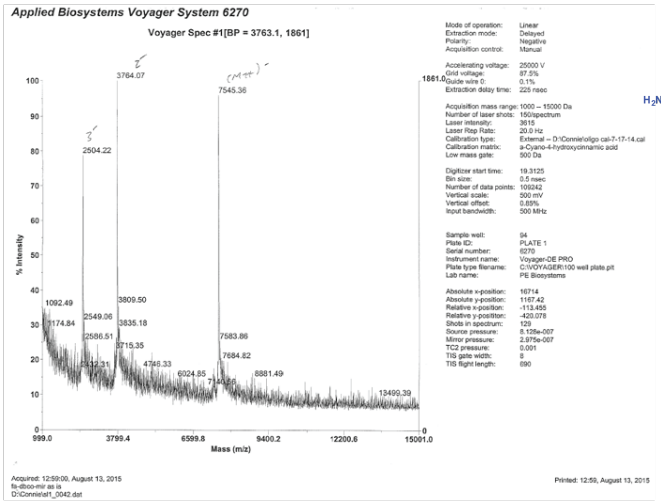
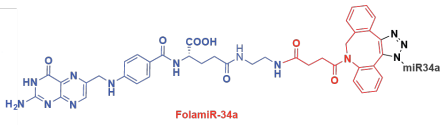


Figure S3. Evaluation of Folate-miRNA conjugation measured by 15% native TAE PAGE. A) In the table, "+" indicates the presence of a particular ligand conjugated to miR-34a-3p. B) Gel red stained polyacrylamide gel. Each lane was loaded with 200 pmols of unconjugated oligo or folate-miRNA. PAGE results suggest successful conjugation as visualized in the mobility shift in conjugated folate-miRNA compounds. The presence of a duplet in lane 3 could suggest premature reduction of the disulfide bond.

Supplemental Figure 4: Verification of folate-miRNA conjugation

A

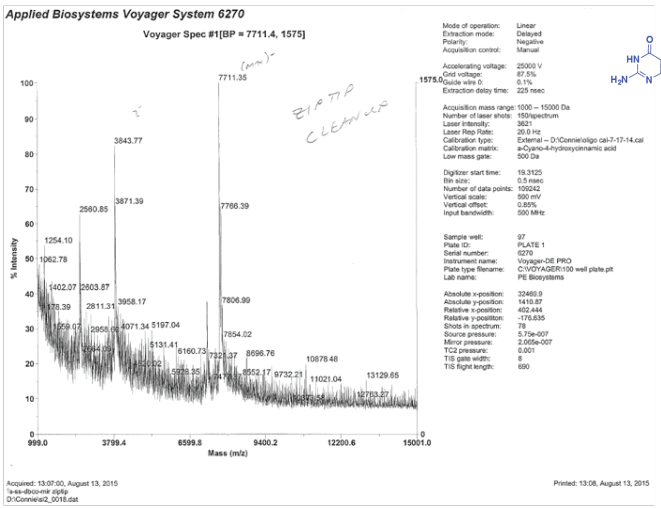


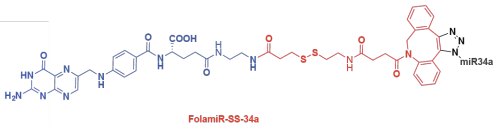


Expected Molecular Weight: 7545.5

Observed : 7545.4 $(M+H)^-$

B





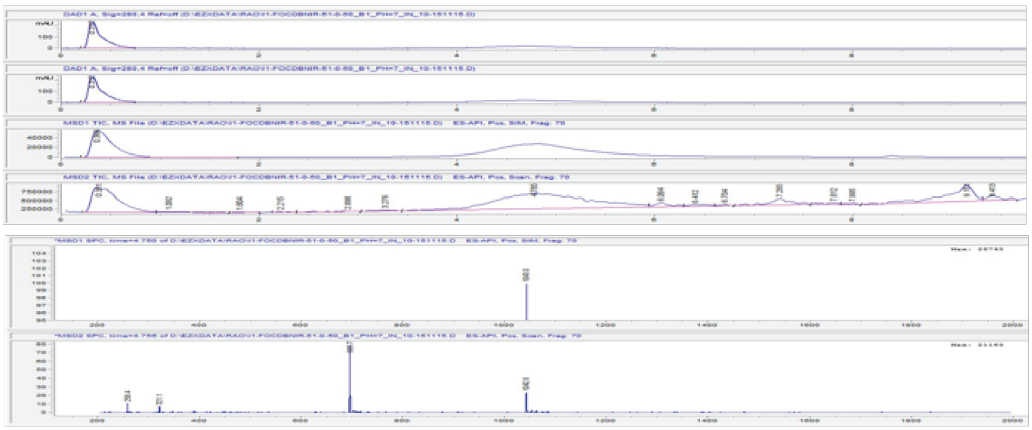
Expected Molecular Weight: 7708.5

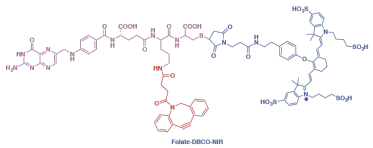
Observed : 7711.35 $(M-H)^-$

Figure S4. MALDI spectra of FolamiRs. A) MALDI spectra of FolamiR-34a conjugate. B) MALDI spectra of FolamiR-SS-34a conjugate.

Supplemental Figure 5: Verification of NIR-folate conjugation

A



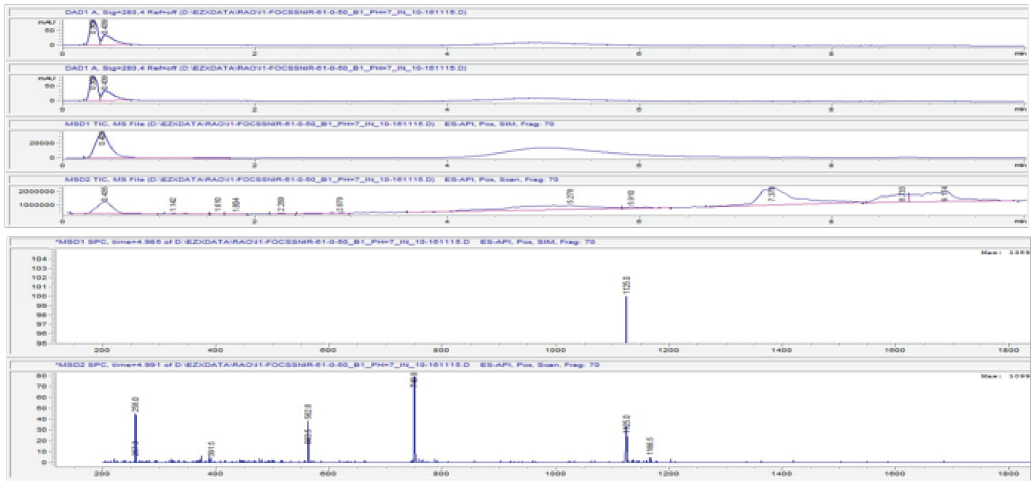


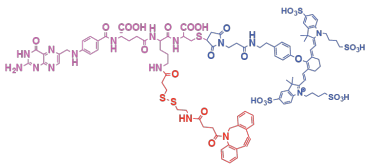
Chemical Formula: $C_{99}H_{110}N_{15}O_{26}S_5^+$

Exact Mass: 2084.63

Molecular Weight: 2086.35

B





Chemical Formula: $C_{104}H_{119}N_{16}O_{27}S_7^+$

Exact Mass: 2247.65

Molecular Weight: 2249.60

Figure S5. LC-MS spectra of folate-NIR conjugates. A) LC-MS of Folate-DBCO-NIR conjugate. B) LC-MS of Folate-SS-DBCO-NIR conjugate.

Supplemental Figure 6: Verification of NIR-folate-miRNA conjugation

A

	miR-34a-3p		
Folate ligand	**1**	**2**	**3**
NIR-Folate-SS		+	
NIR-Folate			+

B

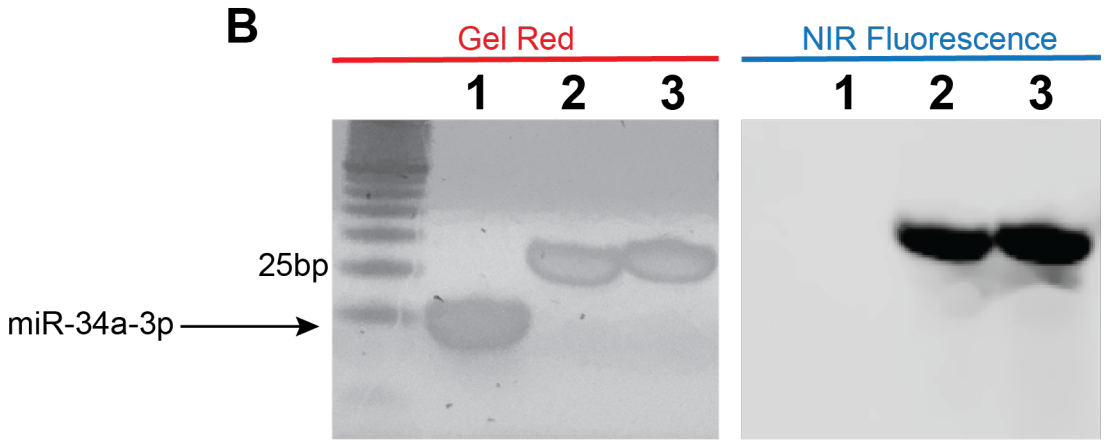


Figure S6. Evaluation of NIR-FolamiR conjugation measured by 15% native TAE PAGE. A) In the table, "+" indicates the presence of a particular ligand conjugated to miR-34a-3p. B) On the left, Gel red stained polyacrylamide gel. On the right, NIR imaging of polyacrylamide gel using the 800 nm channel of Licor Odyssey CLX (Licor). Each lane was loaded with 200 pmols of unconjugated oligo or folate-miRNA. PAGE results confirmed successful conjugation as visualized in the mobility shift in lanes 2 and 3.

Supplemental Figure 7: Verification of NIR-folate-miRNA conjugation

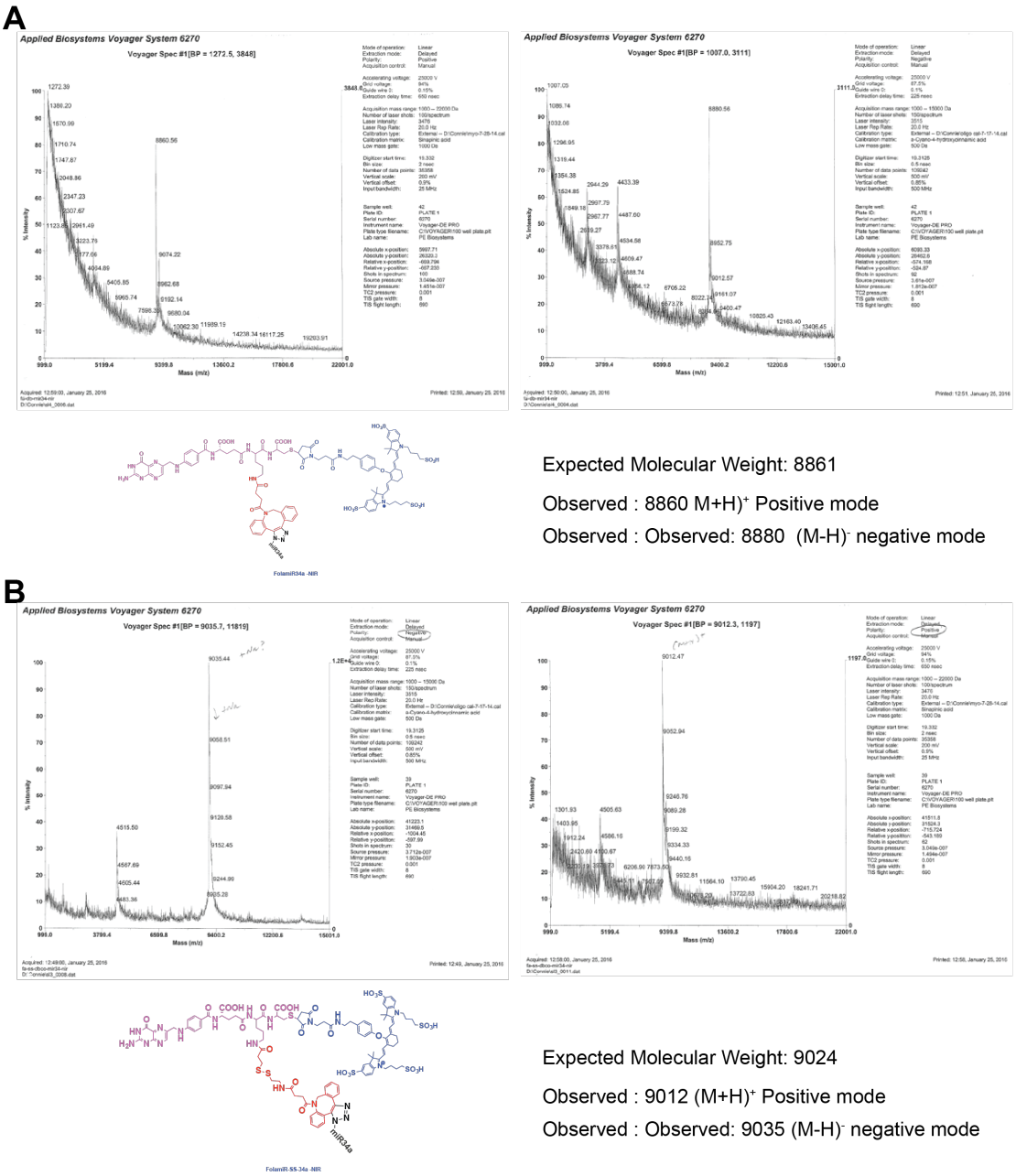


Figure S7. MALDI spectra of FolamiR-NIR conjugates. A) MALDI spectra of FolamiR-34a-NIR dye conjugate. B) MALDI spectra of FolamiR-SS-34a-NIR dye conjugate.

Supplemental Figure 8: miR-34a Renilla sensor response to miR-34a

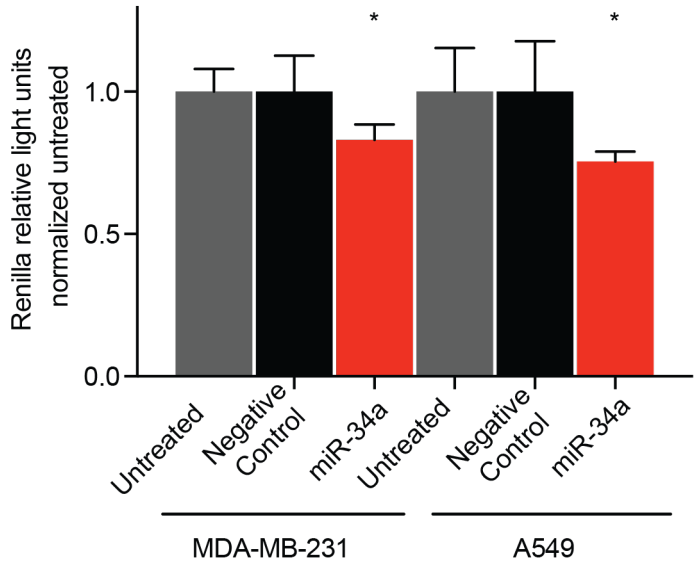


Figure S8. miR34a Renilla sensor response to miRNA mimic transfection. MDA-MB-231 breast cancer cells and A549 lung cancer cells transiently expressing a miR-34a Renilla sensor were used to monitor miR-34a delivery and activity. MDA-MB-231 and A549 cells were transfected with 50nM of miR-34a mimic using Lipofectamine RNAimax (Life Technologies) and Renilla signal was measured 96 hours post treatment.

Supplemental Figure 9: Generation and testing of MDA-MB-231 miR-34a sensor cells

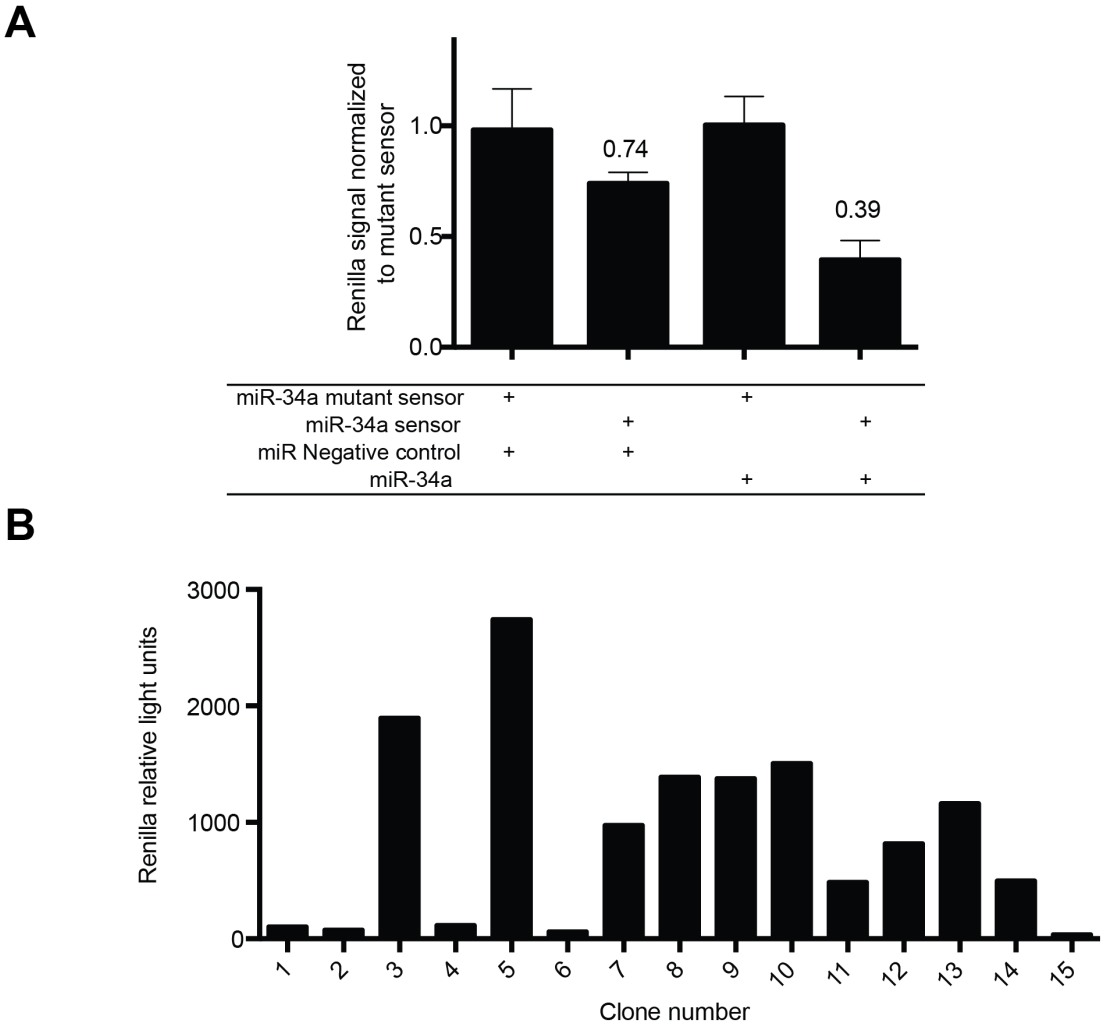


Figure S9. Evaluation of MDA-MB-231 miR-34a sensor cells. A) Specificity of the miRNA sensor was monitored by transiently expressing the miR-34a sensor or a mutated sensor along with a miR-34a mimic or a negative control (scrambled RNA) in MDA-MB-231 breast cancer cells. Cells ($1X10^4$) were seeded in 96-well plates and co-transfected with 25ng of plasmid and 6nM of miRNA mimic using Lipofectamine 2000 (Life Technologies). Renilla activity was measured 48 hours post transfection using the Renilla Glo Luciferase kit (Promega). Error bars represent the mean ± s.d., experiments were performed in triplicate. B) Selection of MB-231 clones based on renilla activity. Renilla readings were performed using $1X10^4$ cells per clone and renilla levels were measured using the Renilla Glo Luciferase kit (Promega).

Supplemental Figure 10: Firefly luciferase response to siLuc2

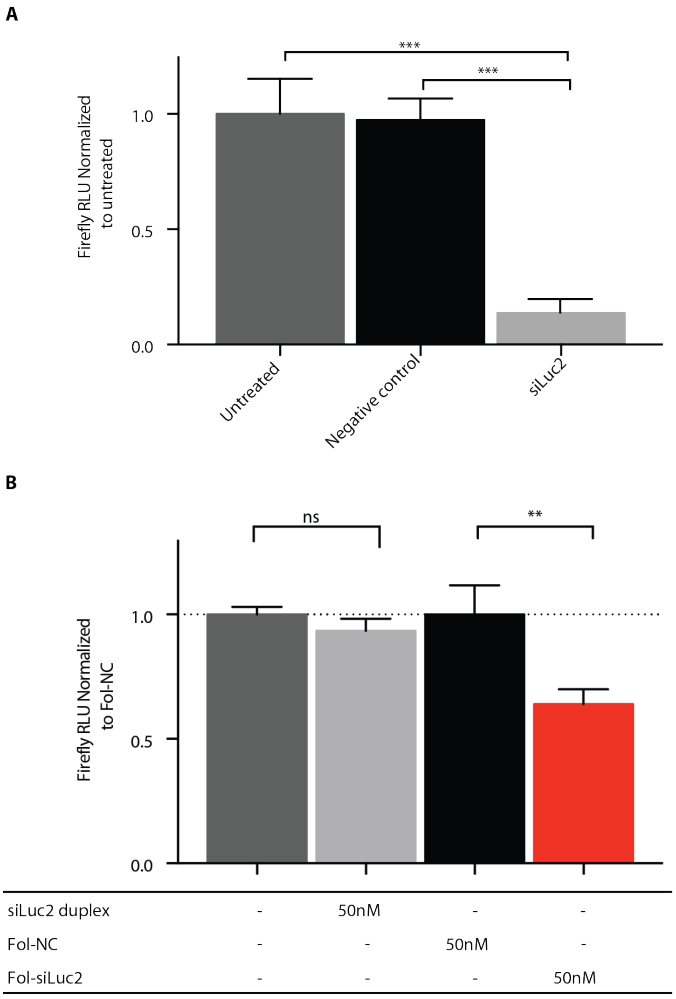


Figure S10. Folate mediated delivery of functional siLuc2. A) Firefly level in MDA-MB-231 Firefly expressing cells is reduced folloingt cells expressing a pmiRGlo plasmid (Promega) respond with firely luciferase (Luc2) signal modulation after transfection with an siRNA against Luc2 suggesting that the firefly signal can be used to monitor siLuc2 uptake and activity. B) Firefly luciferase signal in MDA-MB-231 cells following treatment with folate-siLuc2 or Folate-NC (72 hours post treatment, 50nM final concentration). Untreated and unconjugated duplex siRNA were also included as controls. MDA-MB-231 were transfected with 50nM of siLuc2 using Lipofectamine RNAimax (Life Technologies) and Firefly signal was measured 72 hours post treatment. Luciferase activity levels from MDA-MB-231 cells normalized to negative control. Experiments were performed three times with technical triplicates for each condition. One-way analysis of variance (ANOVA) and Bonferroni post hoc test were used to test for statistical significance. Mean ± S.D., ** $P<0.01$.

Supplemental Figure 11: Serum stability assay

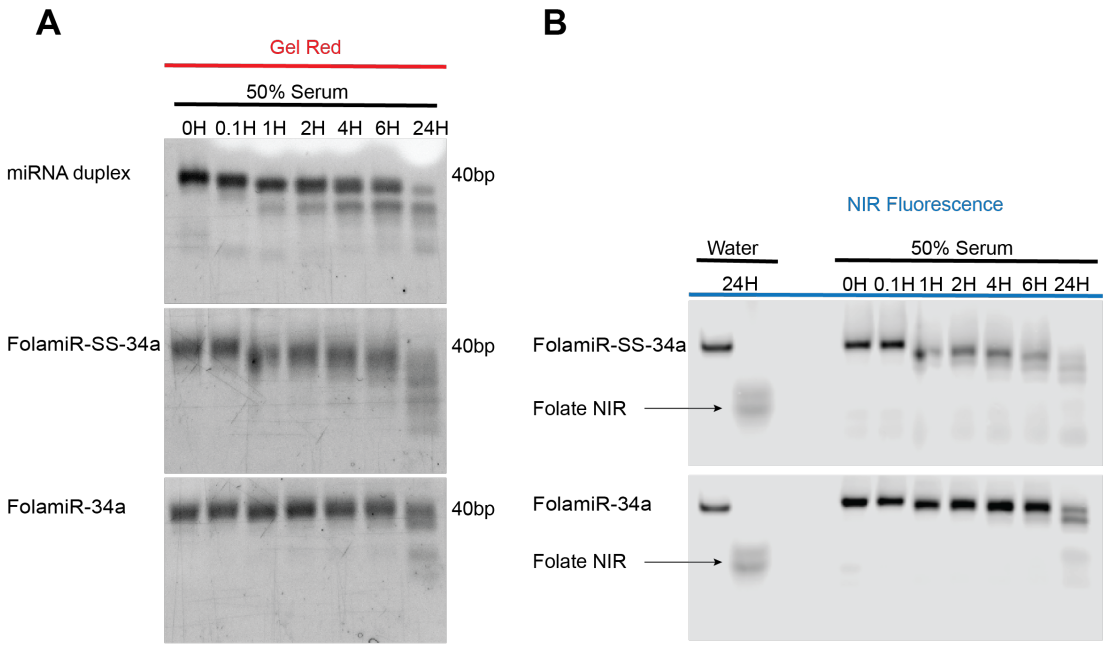


Figure S11. Serum stability of FolamiRs and duplex RNA oligos measured by 15% TAE native PAGE after incubation in 50% serum solution for different periods of time. A) Gel red stained polyacrylamide gel following resolution of miRNAs after serum exposure. B) NIR imaging of polyacrylamide gel using the 800 nm channel of Licor Odyssey CLX (Licor).

Supplemental Figure 12: In vivo dose titration of FolamiR-34a

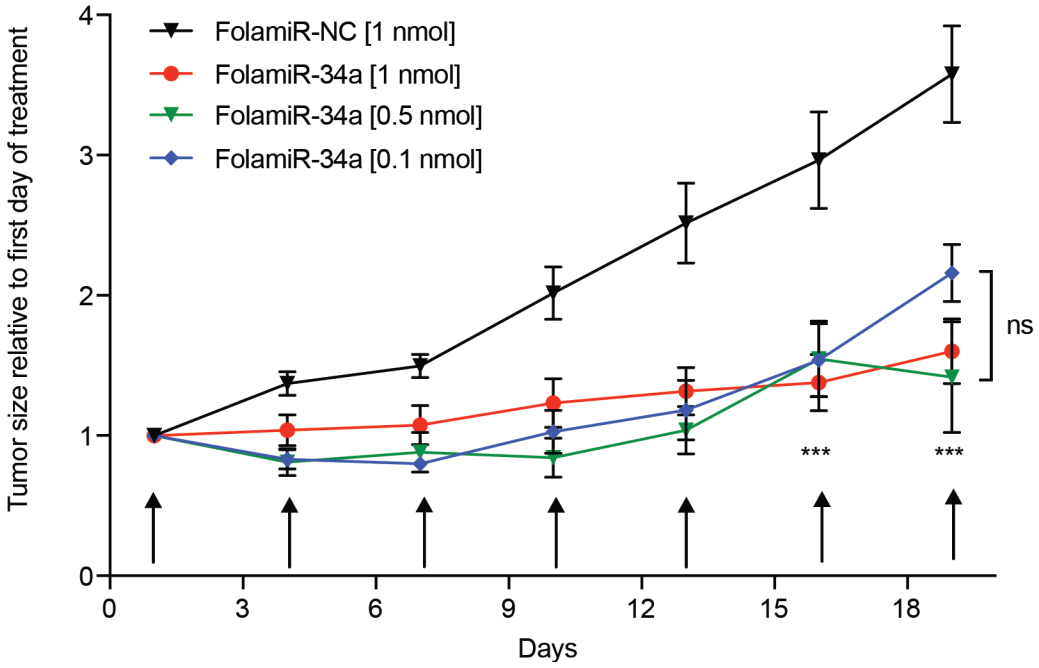


Figure S12. Tumor growth response to increasing doses of FolamiR34a. Tumor size following FolamiR-34a treatment (n = 5, error bars represent mean ± s.e.m., statistical analysis performed with a two-way ANOVA, ***, $P < 0.001$). Arrows represent treatment times (intravenous injection). Tumors were measured with a vernier caliper and tumor volume was calculated by: volume (mm^3) = width × ($length^2$) × 2^{-1}.

Supplemental Figure 13: Tumors of mice treated with FolamiR-34a show high levels of miR-34a

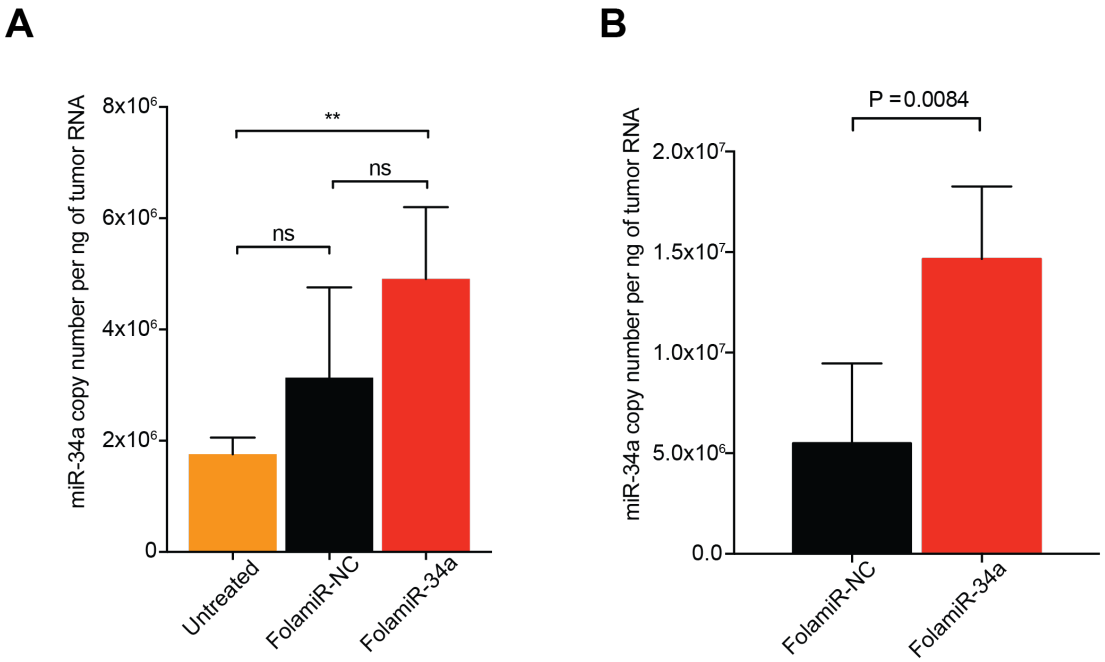


Figure S13. miR34a copy number in tumors treated with FolamiR. MiR-34a levels measured by qRT-PCR from (A) breast cancer xenografted tumors (B) and lung adenocarcinoma *Kras*$^{LSL\text{-}G12D/+}$;*p53*$^{flx/flx}$ tumors at 24 hours post last injection (n = 5; error bars represent mean ± s.d, statistical analysis performed with one-way ANOVA or Student's t-test).

Supplemental Figure 14: FolamiR-34a treatment does not generate a toxic response

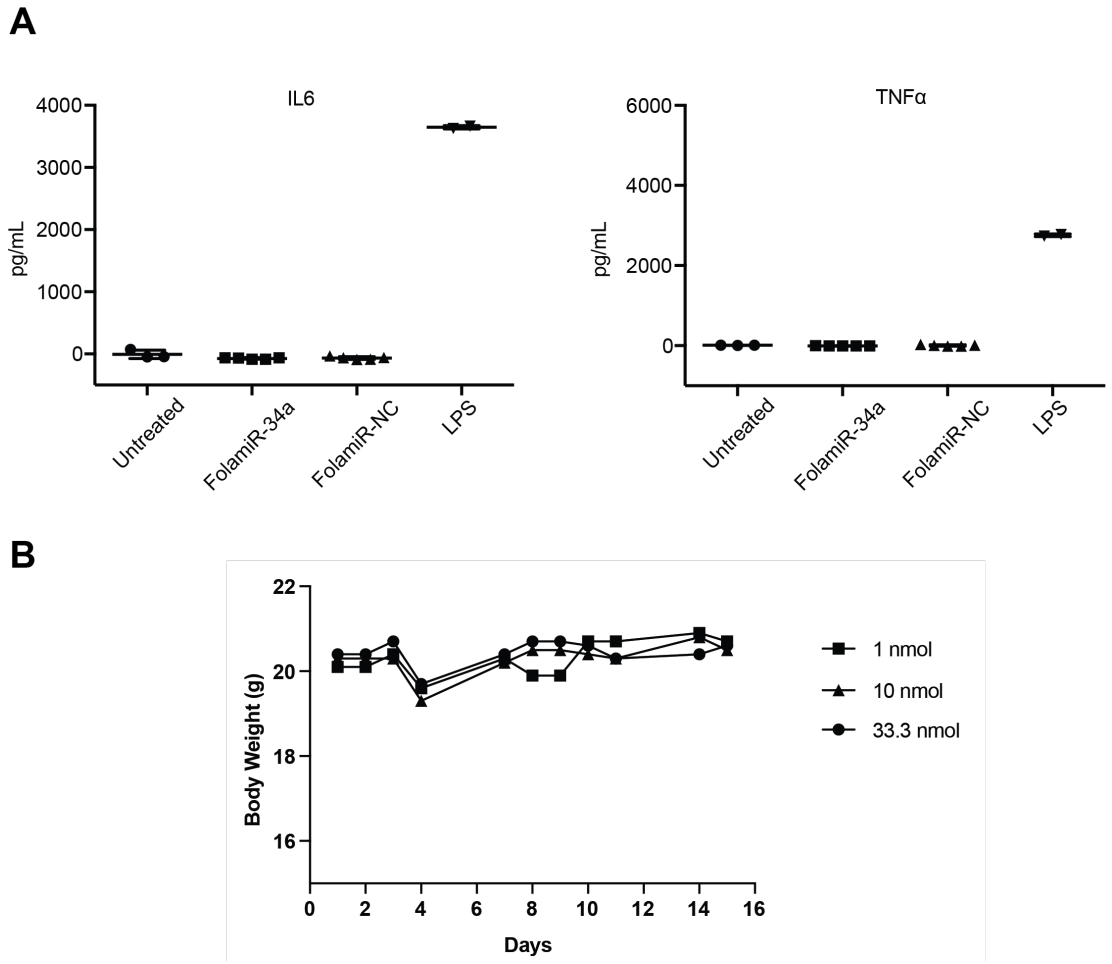


Figure S14. Serum cytokines and Maximum Tolerated Dose Study. A) Serum obtained from FolamiR treated Nu/Nu mice bearing MDA-MB-231 tumors was evaluated for relevant cytokines: tumor necrosis factor (TNF)α, and interleukin (IL)-6 (n=5). Serum from lipopolysaccharides (LPS) treated mice was included as a positive detection control (n=2; statistical analysis was performed with a one-way ANOVA with post hoc Bonferroni correction). B) Body weight before and after intravenous administration of increasing doses of FolamiR-34a. Statistical analysis was performed with a two-way ANOVA with post hoc Bonferroni correction.