

5-HALOURACIL-MODIFIED DOUBLE-STRANDED NUCLEIC ACIDS AND THEIR USE IN THE TREATMENT OF CANCER

CROSS REFERENCE TO RELATED APPLICATIONS

[0001] This application claims the benefit of priority from U.S. Provisional Application No. 63/578,033, filed on August 22, 2023, the entire contents of which are incorporated herein by reference.

INCORPORATION BY REFERENCE OF SEQUENCE LISTING

[0002] The Sequence Listing in the XML, named as 050_9294_US_SequenceListing of 15 KB, created on August 18, 2023, and submitted to the United States Patent and Trademark Office via Patent Center, is incorporated herein by reference.

FIELD OF THE DISCLOSURE

[0003] The present disclosure is generally directed to compositions and methods for treating cancer, and more particularly, to methods in which modified double-stranded nucleic acids alone or in conjunction with 5-fluorouracil are used in treating cancer, particularly pancreatic, ovarian and colorectal cancer.

BACKGROUND

[0004] MicroRNAs (miRNAs, miRs) are a class of highly conserved, non-coding small RNA molecules that mediate translation in a cell or organism by negatively regulating the expression of their target genes and thus causing translational arrest, mRNA cleavage or a combination thereof. See Bartel DP. *Cell*. (2009) 136(2):215-33. By targeting multiple transcripts, miRNAs regulate a wide range of biological processes, including apoptosis, differentiation and cell proliferation, thus aberrant microRNA function can lead to cancer (see Ambros V. *Nature*. (2004) 431(7006):350-5) and as such, miRNAs have recently been identified as biomarkers, oncogenes or tumor suppressors. See, *e.g.*, Croce, CM, *Nat Rev Genet*. (2009) 10:704-714).

[0005] Pancreatic cancer is a deadly cancer that is very difficult to treat. See Siegel, RL et al. *CA Cancer J. Clin.* (2015) 65: 5-29. Unique aspects of pancreatic cancer include a very

low 5-year survival rate of less than 7% (Id.), late presentation, early metastasis and a poor response to chemotherapy and radiation. See Maitra A and Hruban RH, *Annu Rev. Pathol.* (2008) 3:157-188. To date gemcitabine-based chemotherapy (2', 2'-difluoro 2'deoxyctidine) is the gold standard for the treatment of pancreatic cancer, however the effect of therapeutic intervention is limited due to drug resistance. Oettle, H et al. *JAMA* (2013) 310: 1473-1481.

[0006] Ovarian cancer is present in approximately 225,000 women in the United States, with approximately 12/100,000 women being newly diagnosed with ovarian cancer each year. Noone AM, et al. (eds). *SEER Cancer Statistics Review, 1975-2015, National Cancer Institute*. Bethesda, MD (2018). There are three primary forms of ovarian cancer. Namely, ovarian epithelial cancer, fallopian tube cancer, and primary peritoneal cancer, which form in the tissue covering the ovary, lining the fallopian tube or peritoneum, respectively. Many chemotherapeutic agents are used to treat ovarian cancers including, but not limited to, cytotoxic drugs such as taxols (e.g., paclitaxel), doxorubicin hydrochloride, topotecan hydrochloride, gemcitabine hydrochloride, carboplatin, and cisplatin. In addition, many antibody-based therapeutic agents are administered to treat ovarian cancers, such as bevacizumab, olaparib and rucaparib camysylate.

[0007] 5-fluorouracil (i.e., 5-FU, or more specifically, 5-fluoro-1H-pyrimidine-2,4-dione) is a well-known pyrimidine antagonist that is used in many adjuvants chemotherapeutic medicants, such as Carac® cream, Efudex®, Fluoroplex®, and Aduvicol®. It is well established that 5-FU targets a critical enzyme, thymidylate synthase (TYMS or TS), which catalyzes the methylation of deoxyuridine monophosphate (dUMP) to deoxythymidine monophosphate (dTMP) an essential step in DNA biosynthesis. Danenberg P. V., *Biochim. Biophys. Acta*. (1977) 473(2):73-92. However, despite the steady improvement of 5-FU-based therapy, the patient response rate to 5-FU-based chemotherapy remains modest, due to the development of drug resistance. Longley D. B, et al., *Apoptosis, Cell Signaling, and Human Diseases*, (2007) p. 263-78.

[0008] Nevertheless, the existing cancer therapies are still facing with many hurdles including toxicity side effects and resistance. For example, it is well known that, although

5-FU is fairly efficacious in treating a variety of cancers, 5-FU possesses substantial toxicity and can elicit a host of adverse side effects. With respect to miRNAs, these compounds are known to be susceptible to enzymatic degradation when administered, which results in poor stabilities. Moreover, tumor cells have been known to circumvent apoptotic pathways by developing resistance to common therapeutic agents, such as 5-FU and gemcitabine. See Gottesman M. M. et al., *Nature Reviews Cancer*, (2002) 2(1):48-58. Thus, there would be a significant benefit in more efficacious, stable, and less toxic medications for the treatment of cancer.

SUMMARY OF THE DISCLOSURE

[0009] Tumor suppressor miRNA candidates such as miR-15a, modified by pyrimidine analog 5-Fluorouracil (5-FU), has potent efficacy in inhibiting tumor growth of colon cancer, pancreatic cancer, and many other tumor types (Fesler, A., Liu, H., Ju, J., Modified miR-15a has therapeutic potential for improving treatment of advanced stage colorectal cancer through inhibition of BCL2, BMI1, YAP1 and DCLK1, *Oncotarget*, 2018, 9 (2), 2367-2383), (Guo, S., Fesler, A., Huang, W., Wang, Y., Yang, J., Wang, X., Zheng, Y., Hwang, G. R., Wang, H., Ju, J., Functional Significance and Therapeutic Potential of miR-15a Mimic in Pancreatic Ductal Adenocarcinoma, *Mol Ther Nucleic Acids*, 2020, 19, 228-239). In addition, with 5-FU modification, gemcitabine is introduced to the 5-FU-miR-15a by replacing C with gemcitabine on the guide strand of miR-15a. The dual modifications of 5-FU and gemcitabine have further enhanced the therapeutic efficacy of tumor suppressor miR-15a. To further improve tumor specificity of 5-FU modified miR-15a to cancer cells, MTX-miRNA (in this case, 5-FU and gemcitabine modified miR-15a as an example) conjugate is designed and synthesized using click chemistry. While both 5-FU and gemcitabine modifications are on the guide (target) strand of miR-15a, MTX conjugation is on the sense (passenger) strand of miR-15a. The rationale behind this design is that folate receptor/reduce folate carrier (elevated in cancer cells) will be used as a way to improve tumor specificity. MTX is on the passenger strand of miRNA so it will not interfere with mRNA target recognition and binding of the target strand. MTX, once break down from the

miRNA mimetic, will add additional tumor killing effect of the modified miRNA tumor suppressor.

[0010] Therefore, one aspect of the present disclosure is directed to a double-stranded nucleic acid composition comprising a modified double-stranded nucleic acid sequence that comprises uracil, gemcitabine, and methotrexate (MTX), wherein the uracil comprises 5-fluorouracil.

[0011] In some embodiments, said modified double-stranded nucleic acid sequence comprises a modified microRNA nucleotide sequence. In some embodiments, said modified microRNA nucleotide sequence comprises a microRNA nucleotide sequence of miR-15a as set forth in SEQ ID NO.1.

[0012] In some embodiments, said miR-15a microRNA nucleotide sequence comprises a guide strand and a passenger strand.

[0013] In some embodiments, cytidines are replaced with gemcitabines on the guide strand of the miR-15a microRNA nucleotide sequence. In some embodiments, the uracils on the guide strand of the miR-15a microRNA nucleotide sequence are 5-fluorouracils.

[0014] In some embodiments, the methotrexate (MTX) is conjugated on the passenger strand of the miR-15a microRNA nucleotide sequence. In some embodiments, the methotrexate (MTX) is conjugated onto the 5' end of the passenger strand of the miR-15a microRNA nucleotide sequence.

[0015] In some embodiments, dual modifications of miR-15a using 5-fluorouracil and gemcitabine enhance the therapeutic efficacy of tumor suppression compared to unmodified miR-15a.

[0016] In some embodiments, dual modifications of miR-15a using 5-fluorouracil and gemcitabine enhance the therapeutic efficacy of tumor suppression compared to unmodified miR-15a.

[0017] Another aspect of the disclosure is directed to a pharmaceutical composition comprising a double-stranded nucleic acid composition comprising a double-stranded

nucleic acid sequence that comprises uracil, gemcitabine and methotrexate (MTX), wherein the uracil comprises 5-fluorouracil, and a pharmaceutically acceptable carrier.

[0018] In some embodiments, said modified microRNA nucleotide sequence in the pharmaceutical composition, comprises a microRNA nucleotide sequence of miR-15a as set forth in SEQ ID NO. 1.

[0019] In some embodiments, said miR-15a microRNA nucleotide sequence in the pharmaceutical composition, comprises a guide strand and a passenger strand.

[0020] In some embodiments, cytidines are replaced with gemcitabines on the guide strand of the miR-15a microRNA nucleotide sequence in the pharmaceutical composition. In some embodiments, the uracils on the guide strand of the miR-15a microRNA nucleotide sequence in the pharmaceutical composition are 5-fluorouracils.

[0021] In some embodiments, the methotrexate (MTX) is conjugated on the passenger strand of the miR-15a microRNA nucleotide sequence in the pharmaceutical composition. In some embodiments, the methotrexate (MTX) is conjugated onto the 5' end of the passenger strand of miR-15a microRNA nucleotide sequence in the pharmaceutical composition.

[0022] Another aspect of the disclosure is directed to a method for treating cancer comprising administering to a subject an effective amount of a double-stranded nucleic acid composition comprising a modified double-stranded nucleic acid sequence that comprises uracil, gemcitabine and methotrexate (MTX), wherein the uracil comprises 5-fluorouracil.

[0023] In some embodiments, the subject is a mammal. In some embodiments, the mammal is a human.

[0024] In some embodiments, said subject has a cancer selected from the group consisting of colorectal, stomach, esophageal, breast, lung, prostate, ovarian, uterine, pancreatic, liver, skin, blood or cervical cancer. In some embodiments, said subject has pancreatitis or fibrosis.

[0025] In some embodiments, said subject has pancreatic cancer. In some embodiments, said pancreatic cancer is pancreatic ductal adenocarcinoma cancer (PDAC). In some embodiments, said subject has ovarian cancer.

[0026] In some embodiments, said double-stranded nucleic acid composition is administered to the subject by injection.

[0027] In some embodiments, said modified double-stranded nucleic acid sequence for the method for treating cancer comprises a modified microRNA nucleotide sequence. In some embodiments, said modified microRNA nucleotide sequence for the method for treating cancer comprises a microRNA nucleotide sequence of miR-15a as set forth in SEQ ID NO. 1.

[0028] In some embodiments, said miR-15a microRNA nucleotide sequence for the method for treating cancer comprises a guide strand and a passenger strand. In some embodiments, the uracils on the guide strand of the miR-15a microRNA nucleotide sequence for the method for treating cancer, are 5-fluorouracils.

[0029] In some embodiments, cytidines are replaced with gemcitabines on the guide strand of the miR-15a microRNA nucleotide sequence for the method for treating cancer.

[0030] In some embodiments, the modified double-stranded nucleic acid sequence induces apoptosis. In some embodiments, the modified double-stranded nucleic acid sequence comprises MTX-5FU-Gem-miR-15a. In some embodiments, the modified miR-15a exhibits an enhanced potency in inducing apoptosis when compared to both unmodified miR-15a and co-treatment of 5-FU and gemcitabine.

[0031] The data provided herein show an enhanced therapeutic efficacy of tumor suppression and improves tumor specificity of double stranded nucleic acid compositions described herein when compared to known anticancer agents, such as 5-FU, gemcitabine or methotrexate (MTX) alone. As such, the present compositions and methods provide the additional benefit of permitting a lower dosing, which results in lower toxicity and fewer side effects. A further significant advantage exhibited by the described nucleic acid compositions is that the instant compositions have significantly improved stability compared

to unmodified miR-15a sequence. Thus, at least in view of the noted advantages, the nucleic acid compositions disclosed herein represent a substantial advance in the treatment of cancer by suppressing multiple oncogenes and signaling pathways.

BRIEF DESCRIPTION OF THE DRAWINGS

[0032] FIG. 1. MTX-5-FU-GEM-miR-15a sequence and location of its modifications.

MTX-5-FU-GEM-miR-15a has the uracils substituted for 5-fluorouracil (5-FU) and the cytidines substituted for gemcitabine (GEM) its 5' strand. Methotrexate (MTX) is conjugated onto the 5' end of the 3' strand of the modified-miRNA.

[0033] FIGS. 2A-2B. MTX-5-FU-GEM-miR-15a exhibits a dose-dependent inhibition of cancer cell growth without the use of delivery vehicle. **(A)** MTX-5-FU-GEM-miR-15a is able to inhibit proliferation in MD-MBA-231 cells ($IC_{50} = 4.6$ nM) without use of delivery vehicle, whereas miR-15a is unable to enter the cell and inhibit proliferation without delivery vehicle. **(B)** Furthermore, MTX-5-FU-GEM-miR-15a inhibits cancer cell growth without the use of delivery vehicle in A549 non-small cell lung cancer cells, MD-MBA-231 triple-negative breast cancer cells, and in MIA PaCa-2 and PANC-1 pancreatic cancer cells. A549 has a non-detectable level of folate receptor while MD-MBA-231 cells display a relatively high level of folate receptor. Compared to each other, MD-MBA-231 cells are 22.2-fold more sensitive than A549 cells. Similarly, in PDAC, PANC-1 has a very low expression of folate receptor, while MIA PaCa-2 has a higher expression of folate receptor and their relative sensitivities appears to be correlated with folate receptor status, as MIA PaCa-2 cells are 1.8-fold more sensitive.

[0034] FIGS. 3A-3B. MTX-5-FU-GEM-miR-15a acts like a miRNA, retaining target specificity to its miR-15a targets, YAP1 and BMI1. MTX-5-FU-GEM-miR-15a is able to knock down reported targets of miR-15a. MTX-5-FU-GEM-miR-15a **(A)** knocks down YAP1 and BMI1 in MD-MBA-231 triple-negative breast cancer cells and **(B)** knocks down YAP1 in MIA PaCa-2 pancreatic cancer cells.

[0035] FIGS. 4A-4E. MTX-5FU-Gem-miR-15a is able to inhibit PDAC PDX organoids (hF44, hF3, hT89) growth under vehicle free conditions. The IC_{50} of the modified miRNA

is around 2 nM which is ~250-fold lesser than Gemcitabine in treating these organoids. **(A)** Representative images of untreated hF44 PDAC organoids compared to **(B)** those treated with 12.5 nM of MTX-5-FU-GEM-miR-15a. There is a >200-fold enhancement of growth inhibition when compared to treatment with gemcitabine alone in **(C)** hF3, **(D)** hF44, and **(E)** hT89 organoids. Data are presented as mean $\pm$ standard error of the mean ($n = 3$).

[0036] FIG. 5. Dose Response Curves of Modified-miR-15a Mimetics and Gemcitabine in hT89 Human PDAC Organoids. Human PDAC hT89 cells were plated onto 96-well plates and treated with 5-FU-miR-15a ($IC_{50} = 24.7 \pm 3.4$ nM), MTX-5-FU-GEM-miR-15a ($IC_{50} = 1.2 \pm 0.2$ μ M), and gemcitabine ($IC_{50} = 1.2 \pm 0.2$ μ M).

[0037] FIG. 6. PDAC cell lines (PANC-1, MIAPaCa-2 and Hs766T) and Gemcitabine resistant counterpart of Hs766T were subjected to treatment with MTX-5FU-Gem-miR-15a at varying concentrations without using any delivery vehicle. It was observed that the modified miR-15a demonstrates the ability to successfully cross the lipid bilayer without any delivery vehicle and inhibit the cancer cell growth in a dose-dependent manner. The IC_{50} values for the modified miRNA and Gemcitabine is given in the table.

[0038] FIG. 7. (A) MTX-5FU-Gem-15a was found to induce cell cycle arrest in both Hs 766T parental and Gemcitabine resistant cells as measured by propidium iodide (PI) staining. The G2/S ratio is significantly decreased after modified miR-15a treatment, indicating an augmented population of cells in the S phase and a concurrent reduction of cells in G2 phase suggesting that the modified miR-15a induced cell cycle arrest in both parental and Gemcitabine resistant PDAC cells. **(B)** Annexin V/PI staining demonstrated induction of apoptosis in both the cell lines following treatment with MTX-5FU-Gem-miR-15a. The modified miR-15a exhibits an enhanced potency in inducing apoptosis in both when compared to both unmodified miR-15a and the co-treatment of 5-FU and Gem.

[0039] FIG. 8. MTX-5FU-Gem-miR-15a retains the target specificity and downregulate the expression of miR-15a targets with. PDAC cell line (Hs766T) was transfected with unmodified miR-15a and MTX-5FU-Gem-miR-15a using delivery vehicle. It was found that the modified miRNA treatment showed increased downregulation of some key miR-15a

targets CHK1, WEE1, and YAP1, which play crucial role in PDAC progression and drug resistance.

[0040] FIG. 9. The modified miR-15a demonstrated significant inhibition of metastatic pancreatic ductal adenocarcinoma (PDAC) tumor growth in NOD/SCID mice. Xenografts were established by intravenously injecting luciferase-expressing Hs766T cells into the mice via the tail vein. By day 37 post-injection, mice treated with MTX-5FU-Gem-miR-15a at a dosage of 4 mg/kg exhibited a 7.3-fold reduction in tumor growth compared to the control group.

[0041] FIG. 10. (A) The comparative effects of modified and unmodified miR-15a on ovarian cancer cell viability. OVCAR-3 (epithelial ovarian cancer cell line) cells were subjected to treatment with both MTX-5FU-Gem-miR-15a and unmodified miR-15a at varying concentrations without using any delivery vehicle. It was observed that the unmodified miR-15a cannot traverse the lipid bilayer effectively in the absence of a delivery vehicle. In contrast, the modified miR-15a demonstrates the ability to successfully cross the lipid bilayer and inhibit the cancer cell growth in a dose-dependent manner. The IC_{50} value for MTX-5FU-Gem-15a is calculated to be 1.88 ± 0.31 nM in OVCAR-3 parental cells. **(B)** The efficacy of the modified miRNA mimic was also observed in two other epithelial ovarian cancer cell lines – SK-OV-3 and A2780. In SK-OV-3 cells, the efficacy of the miRNA mimic was observed to be even higher with delivery vehicle ($IC_{50} = 5.67 \pm 2.1$ nM) when compared to treatment without delivery vehicle ($IC_{50} = 37.5 \pm 16.9$ nM).

[0042] FIG. 11. (A) Flow cytometry analysis (using Propidium iodide staining) of cell cycle progression in OVCAR-3 ovarian cancer cells treated with MTX-5FU-Gem-miR-15a. The G2/S ratio is significantly decreased after modified miR-15a treatment, indicative of an augmented population of cells within the S phase and a concurrent reduction of cells within the G2 phase suggesting cell cycle arrest in the S-phase. **(B)** Annexin V/PI staining demonstrated an increased potency in inducing apoptosis in MTX-5FU-Gem-miR-15a treated cells when compared to both unmodified miR-15a and the co-treatment of 5FU and Gem. **(C)** The effect of the modified miRNA mimic on cell cycle and apoptosis was also

assessed in other two ovarian cancer cells – SK-OV-3 and A2780. Similar cell cycle arrest and apoptosis induction were observed in both cell lines.

[0043] FIG. 12. (A) Parental OVCAR-3 cells were rendered resistant to Olaparib by gradually exposing them to increasing concentrations of the drug and the efficacy of the MTX-5FU-Gem-miR-15a was evaluated for its cytotoxic effects on these Olaparib-resistant ovarian cancer cells in absence of any delivery vehicle. It was observed that the unmodified miR-15a did not have any effect without any delivery vehicle. However, the modified miR-15a demonstrated significant potential in inhibiting proliferation and inducing cytotoxicity in the drug-resistant cells ($IC_{50} = 6.5$ nM). **(B)** The effect of MTX-5FU-Gem-miR-15a treatment on cell cycle progression and apoptosis induction were also assessed. Consistent with the observations in the parental cell line, the modified miRNA treatment led to a significant decrease in the G2/S ratio, indicative of S phase cell cycle arrest, and demonstrated a heightened apoptosis induction compared to both unmodified miR-15a and the co-treatment with 5-FU and Gemcitabine in the drug-resistant cells too.

[0044] FIG. 13. MTX-5FU-Gem-miR-15a was also found to demonstrate sustained miR-15a functionality and downregulate the expression of WEE1, BMI1, DCLK1 and BCL2, targets of miR-15a in OVCAR-3 cell line. OVCAR-3 cells were transfected with the miRNAs at 50 nM concentration using oligofectamine as a delivery vehicle and the protein samples were collected 3 days post transfection.

DETAILED DESCRIPTION OF THE DISCLOSURE

[0045] The present disclosure provides double stranded nucleic acid compositions that incorporate 5-fluorouracil (5-FU), gemcitabine (GEM) and methotrexate (MTX). Without being bound by any one particular theory, surprisingly the present disclosure reveals that the replacement of uracil nucleotides within a double stranded nucleic acid sequence e.g., microRNA oligonucleotide sequence with a 5-halouracil increases the ability of the microRNA to inhibit cancer, development, progression and tumorigenesis. As chemotherapeutic agents such as 5-fluorouracil, gemcitabine and methotrexate (MTX) are highly toxic and often cause resistance and patient death, it limits their efficacy to benefit patients. microRNA tumor suppressor can suppress multiple oncogenic targets, however

there is always a concern with microRNA off-target effect and toxicity. The present disclosure surprisingly reveals that integration of both 5-FU and gemcitabine to double stranded nucleic acids (e.g., microRNAs) and methotrexate (MTX) conjugation create a new class of anticancer agents with high potency and tumor specificity. As unexpected, due to the concern of toxic side effects by combining these together, 5-FU and/or gemcitabine modified, and methotrexate (MTX) conjugated double stranded nucleic acids are highly tumor specific and effective to eliminate cancer cells without toxic side effect. In addition, the modified double stranded nucleic acids (e.g., miRNAs) can be delivered to cancer cells independent of delivery vehicle, which is another unique feature.

[0046] As such, the present disclosure provides various double stranded nucleic acid (e.g., microRNA) compositions having 5-halouracil molecules, gemcitabine and methotrexate (MTX) incorporated in their nucleic acid sequences and methods for using the same. The present disclosure further provides formulations, such as pharmaceutical compositions comprising the modified nucleic acid compositions, and methods for treating cancers that include administration of the same to a subject in need thereof.

[0047] The term “double stranded nucleic acid” is used interchangeably to refer to microRNA or siRNA (small interfering RNA).

[0048] The term “siRNA (small interfering RNA)” also known as short interfering RNA or silencing RNA, refers to a class of double-stranded RNA at first non-coding RNA molecules, typically 20-24 (normally 21) base pairs in length, similar to miRNA, and operating within the RNA interference (RNAi) pathway.

[0049] The term “microRNA” or “miRNA” or “miR” are used interchangeably to refer to small non-coding ribose nucleic acid (RNA) molecules that are capable of regulating the expression of genes through interacting with messenger RNA molecules (mRNA), DNA or proteins. Typically, microRNAs are composed of nucleic acid sequences of about 19-25 nucleotides (bases) and are found in mammalian cells.

[0050] The term “guide strand” and “passenger strand” refer to the two strands in a miRNA duplex. The guide strand (also known as leading strand or miR) refers to the active

strand that is incorporated into the RISC (RNA-induced silencing complex) and the passenger strand (or miR*) refers to the complementary strand which gets degraded.

[0051] The term “modified microRNA”, “modified miRNA” or “modified miR” are used interchangeably herein to refer to a microRNA that differs from the native or endogenous microRNA (unmodified microRNA). More specifically, in the present disclosure a modified microRNA differs from the unaltered or unmodified microRNA nucleic acid sequence by one or more base. In some embodiments of the present disclosure, a modified microRNA of the present disclosure includes at least one uracil (U) nucleotide base substituted with a 5-halouracil. In some embodiments, a 5-halouracil is a 5-fluorouracil. In other embodiments a modified microRNA includes an additional nucleotide (i.e., adenine (A), cytosine (C), uracil (U), and guanine (G)) and at least one uracil base that is substituted with a 5-halouracil.

[0052] In one aspect of the present disclosure, nucleic acid compositions that include a modified double stranded nucleic acid sequence (e.g., modified microRNA nucleotide sequence) having uracil bases (U, U bases) that have been replaced with 5-halouracils, such as 5-fluorouracils (5-FU); cytidines that have been replaced with gemcitabines and having conjugated methotrexate (MTX), are described. As further discussed herein, the nucleic acid compositions of the present disclosure are useful, at least, in the treatment of cancer, particularly pancreatic cancer.

[0053] In some embodiments, the nucleic acid compositions contain a nucleotide sequence that has been modified by derivatizing at least one of the uracil nucleobases at the 5-position with a group that provides a similar effect as a halogen atom. In some embodiments, the group providing the similar effect has a similar size in weight or spatial dimension to a halogen atom, e.g., a molecular weight of up to or less than 20, 30, 40, 50, 60, 70, 80, 90, or 80 g/mol. In certain embodiments, the group providing a similar effect as a halogen atom may be, for example, a methyl group, trihalomethyl (e.g., trifluoromethyl) group, pseudohalide (e.g., trifluoromethanesulfonate, cyano, or cyanate) or deuterium (D) atom. The group providing a similar effect as a halogen atom may be present in the absence of or in addition to a 5-halouracil base in the miR-15a nucleotide sequence.

[0054] In certain embodiments, the modified microRNA has more than one, or exactly one uracil that has been replaced with a 5-halouracil.

[0055] In some embodiments, the modified microRNA nucleotide sequence includes three, four, five, six, seven, eight or more uracil bases that have been replaced with a 5-halouracil.

[0056] In other embodiments, all of the uracil nucleotide bases of the modified mRNA have been replaced by a 5-halouracil.

[0057] In some embodiments, the 5-halouracil is, for example, 5-fluorouracil, 5-chlorouracil, 5-bromouracil, or 5-iodouracil. In specific embodiments, the 5-halouracil is 5-fluorouracil

[0058] In an exemplary embodiment, the present disclosure is directed to nucleic acid compositions that include a miR-15a nucleotide sequence that has been modified. In some embodiments, the miR-15a nucleotide sequence has been modified by replacing at least one of the U bases with a 5-halouracil.

[0059] The term “miR-15a”, as used herein, is meant to be synonymous with the terms “microRNA-15a” or “miRNA-15a” and refers to an oligonucleotide having the following nucleotide sequence: UAGCAGCACAUAAUGGUUUGUG [SEQ ID NO. 1], where it is understood that A = adenine, C = cytosine, U = uracil, and G = guanine bases. The foregoing nucleotide sequence is herein referred to as a miR-15a unmodified (i.e., “native”) sequence unless otherwise specified. MiR-15a may also be referred to in the field as hsa-miR-15a or hsa-miR-15a-5p, with accession number(s) MI0000069. MiR-15a is well known and has been studied in detail, e.g., Xie T, et al. *Clin Transl Oncol.* (2015) 17(7):504-10; and Acunzo M, and Croce CM, *Clin. Chem.* (2016) 62(4):655-6. Methods for creating a miR-15a mimic are known by those of ordinary skill in the art. Unless otherwise stated, all such modified miR-15a forms are herein considered to be within the scope of the term “miR-15a mimic”, as used herein.

[0060] Generally, a modified miR-15a (i.e., miR-15a mimic) contains no more than one, two, three, four, or five additional nucleotides covalently appended to the miR-15a native

sequence, wherein the additional bases are independently selected from C, U, G, and C, or the additional bases may be exclusively U. Typically, the miR-15a is used in single-strand form, but double-stranded versions are considered herein.

[0061] In some embodiments, at least one of the U bases in the miR-15a sequence, whether in the native and/or in an appended portion, is a 5-halouracil. The 5-halouracil can be, for example, 5-fluorouracil, 5-chlorouracil, 5-bromouracil, or 5-iodouracil.

[0062] In some embodiments, the miR-15a sequence is double stranded comprising a guide strand and a passenger strand. In some embodiments, the guide strand of the miR-15a sequence corresponds to SEQ ID NO. 1. In some embodiments, all of the U bases in the miR-15a guide strand sequence, are 5-halouracils. In a specific embodiment, all of the U bases in the miR-15a guide strand sequence, are 5-fluouracils. In some embodiments, all of the cytidines in the miR-15a guide strand sequence, are replaced with gemcitabines.

[0063] In some embodiments, the passenger strand of the miR-15a sequence has a nucleotide sequence of CAGGCCAUAUUGUGCCUCA [SEQ ID NO. 2]. In some embodiments, the methotrexate (MTX) is conjugated on the passenger strand of the miR-15a microRNA nucleotide sequence. In some embodiments, the methotrexate (MTX) is conjugated onto the 5' end of the passenger strand of the miR-15a microRNA nucleotide sequence.

[0064] In some embodiments, the double stranded MTX-5-FU-GEM-miR-15a sequence has a nucleotide sequence [SEQ ID NO. 3] as depicted in FIG. 1.

[0065] In certain embodiments, the nucleic acid composition contains a miR-15a nucleotide sequence that has been modified by derivatizing at least one of the uracil (U) nucleobases at the 5-position with a group that provides a similar effect as a halogen atom. In some embodiments, the group providing the similar effect has a similar size in weight or spatial dimension to a halogen atom, e.g., a molecular weight of up to or less than 20, 30, 40, 50, 60, 70, 80, 90, or 80 g/mol. The group providing a similar effect as a halogen atom may be, for example, a methyl group, trihalomethyl (e.g., trifluoromethyl) group, pseudohalide (e.g., trifluoromethanesulfonate, cyano, or cyanate) or deuterium (D) atom. The group

providing a similar effect as a halogen atom may be present in the absence of or in addition to a 5-halouracil base in the miR-15a nucleotide sequence. Moreover, the group providing a similar effect as a halogen atom may be located in the native (or seed) portion and/or in an appended portion of the miR-15a nucleotide sequence.

[0066] In some embodiments, the microRNA candidates are 5-FU-miR-129, 5-FU-miR-506, 5-FU-miR-200, 5-FU-miR-200b, 5-FU-miR-200c, 5-FU-miR-140, 5-FU-miR-194, 5-FU-miR-miR-215, 5-FU-miR-34, 5-FU-let-7g, and 5-FU-miR-489.

[0067] The term “miR-129” as used herein, is meant to be synonymous with the terms “microRNA-129” or “miRNA-129” and refers to an oligonucleotide having the following nucleotide sequence: CUUUUUGCGGUCUGGGCUUGC [SEQ ID NO. 4], where it is understood that C = cytosine, U = uracil, and G = guanine bases. The foregoing nucleotide sequence is herein referred to as an unmodified miR-129 (i.e., “native”) sequence unless otherwise specified. MiR-129 may also be referred to in the field as hsa-miR-129 or hsa-miR-129-5p, with accession number(s) MI0000252 and MIMAT0000242. MiR-129 is well known and has been studied in detail. See, e.g., J. Wu et al., *Cell Cycle*, (2010) 9:9, 1809-1818. As also well known in the art, the miR-129 sequence may be modified to produce a “miR-129 mimic”, which has a sequence modified from the native sequence, but that retains the known function or activity of the native miR-129. Unless otherwise stated, all such modified miR-129 compositions are herein considered to be within the scope of the term “miR-129 mimic” as used herein.

[0068] The term “miR-140”, as used herein, is meant to be synonymous with the terms “microRNA-140” or “miRNA-140” and refers to an oligonucleotide having the following nucleotide sequence: CAGUGGUUUUACCCUAUGGUAG [SEQ ID NO. 5], where it is understood that A = adenine, C = cytosine, U = uracil, and G = guanine bases. The foregoing nucleotide sequence is herein referred to as a miR-140 unmodified (i.e., “native”) sequence unless otherwise specified. MiR-140 may also be referred by accession number(s) NT_010498 or by miRBase Accession MI0000456. MiR-140 is well known and has been studied in detail, e.g., Zhai, H. et al., *Oncotarget*. (2015) 6: 19735-46. As stated above for exemplary mimics miR-129 and miR-15a, methods for creating a miR-140 mimic are known

by those of ordinary skill in the art. Unless otherwise stated, all such modified miR-140 forms are herein considered to be within the scope of the term “miR-140 mimic”, as used herein.

[0069] The term “miR-192”, as used herein, is meant to be synonymous with the terms “microRNA-192” or “miRNA-192” and refers to an oligonucleotide having the following nucleotide sequence: CUGACCUAUGAAUUGACAGCC [SEQ ID NO. 6], where it is understood that A = adenine, C = cytosine, U = uracil, and G = guanine bases. The foregoing nucleotide sequence is herein referred to as a miR-192 unmodified (i.e., “native”) sequence unless otherwise specified. MiR-192 may also be referred as hsa-mir-192 or by miRBase Accession MI0000234, or MIMAT0000222. MiR-192 is well known and has been studied in detail, e.g., Song, B. et al., Clin. Cancer Res. (2008), 14: 8080-8086. As stated above for exemplary mimics miR-129, miR-140 and miR-15a, methods for creating a miR-192 mimics are known by those of ordinary skill in the art. Unless otherwise stated, all such modified miR-192 forms are herein considered to be within the scope of the term “miR-192 mimic”, as used herein.

[0070] The term “miR-502”, as used herein, is meant to be synonymous with the terms “microRNA-502” or “miRNA-502” and refers to an oligonucleotide having the following nucleotide sequence: AUCCUUGCUAUCUGGGUGCUA [SEQ ID NO. 7], where it is understood that A = adenine, C = cytosine, U = uracil, and G = guanine bases. The foregoing nucleotide sequence is herein referred to as a miR-502 unmodified (i.e., “native”) sequence unless otherwise specified. MiR-502 may also be referred as hsa-mir-502 or by miRBase Accession MI0003186, or MIMAT0002873. MiR-502 is well known and has been studied in detail, e.g., Zhai, H, et al., Oncogene. (2013), 32:12 pp. 1570-1579. As stated above for exemplary mimics miR-129, miR-140, miR-192 and miR-15a, methods for creating a miR-502 mimics are known by those of ordinary skill in the art. Unless otherwise stated, all such modified miR-502 forms are herein considered to be within the scope of the term “miR-502 mimic”, as used herein.

[0071] The term “miR-506”, as used herein, is meant to be synonymous with the terms “microRNA-506” or “miRNA-506” and refers to an oligonucleotide having the following nucleotide sequence: UAUUCAGGAAGGUGUUACUUA [SEQ ID NO. 8], where it is

understood that A = adenine, C = cytosine, U = uracil, and G = guanine bases. The foregoing nucleotide sequence is herein referred to as a miR-506 unmodified (i.e., “native”) sequence unless otherwise specified. MiR-506 may also be referred as hsa-mir-506 or by miRBase Accession MI0003193, or MIMAT0022701. MiR-506 is well known and has been studied in detail, e.g., Li, J, et al., *Oncotarget*. (2016), 7:38 pp. 62778-62788, and Li, J. et al., *Oncogene*. (2016) 35 pp. 5501-5514. As stated above for exemplary mimics miR-129, miR-140, miR-502, miR-192 and miR-15a, methods for creating a miR-506 mimics are known by those of ordinary skill in the art. Unless otherwise stated, all such modified miR-506 forms are herein considered to be within the scope of the term “miR-506 mimic”, as used herein.

[0072] In some embodiments, the nucleic acid compositions of the present disclosure may be produced biosynthetically, such as by using *in vitro* RNA transcription from plasmid, PCR fragment, or synthetic DNA templates, or by using recombinant (*in vivo*) RNA expression methods. See, e.g., C. M. Dunham et al., *Nature Methods*, (2007) 4(7), pp. 547-548. The microRNA sequence (e.g., miR-15a sequence, miR-140 sequence, miR-192 sequence, miR-502 sequence, miR-506 sequence or miR-129 sequence) may be further chemically modified such as by functionalizing with polyethylene glycol (PEG) or a hydrocarbon or a targeting agent, particularly a cancer cell targeting agent, such as folate, by techniques well known in the art. To include such groups, a reactive group (e.g., amino, aldehyde, thiol, or carboxylate group) that can be used to append a desired functional group may first be included in the oligonucleotide sequence. Although such reactive or functional groups may be incorporated onto the as-produced nucleic acid sequence, reactive or functional groups can be more facilely included by using an automated oligonucleotide synthesis in which non-nucleoside phosphoramidites containing reactive groups or reactive precursor groups are included.

[0073] The term “methotrexate (MTX)”, as used herein, is a folic acid analog acting as a competitive inhibitor to inhibit dihydrofolate reductase (DHFR), an enzyme that participates in the tetrahydrofolate synthesis (Bertino, J. R., *Cancer research: from folate antagonism to molecular targets*, *Best Pract Res Clin Haematol*, 2009, 22 (4), 577-82). The affinity of

MTX for DHFR is about 1000-fold that of folate (Bertino, J. R., Cancer research: from folate antagonism to molecular targets, *Best Pract Res Clin Haematol*, 2009, 22 (4), 577-82). DHFR catalyzes the conversion of dihydrofolate to the active tetrahydrofolate (Schweitzer, B. I., Dicker, A. P., Bertino, J. R., Dihydrofolate reductase as a therapeutic target, *FASEB J*, 1990, 4 (8), 2441-52). Folic acid is needed for the de novo synthesis of the nucleoside thymidine, required for DNA synthesis (Schweitzer, B. I., Dicker, A. P., Bertino, J. R., Dihydrofolate reductase as a therapeutic target, *FASEB J*, 1990, 4 (8), 2441-52). Also, folate is essential for purine and pyrimidine base biosynthesis, so synthesis will be inhibited. MTX, therefore, inhibits the synthesis of DNA, RNA, thymidylates, and proteins. As a result, MTX is one of the key anti-cancer agents (Schweitzer, B. I., Dicker, A. P., Bertino, J. R., Dihydrofolate reductase as a therapeutic target, *FASEB J*, 1990, 4 (8), 2441-52).

[0074] MTX binds to folate receptor and/or reduced folate carrier (Brzezinska, A., Winska, P. Balinska, M., Cellular aspects of folate and antifolate membrane transport, *Acta Biochim Pol*, 2000, 47 (3), 735-49). Elevated folate receptor levels have been found in many tumor types, as a result, folate receptor can be utilized as a way of target delivery of anticancer therapeutics to cancer cells (Li, M. H., Choi, S. K., Thomas, T. P., Desai, A., Lee, K. H., Kotlyar, A., Banaszak Holl, M. M., Baker, J. R., Jr., Dendrimer-based multivalent methotrexates as dual acting nanoconjugates for cancer cell targeting, *Eur J Med Chem* 2012, 47 (1), 560-72).

[0075] Methotrexate is one of the mainstays of treatment for inflammatory forms of arthritis. It reduces pain and swelling and can actually slow joint damage and disease progression over time. For this reason, methotrexate is known as a disease-modifying antirheumatic drug (DMARD). Methotrexate is used as first-line therapy for patients with rheumatoid arthritis (RA), psoriatic arthritis (PsA) and juvenile idiopathic arthritis (JIA).

[0076] Gemcitabine (i.e., 2',2'-difluoro 2'deoxyctidine, dFdC, dFdCyd, difluorodeoxycytidine hydrochloride or more specifically, gemcitabine hydrochloride) is a well known pyrimidine nucleoside. Gemcitabine is a hydrochloride salt of an analogue of the antimetabolite nucleoside deoxycytidine, which possesses anti-neoplastic activity. Gemcitabine is converted intracellularly to the active metabolites difluorodeoxycytidine di-

and triphosphate (dFdCDP, dFdCTP). dFdCDP inhibits ribonucleotide reductase, thereby decreasing the deoxynucleotide pool available for DNA synthesis; dFdCTP is incorporated into DNA, resulting in DNA strand termination and apoptosis. Gemcitabine has the chemical structure 1-(2-oxo-4-amino-1,2-dihydropyrimidin-1-yl)-2-deoxy-2,2-difluororibose hydrochloride. To date gemcitabine-based chemotherapy (2',2'-difluoro 2'deoxyctidine) is the gold standard for the treatment of pancreatic cancer, however the effect of therapeutic intervention is limited due to drug resistance. (Oettle, H et al. *JAMA* (2013) 310 pp. 1473-1481).

[0077] Pancreatic ductal adenocarcinoma cancer (PDAC) has the worst prognosis of any major malignancy with over 45,000 deaths in the United States. Despite all the advancement in pancreatic cancer research on early detection and treatment, limited impact has been achieved on the improvement of patient outcome. New strategies to tackle this disease are urgently needed. Resistance to 5-fluorouracil (5-FU) or Gemcitabine based chemotherapy is one of the major causes for the failure of treating advanced PDAC. Resistance to chemotherapy is highly complex for PDAC including protein coding gene mutations such as KRAS, TP53, and tumor associated fibroblasts. In addition, it has been well recognized recently that epigenetic alterations play a key role in tumorigenesis and resistance to 5-fluorouracil (5-FU) based chemotherapy. Recent studies have shown that epigenetic alterations such as changes in expression of non-coding miRNAs are major contributors to 5-FU and Gemcitabine resistance by providing acute changes in protein synthesis at the post-transcriptional and translational levels. miRNAs are a class of small non-coding RNAs to modulate protein expression by promoting RNA degradation, and inhibiting mRNA translation.

[0078] miR-15a has been identified with tumor suppressive function in PDAC by inhibiting the expression of several major therapeutic target genes (Wee1, Chk1, BMI1, and YAP1). More importantly, a novel strategy has been developed to create modified miR-15a mimics with enhanced efficacy for eliminating 5-FU and Gemcitabine resistant PDAC cells while retaining target specificity. miR-15a mimics were designed by modifying the target strand of miR-15a by replacing uracil (U) bases with 5-FU at various locations to combine

the power of 5-FU and therapeutic miRNA into one entity to create therapeutic synergy. A unique feature of the 5-FU modified miRNA is that it can be internalized by pancreatic cancer cells without any delivery vehicle (e.g., oligofectamine, lipid nanoparticle, magnetic nanoparticle). This represents a major advancement and a paradigm shift in miRNA based therapeutic development. This modification improves the potency and stability of the miR-15a mimic and enhances its ability to inhibit PDAC cancer metastasis in vivo. Cytidines are replaced with gemcitabines on the guide (target) strand of the 5-FU modified miR-15a microRNA nucleotide sequence. These dual modifications of miR-15a using 5-fluorouracil and gemcitabine enhance the therapeutic efficacy of tumor suppression compared to unmodified miR-15a. Further the methotrexate (MTX) is conjugated onto the 5' end of the passenger strand of the double stranded 5-FU-GEM-miR-15a sequence. The MTX-5-FU-GEM-miR-15a sequence improves tumor specificity compared to unconjugated modified miR-15a and adds additional tumor killing effect of the modified miR-15a miRNA tumor suppressor once the methotrexate (MTX) breaks down from the modified miR-15a miRNA.

[0079] In another aspect, the present disclosure is directed to formulations of the nucleic acid compositions described herein. For example, the present nucleic acid compositions can be formulated for pharmaceutical uses. In certain embodiments, a formulation is a pharmaceutical composition containing a nucleic acid composition described herein and a pharmaceutically acceptable carrier.

[0080] The term “pharmaceutically acceptable carrier” is used herein as synonymous with a pharmaceutically acceptable diluent, vehicle, or excipient. Depending on the type of pharmaceutical composition and intended mode of administration, the nucleic acid composition may be dissolved or suspended (e.g., as an emulsion) in the pharmaceutically acceptable carrier. The pharmaceutically acceptable carrier can be any of those liquid or solid compounds, materials, compositions, and/or dosage forms which are, within the scope of sound medical judgment, suitable for use in contact with tissues of a subject. The carrier should be “acceptable” in the sense of being not injurious to the subject it is being provided to and is compatible with the other ingredients of the formulation, i.e., does not alter their biological or chemical function.

[0081] Some, non-limiting examples, of materials which can serve as pharmaceutically acceptable carriers include: sugars, such as lactose, glucose and sucrose; starches, such as corn starch and potato starch; cellulose and its derivatives, such as sodium carboxymethyl cellulose, ethyl cellulose and cellulose acetate; gelatin; talc; waxes; oils, such as peanut oil, cottonseed oil, safflower oil, sesame oil, olive oil, corn oil and soybean oil; glycols, such as ethylene glycol and propylene glycol; polyols, such as glycerin, sorbitol, mannitol and polyethylene glycol; esters, such as ethyl oleate and ethyl laurate; agar; buffering agents; water; isotonic saline; pH buffered solutions; and other non-toxic compatible substances employed in pharmaceutical formulations. The pharmaceutically acceptable carrier may also include a manufacturing aid (e.g., lubricant, talc magnesium, calcium or zinc stearate, or stearic acid), a solvent, or encapsulating material. If desired, certain sweetening and/or flavoring and/or coloring agents may be added. Other suitable excipients can be found in standard pharmaceutical texts, e.g., in "Remington's Pharmaceutical Sciences", The Science and Practice of Pharmacy, 19th Ed. Mack Publishing Company, Easton, Pa., (1995).

[0082] In some embodiments, the pharmaceutically acceptable carrier may include diluents that increase the bulk of a solid pharmaceutical composition and make the pharmaceutical dosage form easier for the patient and caregiver to handle. Diluents for solid compositions include, for example, microcrystalline cellulose (*e.g.*, Avicel[®]), microfine cellulose, lactose, starch, pregelatinized starch, calcium carbonate, calcium sulfate, sugar, dextrates, dextrin, dextrose, dibasic calcium phosphate dihydrate, tribasic calcium phosphate, kaolin, magnesium carbonate, magnesium oxide, maltodextrin, mannitol, polymethacrylates (*e.g.*, Eudragit[®]), potassium chloride, powdered cellulose, sodium chloride, sorbitol and talc.

[0083] The nucleic acid compositions of the present disclosure may be formulated into compositions and dosage forms according to methods known in the art. In certain embodiments, the formulated compositions may be specially formulated for administration in solid or liquid form, including those adapted for the following: (1) oral administration, for example, tablets, capsules, powders, granules, pastes for application to the tongue, aqueous or non-aqueous solutions or suspensions, drenches, or syrups; (2) parenteral administration,

for example, by subcutaneous, intramuscular or intravenous injection as, for example, a sterile solution or suspension; (3) topical application, for example, as a cream, ointment or spray applied to the skin, lungs, or mucous membranes; or (4) intravaginally or intrarectally, for example, as a pessary, cream or foam; (5) sublingually or buccally; (6) ocularly; (7) transdermally; or (8) nasally.

[0084] In some embodiments, the formulations of the present disclosure include a solid pharmaceutical agent that is compacted into a dosage form, such as a tablet, may include excipients whose functions include helping to bind the active ingredient and other excipients together after compression. Binders for solid pharmaceutical compositions include acacia, alginic acid, carbomer (*e.g.* carbopol), carboxymethylcellulose sodium, dextrin, ethyl cellulose, gelatin, guar gum, hydrogenated vegetable oil, hydroxyethyl cellulose, hydroxypropyl cellulose (*e.g.* Klucel[®]), hydroxypropyl methyl cellulose (*e.g.* Methocel[®]), liquid glucose, magnesium aluminum silicate, maltodextrin, methylcellulose, polymethacrylates, povidone (*e.g.* Kollidon[®], Plasdone[®]), pregelatinized starch, sodium alginate and starch.

[0085] The dissolution rate of a compacted solid pharmaceutical composition in a subject's stomach may be increased by the addition of a disintegrant to the composition. Disintegrants include alginic acid, carboxymethylcellulose calcium, carboxymethylcellulose sodium (*e.g.*, Ac-Di-Sol[®], Primellose[®]), colloidal silicon dioxide, croscarmellose sodium, crospovidone (*e.g.*, Kollidon[®], Polyplasdone[®]), guar gum, magnesium aluminum silicate, methyl cellulose, microcrystalline cellulose, polacrillin potassium, powdered cellulose, pregelatinized starch, sodium alginate, sodium starch glycolate (*e.g.* Explotab[®]) and starch.

[0086] Therefore, in certain embodiments, glidants can be added to formulations to improve the flowability of a non-compacted solid agent and to improve the accuracy of dosing. Excipients that may function as glidants include colloidal silicon dioxide, magnesium trisilicate, powdered cellulose, starch, talc and tribasic calcium phosphate.

[0087] When a dosage form such as a tablet is made by the compaction of a powdered composition, the composition is subjected to pressure from a punch and dye. Some excipients and active ingredients have a tendency to adhere to the surfaces of the punch and

dye, which can cause the product to have pitting and other surface irregularities. A lubricant can be added to the composition to reduce adhesion and ease the release of the product from the dye. Lubricants include magnesium stearate, calcium stearate, glyceryl monostearate, glyceryl palmitostearate, hydrogenated castor oil, hydrogenated vegetable oil, mineral oil, polyethylene glycol, sodium benzoate, sodium lauryl sulfate, sodium stearyl fumarate, stearic acid, talc and zinc stearate.

[0088] A formulated pharmaceutical composition for tableting or capsule filling can be prepared by wet granulation. In wet granulation, some or all of the active ingredients and excipients in powder form are blended and then further mixed in the presence of a liquid, typically water, that causes the powders to clump into granules. The granulate is screened and/or milled, dried and then screened and/or milled to the desired particle size. The granulate may then be tableted, or other excipients may be added prior to tableting, such as a glidant and/or a lubricant. A tableting composition may be prepared conventionally by dry blending. For example, the blended composition of the actives and excipients may be compacted into a slug or a sheet and then comminuted into compacted granules. The compacted granules may subsequently be compressed into a tablet.

[0089] In other embodiments, as an alternative to dry granulation, a blended composition may be compressed directly into a compacted dosage form using direct compression techniques. Direct compression produces a more uniform tablet without granules. Excipients that are particularly well suited for direct compression tableting include microcrystalline cellulose, spray dried lactose, dicalcium phosphate dihydrate and colloidal silica. The proper use of these and other excipients in direct compression tableting is known to those in the art with experience and skill in particular formulation challenges of direct compression tableting. A capsule filling may include any of the aforementioned blends and granulates that were described with reference to tableting; however, they are not subjected to a final tableting step.

[0090] In liquid pharmaceutical compositions of the present disclosure, the agent and any other solid excipients are dissolved or suspended in a liquid carrier such as water, water-for-injection, vegetable oil, alcohol, polyethylene glycol, propylene glycol or glycerin. Liquid

pharmaceutical compositions may contain emulsifying agents to disperse uniformly throughout the composition an active ingredient or other excipient that is not soluble in the liquid carrier. The liquid formulation may be used as an injectable, enteric, or emollient type of formulation. Emulsifying agents that may be useful in liquid compositions of the present invention include, for example, gelatin, egg yolk, casein, cholesterol, acacia, tragacanth, chondrus, pectin, methyl cellulose, carbomer, cetostearyl alcohol and cetyl alcohol.

[0091] In some embodiments, liquid pharmaceutical compositions of the present disclosure may also contain a viscosity enhancing agent to improve the mouth-feel of the product and/or coat the lining of the gastrointestinal tract. Such agents include acacia, alginic acid bentonite, carbomer, carboxymethylcellulose calcium or sodium, cetostearyl alcohol, methyl cellulose, ethylcellulose, gelatin guar gum, hydroxyethyl cellulose, hydroxypropyl cellulose, hydroxypropyl methyl cellulose, maltodextrin, polyvinyl alcohol, povidone, propylene carbonate, propylene glycol alginate, sodium alginate, sodium starch glycolate, starch tragacanth and xanthan gum. In other embodiments, the liquid composition of the present disclosure may also contain a buffer, such as gluconic acid, lactic acid, citric acid or acetic acid, sodium gluconate, sodium lactate, sodium citrate, or sodium acetate.

[0092] Sweetening agents, such as sorbitol, saccharin, sodium saccharin, sucrose, aspartame, fructose, mannitol and invert sugar, may be added to certain formulations of the present disclosure to improve the taste. Flavoring agents and flavor enhancers may make the dosage form more palatable to the patient. Common flavoring agents and flavor enhancers for pharmaceutical products that may be included in the composition of the present disclosure include maltol, vanillin, ethyl vanillin, menthol, citric acid, fumaric acid, ethyl maltol and tartaric acid.

[0093] Preservatives and chelating agents, such as alcohol, sodium benzoate, butylated hydroxy toluene, butylated hydroxyanisole and ethylenediamine tetraacetic acid, may be added at levels safe for ingestion to improve storage stability. Solid and liquid compositions may also be dyed using any pharmaceutically acceptable colorant to improve their appearance and/or facilitate patient identification of the product and unit dosage level.

[0094] A dosage formulation of the present disclosure may be a capsule containing the composition, for example, a powdered or granulated solid composition of the disclosure, within either a hard or soft shell. The shell may be made from gelatin and optionally contain a plasticizer such as glycerin and sorbitol, and an opacifying agent or colorant.

[0095] As stated above, the nucleic acid compositions of the present disclosure show unexpected and exceptional anticancer activity when compared to that exhibited by a native microRNA and/or a known cancer therapy (chemotherapy), such as 5-fluorouracil. Therefore, another aspect of the present disclosure provides a method for treating cancer in a mammal by administering to the mammal an effective amount of the nucleic acid compositions described herein.

[0096] Generally, the methods for treating cancer of the present disclosure include administering a nucleic acid composition of the present disclosure (e.g., a modified double-stranded nucleic acid, such as modified miR-15a) to a subject. In certain embodiments, the nucleic acid composition can be administered as a formulation that includes a nucleic acid composition and a carrier. In other embodiments, the nucleic acid composition of the present disclosure can be administered in the absence of a carrier (i.e., naked) and/or with a carrier such as Polyethyleneimine (PEI), lipid nanoparticle, gold nanoparticle to further improve efficacy.

[0097] The term “subject” as used herein refers to any mammal. The mammal can be any mammal, although the methods herein are more typically directed to humans. The phrase “subject in need thereof” as used herein is included within the term subject and refers to any mammalian subject in need of a treatment, particularly cancer or has a medically determined elevated risk of a cancerous or pre-cancerous condition. In specific embodiments, the subject includes a human cancer patient. In some embodiments, the subject has colorectal cancer or has a medically determined elevated risk of getting colorectal cancer. In other embodiments, the subject has pancreatic cancer, or has a medically determined elevated risk of getting pancreatic cancer such as, for example, being diagnosed with chronic pancreatitis. In yet other embodiments, a subject of the present disclosure has lung cancer, or has a medically determined elevated risk of getting lung cancer.

[0098] The terms “treatment” “treat” and “treating” mean the medical management of a subject with the intent to cure, ameliorate, stabilize, or prevent a disease, pathological condition, or disorder such as cancer. These terms are used interchangeably and include the active treatment, that is, treatment directed specifically toward the improvement of a disease, pathological condition, or disorder, and also include causal treatment, that is, treatment directed toward removal of the cause of the associated disease, pathological condition, or disorder. In addition, treating includes palliative treatment, that is, treatment designed for the relief of symptoms rather than the curing of the disease, pathological condition, or disorder; preventative treatment, that is, treatment directed to minimizing or partially or completely inhibiting the development of the associated disease, pathological condition, or disorder; and supportive treatment, that is, treatment employed to supplement another specific therapy directed toward the improvement of the associated disease, pathological condition, or disorder. It is understood that treatment, while intended to cure, ameliorate, stabilize, or prevent a disease, pathological condition, or disorder, need not actually result in the cure, ameliorization, stabilization or prevention. The effects of treatment can be measured or assessed as described herein and as known in the art as is suitable for the disease, pathological condition, or disorder involved. Such measurements and assessments can be made in qualitative and/or quantitative terms. Thus, for example, characteristics or features of a disease, pathological condition, or disorder and/or symptoms of a disease, pathological condition, or disorder can be reduced to any effect or to any amount.

[0099] In certain embodiments, the nucleic acid compositions of the present disclosure are used to treat cancer, such as pancreatic cancer, more specifically pancreatic ductal adenocarcinoma cancer (PDAC).

[00100] In certain embodiments, the nucleic acid compositions of the present disclosure are used to treat pancreatitis or fibrosis.

[00101] In certain embodiments, the nucleic acid compositions of the present disclosure are used to treat autoimmune diseases or hepatocellular carcinoma. MicroRNA 15a/16-1 have been shown to prevent hepatocellular carcinoma (Liu et al, *Gastroenterology*, 2022, 162, pp 575-589) and autoimmune diseases (Johansson et al, bioRxiv preprint).

[00102] The term “organoids” refers to tiny, self-organized three-dimensional tissue cultures that are derived from stem and progenitor cells. Such cultures can be crafted to replicate much of the complexity of an organ, or to express selected aspects of it like producing only certain types of cells. An organoid mimics its corresponding in vivo organ, such that it can be used to study aspects of that organ in the tissue culture dish.

[00103] In certain embodiments, the organoids are human PDAC Organoids.

[00104] The term “cancer”, as used herein, includes any disease caused by uncontrolled division and growth of abnormal cells, including, for example, the malignant and metastatic growth of tumors. The term “cancer” also includes pre-cancerous conditions or conditions characterized by an elevated risk of a cancerous or pre-cancerous condition. Thus, the treatment of cancer is herein also considered to include a method for the prevention of cancer or a method for preventing a pre-cancerous condition from transforming into a cancerous condition or into a completely non-cancerous condition. The cancer or pre-cancer (neoplastic condition) can be located in any part of the body, including the internal organs and skin. Some examples of applicable body parts containing cancer cells include the colon, rectum (including anus), stomach, esophageal, digestive organs, lungs, pancreas, and liver. The cancer or neoplasm can also include the presence of one or more carcinomas, sarcomas, lymphomas, blastomas, or teratomas (germ cell tumors). In some embodiments, the cancer may also be a form of leukemia.

[00105] In some embodiments, the nucleic acid compositions described herein are used to treat pancreatic, colorectal, stomach, esophageal, breast, lung, prostate, ovarian, uterine, pancreatic, liver, skin, blood or cervical cancer in any of its stages, as further described below. As is well known, cancer spreads through a subject by invading the normal, non-cancerous tissue surrounding the tumor, via the lymph nodes and vessels, and by blood after the tumor invades the veins, capillaries and arteries of a subject. When cancer cells break away from the primary tumor (“metastasize”), secondary tumors arise throughout an afflicted subject forming metastatic lesions.

[00106] In some embodiments, the treatment methods of the present disclosure are more particularly directed to cancer subjects exhibiting reduced levels of miR-15a expression,

miR-129 expression, miR-506 expression, miR-502, miR-140 or a combination thereof. In this respect, it is known that miR-15a is down-regulated in cancers. See, for example, R I Aqeilan, et al., *Cell Death and Differentiation* (2010) 17, pp. 215–220. Further, it is known that cancerous cells having reduced levels of miR-129 expression are resistant to 5-fluorouracil, as described, e.g., in U.S. Application Pub. No. 2016/0090636, the contents of which are incorporated by reference in their entirety. Additionally, it is known that pancreatic cancer cells exhibit reduced levels of miR-506. See, e.g., Li, J, et al. *Oncogene*. 35 pp. 5501-5514.

[00107] The nucleic acid compositions according to the present disclosure may be administered by any of the routes commonly known in the art. This includes, for example, (1) oral administration; (2) parenteral administration, for example, by subcutaneous, intramuscular or intravenous injection; (3) topical administration; or (4) intravaginal or intrarectal administration; (5) sublingual or buccal administration; (6) ocular administration; (7) transdermal administration; (8) nasal administration; and (9) administration directly to the organ or cells in need thereof.

[00108] The amount (dosage) of nucleic acid compositions of the present disclosure being administered depends on several factors, including the type and stage of the cancer, presence or absence of an auxiliary or adjuvant drug, and the subject's weight, age, health, and tolerance for the agent. Depending on these various factors, the dosage may be, for example, about 2 mg/kg of body weight, about 5 mg/kg of body weight, about 10 mg/kg of body weight, about 15 mg/kg of body weight, about 20 mg/kg of body weight, about 25 mg/kg of body weight, about 30 mg/kg of body weight, about 40 mg/kg of body weight, about 50 mg/kg of body weight, about 60 mg/kg of body weight, about 70 mg/kg of body weight, about 80 mg/kg of body weight, about 90 mg/kg of body weight, about 100 mg/kg of body weight, about 125 mg/kg of body weight, about 150 mg/kg of body weight, about 175 mg/kg of body weight, about 200 mg/kg of body weight, about 250 mg/kg of body weight, about 300 mg/kg of body weight, about 350 mg/kg of body weight, about 400 mg/kg of body weight, about 500 mg/kg of body weight, about 600 mg/kg of body weight, about 700 mg/kg of body weight, about 800 mg/kg of body weight, about 900 mg/kg of body

weight, or about 1000 mg/kg of body weight, wherein the term "about" is generally understood to be within $\pm$ 10%, 5%, 2%, or 1% of the indicated value. The dosage may also be within a range bounded by any two of the foregoing values. Routine experimentation may be used to determine the appropriate dosage regimen for each patient by monitoring the compound's effect on the cancerous or pre-cancerous condition, or effect on microRNA expression level or activity (e.g., miR-15a, miR-129, miR-140, miR-192, miR-502, miR-506), or effect on BCL2 level or activity, or effect on TS level or activity, or effect on E2F3 level or the disease pathology, all of which can be frequently and easily monitored according to methods known in the art. Depending on the various factors discussed above, any of the above exemplary doses of nucleic acid can be administered once, twice, or multiple times per day.

[00109] The ability of the nucleic acid compositions described herein, and optionally, any additional chemotherapeutic agent for use with the current methods can be determined using pharmacological models well known in the art, such as cytotoxic assays, apoptosis staining assays, xenograft assays, and binding assays.

[00110] The nucleic acid compositions described herein may or may not also be co-administered with one or more chemotherapeutic agents, which may be auxiliary or adjuvant drugs different from a nucleic composition described herein.

[00111] As used herein, "chemotherapy" or the phrase a "chemotherapeutic agent" is an agent useful in the treatment of cancer. Chemotherapeutic agents useful in conjunction with the methods described herein include any agent that modulates BCL2, E2F3 or TS, either directly or indirectly. Examples of chemotherapeutic agents include: anti-metabolites such as methotrexate and fluoropyrimidine-based pyrimidine antagonist, 5-fluorouracil (5-FU) (Carac® cream, Efudex®, Fluoroplex®, Adrucil®) and S-1; antifolates, including polyglutamatable antifolate compounds; raltitrexed (Tomudex®), GW1843 and pemetrexed (Alimta®) and non-polyglutamatable antifolate compounds; nolatrexed (Thymitaq®), plevitrexed, BGC945; folic acid analogs such as denopterin, methotrexate, pteropterin, trimetrexate; and purine analogs such as fludarabine, 6-mercaptopurine, thiamiprine, thioguanine; pyrimidine analogs such as ancitabine, azacitidine, 6-azauridine, carmofur,

cytarabine, dideoxyuridine, doxifluridine, enocitabine, floxuridine. In a specific embodiment of the current disclosure, the chemotherapeutic agent is a compound capable of inhibiting the expression or activity of genes, or gene products involved in signaling pathways implicated in aberrant cell proliferation or apoptosis, such as, for example, YAP1, BMI1, DCLK1, BCL2, thymidylate synthase or E2F3; and pharmaceutically acceptable salts, acids or derivatives of any of the above.

[00112] In some embodiments, the chemotherapeutic agent is an anti-cancer drug, or a tissue sensitizer or other promoter for an anti-cancer drug. In some embodiments, the co-drug may be another nucleic acid, or another miRNA, such as a microRNA mimic of the present disclosure, gemcitabine or free 5-FU.

[00113] In a specific embodiment, the other nucleic acid is a short hairpin RNA (shRNA), siRNA, or nucleic acid complementary to a portion of the BCL2 3'UTR.

[00114] In other embodiments, the chemotherapy may be any of the following cancer drugs, such as one or more of methotrexate, doxorubicin, cyclophosphamide, cis-platin, oxaliplatin, bleomycin, vinblastine, gemcitabine, vincristine, epirubicin, folinic acid, paclitaxel, and docetaxel. The chemotherapeutic agent may be administered before, during, or after commencing therapy with the nucleic acid composition.

[00115] In some embodiments, the chemotherapeutic agent is a co-drug.

[00116] E2F transcription factor 3, E2F3 (RefSeq NG_029591.1, NM_001243076.2, NP_001230005.1) is a transcription factor that binds DNA and interacts with effector proteins, including but not limited to, retinoblastoma protein to regulate the expression of genes involved in cell cycle regulation. Therefore, any drug that inhibits the expression of E2F3 may be considered herein as a co-drug.

[00117] B-cell lymphoma 2 (BCL2), (RefSeq NG_009361.1, NM_000633, NP_000624) including isoform α (NM_000633.2, NP_000624.2) and β NM_000657.2, NP_000648.2 thereof, are encoded by the Bcl-2 gene, which is a member of the BCL2 family of regulator proteins that regulate mitochondria regulated cell death via the intrinsic apoptosis pathway. BCL2 is an integral outer mitochondrial membrane protein that blocks the apoptotic death of

cell cells by binding BAD and BAK proteins. Non-limiting examples of BCL2 inhibitors include antisense oligonucleotides, such as Oblimersen (Genasense; Genta Inc.), BH3 mimetic small molecule inhibitors including, ABT-737 (Abbott Laboratories, Inc.), ABT-199 (Abbott Laboratories, Inc.), and Obatoclax (Cephalon Inc.). Any drug that inhibits the expression of BCL2 may be considered herein as a co-drug.

[00118] Thymidylate synthase (RefSeq: NG_028255.1, NM_001071.2, NP_001062.1) is a ubiquitous enzyme, which catalyzes the essential methylation of dUMP to generate dTMP, one of the four bases which make up DNA. The reaction requires CH₃H₄-folate as a cofactor, both as a methyl group donor, and uniquely, as a reductant. The constant requirement for CH₃H₄-folate means that thymidylate synthase activity is strongly linked to the activity of the two enzymes responsible for replenishing the cellular folate pool: dihydrofolate reductase and serine transhydroxymethylase. Thymidylate synthase is a homodimer of 30-35kDa subunits. The active site binds both the folate cofactor and the dUMP substrate simultaneously, with the dUMP covalently bonded to the enzyme via a nucleophilic cysteine residue (See, Carreras et al, Annu. Rev. Biochem., (1995) 64:721-762). The thymidylate synthase reaction is a crucial part of the pyrimidine biosynthesis pathway which generates dCTP and dTTP for incorporation into DNA. This reaction is required for DNA replication and cell growth. Thymidylate synthase activity is therefore required by all rapidly dividing cells such as cancer cells. Due to its association with DNA synthesis, and therefore, cellular replication, thymidylate synthase has been the target for anti-cancer drugs for many years. Non-limiting examples of thymidylate synthase inhibitors include folate and dUMP analogs, such as 5-fluorouracil (5-FU). Any drug that inhibits the expression of thymidylate synthase may be considered herein as a co-drug.

[00119] If desired, the administration of the nucleic acid composition described herein may be combined with one or more non-drug therapies, such as, for example, radiotherapy, and/or surgery. As well known in the art, radiation therapy and/or administration of the chemotherapeutic agent (in this case, the nucleic acid composition described herein, and optionally, any additional chemotherapeutic agent) may be given before surgery to, for example, shrink a tumor or stop the spread of the cancer before the surgery. As also well

known in the art, radiation therapy and/or administration of the chemotherapeutic agent may be given after surgery to destroy any remaining cancer.

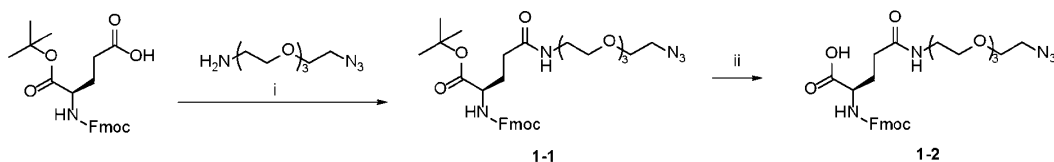
[00120] Examples have been set forth below for the purpose of illustration and to describe certain specific embodiments of the disclosure. However, the scope of this disclosure is not to be in any way limited by the examples set forth herein.

EXAMPLES

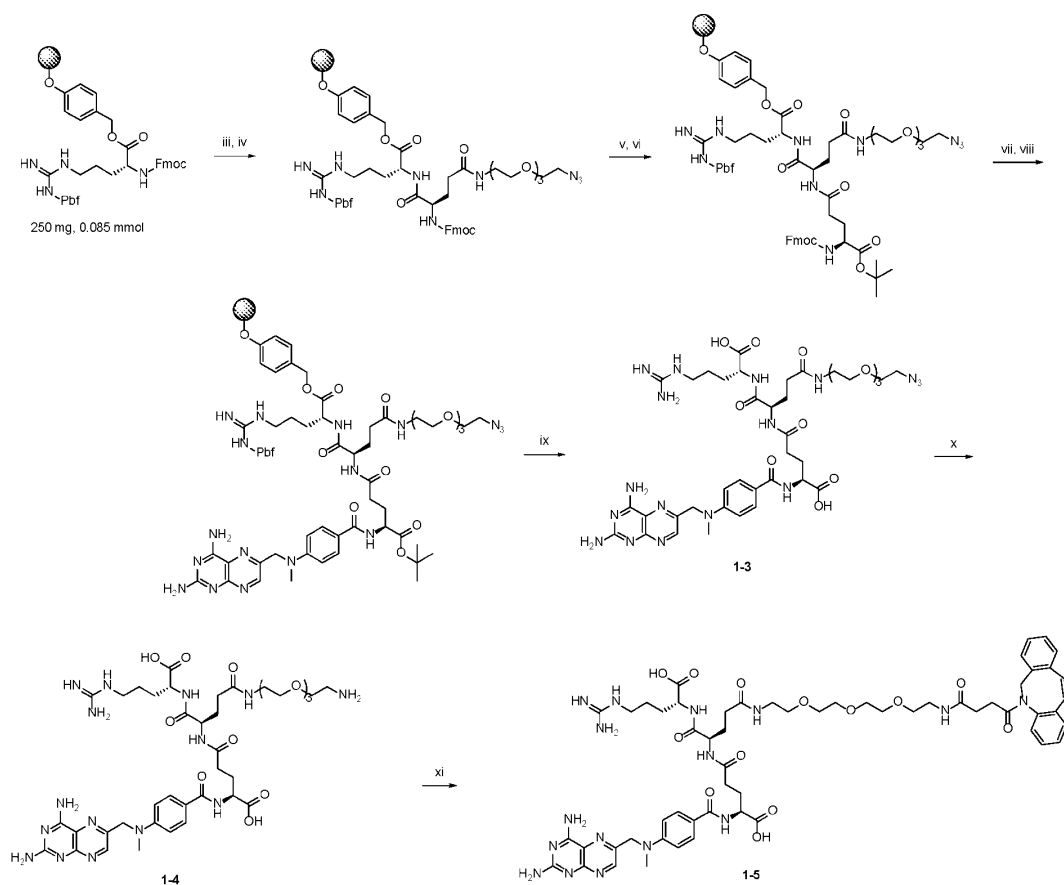
Example 1. Synthesis of Methotrexate-miRNA mimic (MTX-5-FU-Gem-miR-15a) Conjugate:

The following described synthesis and purification of MTX-5-FU-Gem-miR-15a in detail:

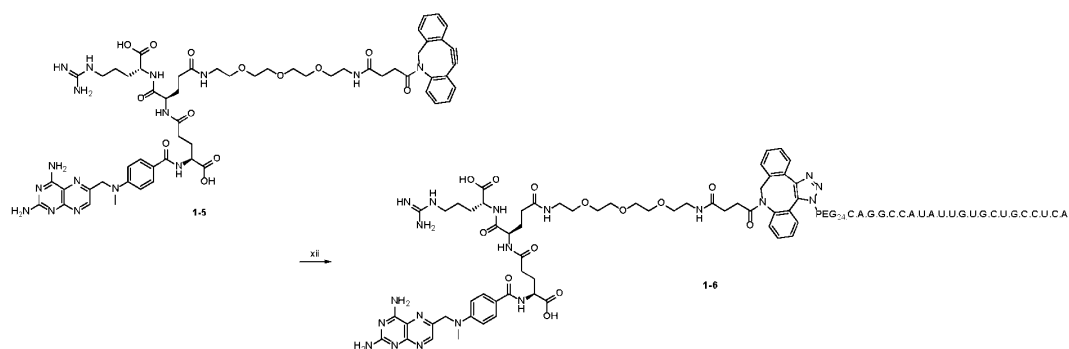
[00121] Scheme 1. Synthesis of Methotrexate-miRNA conjugate



Reagents and Conditions: (i) azido-peg3-amine (1.2 equiv.), EDC HCl (1.3 equiv.), CH₂Cl₂, r.t., overnight, 94%; (ii) TFA (excess), CH₂Cl₂, 0 °C to r.t., overnight, 90%.



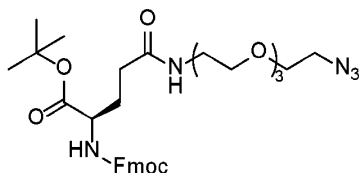
Reagents and Conditions: (iii) 20% piperidine, DMF, r.t., 30 min; (iv) **1-2** (3.0 equiv.), HOBt (3.0 equiv.), HBTU (3.0 equiv.), DIPEA (3.0 equiv.), DMF, r.t., 4 hr; (v) 20% piperidine, DMF, r.t., 30 min; (vi) Fmoc-Glu(OH)-OtBu (3.0 equiv.), HOBt (3.0 equiv.), HBTU (3.0 equiv.), DIPEA (3.0 equiv.), DMF, r.t., 4 hr; (vii) 20% piperidine, DMF, r.t., 30 min; (viii) 2,4-Diamino-N¹⁰-methylptericoic Acid (3.0 equiv.), HOBt (3.0 equiv.), HBTU (3.0 equiv.), DIPEA (3.0 equiv.), DMSO, r.t., 4 hr; (ix) TFA:TIPS:H₂O = 95:2.5:2.5, r.t., 2 hrs; 91% for 7 steps; (x) Pd/C (20 mol%), H₂, EtOH : H₂O = 3 : 1, r.t., overnight, 81%; (xi) DBCO-NHS (1.1 equiv.), DIPEA (1.5 equiv.), DMSO, r.t., overnight, 65%.



Reagents and Conditions: (xii) **1-5** (10.0 equiv.), Azido-PEG24 5-Fu-Gem-miR-15a (1.0 equiv.), water, r.t, 8 hrs, 4 °C, 4 hr

[00122] Experimental Section:

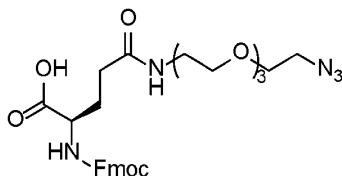
[00123] Fmoc-Glu(PEG₃N₃)-O^tBu (1-1)



[00124] To a solution of Fmoc-Glu-O^tBu (600 mg, 1.41 mmol) and azido-peg3-amine (1.2 equiv.) in CH₂Cl₂ (20 - 30 mL) was added a suspension of EDC·HCl (1.3 equiv.) in CH₂Cl₂ (15 mL). The solution was stirred for at room temperature for 4 hours and the reaction progress was monitored by TLC. Upon completion of the reaction, the reaction was diluted with H₂O, extracted with CH₂Cl₂ (3 x 20 mL), washed with brine (3 x 15 mL), dried over MgSO₄, filtered and the filtrate concentrated in vacuo. Purification was performed by column chromatography on silica gel with increasing amounts of methanol in CH₂Cl₂ to afford **1-1** (827 mg, 94%) as yellow oil. The product was characterized by ¹H NMR, ¹³C NMR and FIA. ¹H NMR (700 MHz, CDCl₃) δ 7.78 (d, *J* = 7.6 Hz, 2H), 7.63 (t, *J* = 7.3 Hz, 2H), 7.42 (t, *J* = 7.5 Hz, 2H), 7.35 – 7.32 (m, 2H), 6.39 (s, 1H), 5.72 (d, *J* = 6.5 Hz, 1H), 4.41 (ddd, *J* = 25.2, 10.6, 7.3 Hz, 2H), 4.24 (t, *J* = 7.0 Hz, 2H), 3.66 – 3.61 (m, 10H), 3.57 (dd, *J* = 7.9, 3.1 Hz, 2H), 3.47 (tdd, *J* = 19.0, 9.0, 5.3 Hz, 2H), 3.39 – 3.33 (m, 2H), 2.34 – 2.26 (m, 2H), 2.25 – 2.18 (m, 1H), 1.98 (dt, *J* = 14.3, 7.7 Hz, 1H), 1.49 (s, 9H). ¹³C NMR (176 MHz, CDCl₃) δ 172.14, 171.16, 156.30, 143.96, 143.75, 141.33, 141.29, 132.15,

132.10, 132.04, 132.03, 128.59, 128.52, 127.74, 127.10, 125.17, 120.00, 119.99, 82.42, 72.48, 72.46, 71.31, 70.72, 70.69, 70.68, 70.67, 70.66, 70.63, 70.62, 70.61, 70.59, 70.58, 70.57, 70.56, 70.55, 70.55, 70.54, 70.54, 70.53, 70.52, 70.51, 70.46, 70.42, 70.38, 70.37, 70.27, 70.09, 70.08, 70.02, 70.02, 69.71, 66.95, 61.79, 61.77, 54.09, 53.47, 50.67, 50.64, 47.20, 42.76, 39.39, 32.44, 28.76, 28.01. ESI-MS m/z : 626.4 $[M+H]^+$. All data were in agreement with literature values (Willibald, J., Harder, J., Sparrer, K., Conzelmann, K. K., Carell, T., Click-modified anandamide siRNA enables delivery and gene silencing in neuronal and immune cells, *J Am Chem Soc* 2012, 134 (30), 12330-3).

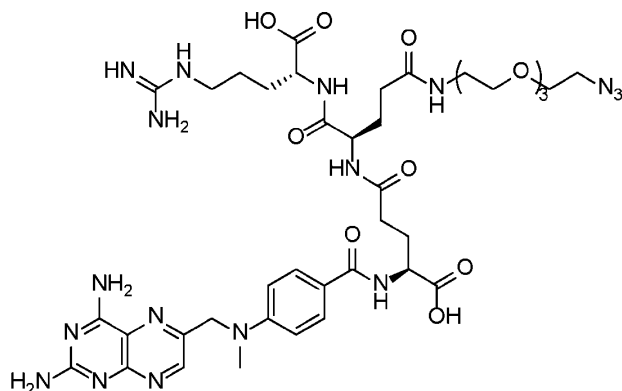
[00125] Fmoc-Glu(PEG₃N₃)-OH (1-2)



[00126] A solution of 1-1 (280 mg, 0.45 mmol) in CH₂Cl₂ (20 mL) was cooled to 0°C and TFA (5 mL) was added dropwise. The reaction mixture was stirred at 0°C overnight and the reaction progress was monitored by TLC. Upon completion of the reaction, the mixture was diluted with CH₂Cl₂ (30 mL) washed with H₂O (4 x 20 mL) until the pH of the aqueous was no longer acidic. The organic layer was then dried over MgSO₄, filtered and the filtrate concentrated in vacuo to afford a brownish oil. Then the TFA leftover was flashed with nitrogen for 1hr and redissolved with distilled water and put under lyophilizer overnight to obtain 1-2 (230 mg, 90%) as brownish solid. The product was directly used for the further steps without chromatographic purification. The product was characterized by ¹H NMR, ¹³C NMR and FIA. ¹H NMR (500 MHz, CDCl₃) δ 7.77 (d, J = 7.5 Hz, 2H), 7.62 (t, J = 7.4 Hz, 2H), 7.41 (t, J = 7.4 Hz, 2H), 7.33 (t, J = 7.3 Hz, 2H), 6.75 (s, 1H), 6.07 (d, J = 6.4 Hz, 1H), 4.44 – 4.34 (m, 3H), 4.23 (t, J = 6.9 Hz, 1H), 3.64 (d, J = 6.4 Hz, 10H), 3.57 (d, J = 4.5 Hz, 2H), 3.49 (dd, J = 15.1, 5.3 Hz, 2H), 3.39 – 3.36 (m, 2H), 2.53 – 2.36 (m, 2H), 2.24 (dd, J = 13.5, 6.5 Hz, 1H), 2.12 (dd, J = 13.9, 6.7 Hz, 1H). ¹³C NMR (126 MHz, CDCl₃) δ 173.64, 173.35, 156.20, 143.92, 143.72, 141.32, 132.12, 128.70, 128.61, 127.75, 127.13, 125.15, 119.99, 70.58, 70.43, 70.15, 69.96, 69.41, 67.08, 53.35, 50.63, 47.13, 39.66, 32.34, 28.77,

15.26. ESI-MS m/z : 570.3 $[M+H]^+$. All data were in agreement with literature values ((Willibald, J., Harder, J., Sparrer, K., Conzelmann, K. K., Carell, T., Click-modified anandamide siRNA enables delivery and gene silencing in neuronal and immune cells, *J Am Chem Soc* 2012, 134 (30), 12330-3).

[00127] Methotrexate-Glu(PEG₃N₃)-Arg-OH (1-3)



[00128] Fmoc-Arg(Pbf)-Wang Resin (250 mg, 85 μ mol) was suspended in 2 mL DMF, swollen for 1 hour, and the solvent was filtered off. To the beads was added a 20% solution of piperidine in DMF (2 mL) and the mixture was bubbling with nitrogen for 30 minutes. The solvent was filtered off, the beads were washed with DMF (3 x 2 mL). Kaiser test was performed after each step.

[00129] A solution of 1-2 (90 mg, 3.0 equiv.), DIPEA (3.0 equiv.), HOBt (3.0 equiv.) and HBTU (3.0 equiv.) in DMF (2 mL) was added to the beads and the reaction mixture was bubbling with nitrogen for 4 hours. The solvent was filtered off and the beads were washed with DMF (3 x 2 mL).

[00130] To the beads was added a 20% solution of piperidine in DMF (2 mL) and the mixture was bubbling with nitrogen for 30 minutes. The solvent was filtered off, the beads were washed with DMF (3 x 2 mL).

[00131] A solution of Fmoc-Glu-OtBu (3.0 equiv.), DIPEA (3.0 equiv.), HOBt (3.0 equiv.) and HBTU (3.0 equiv.) in DMF (2 mL) was added to the beads and the reaction mixture was

bubbling with nitrogen for 4 hours. The solvent was filtered off and the beads were washed with DMF (3 x 2 mL).

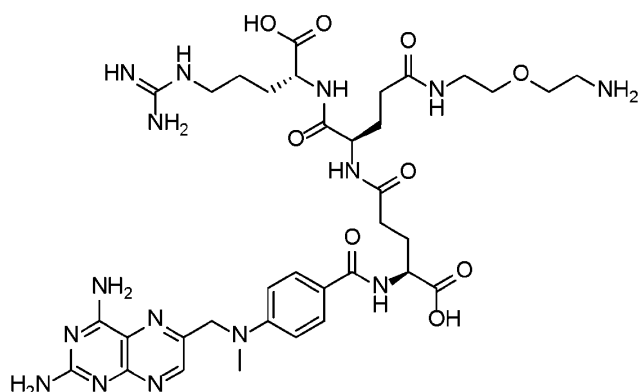
[00132] To the beads was added a 20% solution of piperidine in DMF (2 mL) and the mixture was bubbling with nitrogen for 30 minutes. The solvent was filtered off, the beads were washed with DMF (3 x 2 mL).

[00133] A solution of N¹⁰-methylpteroic acid (50 mg, 3.0 equiv.), DIPEA (3.0 equiv.), HOBt (3.0 equiv.) and HBTU (3.0 equiv.) in DMF (2 mL) was added to the beads and the reaction mixture was bubbling with nitrogen for 4 hours. The solvent was filtered off and the beads were washed with DMF (3 x 2 mL) and IPA (3 x 2 mL). The beads were put under vacuum to dry for 1 hour to get rid of DMF as much as possible.

[00134] A 95:2.5:2.5 mixture of TFA:TIPS:H₂O was added to the dried beads and bubbling with nitrogen for 2 hours. The yellow solution was separated from the beads by filtration and the liquid was concentrated through rotavap. The solution was flashed with nitrogen for 1 to 2 hours to get rid of most TFA, and then the mixture was dissolved in distilled water and lyophilized to afford brown crude solid. The purification was done by reverse phase Yamazen (5% ACN in water to 65% ACN in water) to afford the final product **1-3** (72 mg, 91%) as pale-yellow solid.

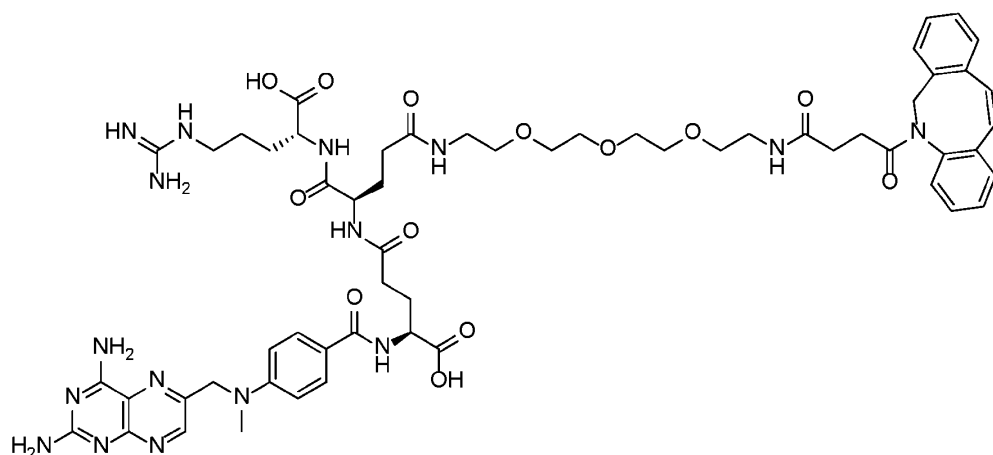
[00135] The product was characterized by ¹H NMR, ¹³C NMR and HRMS. ¹H NMR (700 MHz, MeOD) δ 8.35 (s, 1H), 7.39 (s, 2H), 6.45 (s, 2H), 4.55 (s, 2H), 4.25 (s, 1H), 4.17 – 3.99 (m, 2H), 3.52 (dd, *J* = 14.0, 7.1 Hz, 10H), 3.43 (s, 2H), 3.32 (s, 2H), 3.20 (s, 2H), 2.99 (s, 5H), 2.31 (d, *J* = 7.7 Hz, 2H), 2.14 (d, *J* = 23.2 Hz, 3H), 2.00 – 1.89 (m, 2H), 1.79 (d, *J* = 6.1 Hz, 1H), 1.71 (d, *J* = 7.3 Hz, 1H), 1.54 (d, *J* = 6.6 Hz, 1H), 1.42 (d, *J* = 6.7 Hz, 2H). ¹³C NMR (176 MHz, D₂O) δ 175.38, 174.82, 172.59, 168.31, 162.72, 156.54, 151.40, 151.17, 148.92, 128.59, 121.78, 120.18, 111.25, 70.72, 69.52, 69.51, 69.44, 69.33, 69.14, 68.62, 54.74, 53.26, 50.07, 43.15, 40.53, 38.88, 38.82, 31.76, 28.73, 27.54, 26.88, 24.36. HRMS (TOF) *m/z*: Calcd. For C₃₉H₅₇N₁₇O₁₁, 939.4423. Found, 940.4517.

[00136] Methotrexate-Glu(PEG₃NH₂)-Arg-OH (1-4)



[00137] To a solution of 1-3 (23 mg, 0.025 mmol) in solvent (EtOH : H₂O = 3 : 1, 2 mL) was added to 20 mol% Pd/C catalyst under hydrogen. The reaction mixture was stirred at room temperature overnight and the reaction progress was monitored by TLC. Upon completion of the reaction, the mixture was filtered through celite and washed with water. The crude mixture was concentrated in vacuo to remove ethanol. Then the crude was put under lyophilizer overnight to remove water to obtain 1-4 (18.3 mg, 81%) as pale yellow solid. The crude product was directly used for the further steps without chromatographic purification. The product was characterized by ¹H NMR, ¹³C NMR and FIA. ¹H NMR (700 MHz, D₂O) δ 8.35 (s, 1H), 7.50 (s, 2H), 6.59 (d, *J* = 7.4 Hz, 2H), 4.50 (s, 2H), 4.23 (s, 1H), 4.06 (dd, *J* = 16.2, 8.8 Hz, 2H), 3.53 – 3.39 (m, 10H), 3.17 (d, *J* = 24.5 Hz, 3H), 3.05 (s, 3H), 3.00 (s, 3H), 2.98 – 2.93 (m, 2H), 2.29 (dd, *J* = 14.2, 6.9 Hz, 2H), 2.21 – 2.09 (m, 3H), 1.93 (d, *J* = 5.5 Hz, 2H), 1.80 (d, *J* = 9.4 Hz, 1H), 1.69 (s, 1H), 1.52 (s, 1H), 1.40 (d, *J* = 7.0 Hz, 2H). ¹³C NMR (176 MHz, D₂O) δ 178.32, 178.16, 175.38, 174.87, 174.84, 172.58, 169.43, 168.87, 168.78, 162.76, 161.95, 160.42, 157.37, 156.49, 154.13, 153.39, 151.50, 149.04, 148.06, 128.85, 128.71, 122.14, 120.18, 120.04, 112.16, 111.55, 111.26, 98.99, 70.68, 69.54, 69.52, 69.49, 69.45, 69.39, 69.35, 69.34, 69.26, 69.21, 69.18, 68.63, 68.60, 66.52, 57.13, 54.79, 54.47, 53.18, 42.41, 40.51, 39.05, 38.98, 38.93, 38.86, 38.82, 31.78, 29.44, 28.78, 27.57, 26.82, 24.39. ESI-MS *m/z*: 914.3 [M+H]⁺.

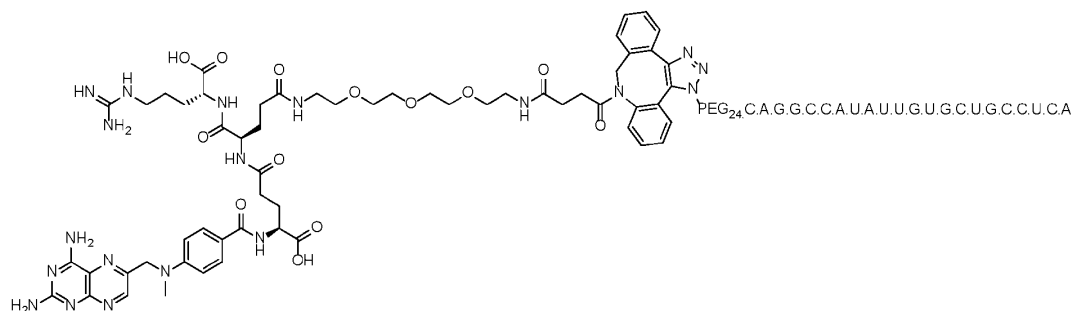
[00138] Methotrexate-DBCO (1-5)



[00139] To a solution of 1-4 (18 mg, 0.02 mmol) and DBCO-NHS ester (9 mg, 1.1 equiv.) in DMSO (1 mL) was added DIPEA (1.5 equiv.) dropwise. The solution was stirred for at room temperature overnight. Upon completion of the reaction, the reaction was directly loaded to the reverse phase YAMAZEN column and the purification was performed by column chromatography on ODS gel with increasing amounts of acetonitrile in water to afford crude 1-5 as orange solid. Then the crude was purified through semi-prep HPLC with acetonitrile/water system to obtain 1-5 (15.3 mg, 65%) as pale-yellow solid. The purity was confirmed by HPLC (Rt: 13.51 min, 95% at 210 nm, 96% at 254 nm). The product was characterized by ^1H NMR, ^{13}C NMR and HRMS. ^1H NMR (700 MHz, D_2O) δ 8.25 (d, J = 6.6 Hz, 1H), 7.45 (t, J = 7.5 Hz, 2H), 7.22 (dd, J = 20.7, 7.4 Hz, 1H), 7.13 – 7.02 (m, 4H), 6.97 (s, 1H), 6.94 – 6.86 (m, 1H), 6.76 (t, J = 7.3 Hz, 1H), 6.52 (d, J = 7.9 Hz, 2H), 4.60 – 4.50 (m, 1H), 4.43 – 4.32 (m, 2H), 4.25 (ddd, J = 6.7, 3.9, 1.3 Hz, 1H), 4.06 (dt, J = 14.3, 6.8 Hz, 2H), 3.45 – 3.33 (m, 10H), 3.26 (dt, J = 10.1, 5.1 Hz, 1H), 3.22 – 3.09 (m, 3H), 3.07 – 2.95 (m, 5H), 2.87 (s, 3H), 2.25 (dd, J = 16.3, 7.8 Hz, 3H), 2.17 – 2.06 (m, 3H), 2.05 – 1.98 (m, 1H), 1.97 – 1.86 (m, 3H), 1.73 (ddd, J = 21.4, 15.9, 8.0 Hz, 3H), 1.54 (dd, J = 14.1, 7.1 Hz, 1H), 1.42 (dt, J = 14.5, 7.0 Hz, 2H). ^{13}C NMR (176 MHz, D_2O) δ 178.11, 175.20, 174.71, 173.95, 173.42, 172.55, 168.30, 162.43, 156.51, 151.33, 150.12, 147.34, 131.58, 128.73, 127.75, 126.63, 125.23, 121.96, 121.14, 120.39, 113.97, 111.47, 107.38, 69.51, 69.30, 68.61, 68.58, 54.71, 54.40, 53.22, 40.54, 38.90, 38.83, 38.79, 38.73, 31.79, 31.67,

30.69, 29.94, 28.88, 26.82, 24.43. HRMS (TOF) m/z: Calcd. For $C_{58}H_{72}N_{16}O_{13}$, 1200.5465. Found, 1201.5577.

[00140] Methotrexate-miRNA Conjugate (1-6)



[00141] The copper free click reaction was performed between Methotrexate-DBCO and Azido-PEG₂₄ 5-Fu-Gem-miR-15a. Click reaction was performed at a 1:10 molar ratio (azide oligo: Methotrexate-DBCO = 464 ug: 700 ug) at room temperature in water for 8 hours and then cooled to 4°C for 4 hours. Unconjugated Methotrexate-DBCO was removed from the reaction using Oligo Clean & Concentrator (Zymo Research) per the manufacturer's instructions. Conjugation was verified using MALDI spectral analysis. Target MW 9489.1, found 9491.918.

Example 2. Preparation of Double-stranded Methotrexate-conjugated-modified-microRNAs

[00142] The crude sample of single-stranded methotrexate (MTX) conjugated-miR-15a (ssMTX-miR-15a) was purified with the Zymo Oligo Clean & Concentrator™ kit. The crude ssMTX-miR-15a sample was diluted to 200 ng/μL and aliquoted into 50 μL aliquots before beginning clean-up (i.e., 10 μg in 50 μL). 100 μL of Oligo Binding Buffer and 400 μL 100% ethanol was added to each 50 μL sample. The samples were transferred to the Zymo-Spin™ IC Column in a Collection Tube and centrifuge at max speed (13,000 x g) for 30 seconds. Flow through was discarded. Next, 750 μL of DNA Wash Buffer was added to the column and was centrifuged at max speed for 1 minute. The column was transferred into a nuclease-free tube and the purified sample was eluted by adding 15 μL water directly to the

column matrix and centrifuging at max speed for 30 seconds. The purified samples were combined purified RNA samples and quantitate on a Nanodrop.

[00143] To anneal the sample, sterile 2'-Deprotection Buffer was prepared (571 μ L glacial acetic acid with 99.4 mL RNase-free water to make 100 mL of 100 mM acetic acid. Adjust the pH of the 100 mM acetic acid to 3.4–3.8 using TEMED. Sterile filter.) The modified-miR-15a 5' strand was resuspended to 100 μ M in 2'-Deprotection Buffer. The ssMTX-miR-15a was combined with the modified-miR-15a 5' at a 1:1 ratio (by concentration). The combined sample was incubated at 60° C for 45 min, then at room temperature for 30 min. Add 25 μ L 3M sodium acetate and 750 μ L 100% ethanol was added per 200 μ L of combined miRNA solution. The solution was mixed and placed at -20°C overnight or -80°C for 2 hours to precipitate RNA. After, the sample was centrifuged at 13,000 x g for 20 min at 4°C. The supernatant was discarded and then washed with 200 μ L of 95% ethanol. The supernatant was discarded again, and the pellet was dried in the sterile hood for 5-10 min. The pellet was resuspended in water and then adjusted to their respective working concentrations.

Example 3. Materials and Methods

[00144] Cell Culture: MD-MBA-231, MIA PaCa-2, and PANC-1 cells were all cultured in Dulbecco's Modified Eagle Medium (DMEM) supplemented with 10% fetal bovine serum. A549 cells were cultured in Ham's F-12K (Kaighn's) Medium supplemented with 10% fetal bovine serum (FBS).

[00145] Cell Lines and Tissue Culture: Human ovarian cancer cell lines, OVCAR-3, SK-OV-3 and A2780, were purchased from the American Type Culture Collection (ATCC, Manassas, VA, USA) and MilliporeSigma (St. Louis, MO, USA). All the cells were cultured using a specific medium (Table) at 37°C in a humidified atmosphere of 5% (V/V) CO₂ incubator.

Cell Line	Culture Medium	Supplements
SK-OV-3	McCoy's 5A	FBS (10%)
OVCAR-3	RPMI 1640	FBS (10%), Bovine insulin (0.01 mg/ml)
A2780	RPMI 1640	FBS (10%), Glutamine (2 mM)

[00146] Western Blotting: MD-MBA-231 and MIA PaCa-2 cells were seeded onto 6-well plates at a cell density of 100,000 cells per well. 24 hours later, the cells were incubated with Oligofectamine and 50 nM of MTX-5-FU-GEM-miR-15a. 5 hours later, the media was changed to DMEM supplemented with 10% dialyzed-fetal bovine serum. 72 hours later, cells were lysed with RIPA buffer and the protein samples were used for western immunoblotting. Proteins were probed with anti-BMI1 antibody (Cell Signaling, 1:10,000), anti-YAP1 antibody (Cell Signaling, 1:10,000), and anti-GAPDH antibody (Santa Cruz, 1:200,000). Protein bands were visualized using a LI-COR Biosciences Odyssey FC imaging system and Super Signal West Pico chemiluminescent substrate (Thermo Fisher Scientific).

[00147] Design and Synthesis of Gemcitabine-modified miRNA Mimics: MTX-5FU-Gem-miR-15a mimic was designed and synthesized by incorporating 5-FU and Gemcitabine into the guide/antisense strands of hsa-miR-15a, substituting uridine and cytidine, respectively. The passenger/sense strand was conjugated with Methotrexate (MTX). The guide strand, modified with 5-FU and Gem, and the unconjugated passenger strand with an azide linker on the 5' end were purchased from Horizon Discovery (Horizon Discovery, Cambridge, UK). Both strands of oligonucleotides were HPLC purified. Methotrexate-dibenzocyclooctyne (MTX-DBCO) was synthesized at the Ojima Lab at Stony Brook University and was used to conjugate MTX to the azide-linked passenger strand via a copper-free, biorthogonal click reaction. The guide strands and passenger strands were then annealed before use.

[00148] miRNA Transfection: A non-specific scramble miRNA, Pre-miR Negative Control #2 (negative control) (Thermo Fisher Scientific, Waltham, MA, USA) was used to

represent the negative control. Pre-miR hsa-miR-15a-5p, miRNA mimics identical to the miR-15a-5p (miR-15a) strands, respectively, were purchased from Thermo Fisher Scientific (Thermo Fisher Scientific, Waltham, MA, USA). Cells were transfected with oligonucleotides at a concentration of 50 nM (negative control, miR-15a, and MTX-5FU-Gem-miR-15a).

[00149] For vehicle-mediated transfections, cell lines were plated in 6-well plates at a density of 100,000 cells per well. After 24 hours, cells were transfected using a mixture of Oligofectamine (Thermo Fisher Scientific, Waltham, MA, USA) and the respective oligonucleotides according to the manufacturer's instructions. Five hours post-transfection, the media was replaced with fresh media supplemented with 10% dialyzed fetal bovine serum (DFBS) (Thermo Fisher Scientific).

[00150] For vehicle-free transfections, cell lines were seeded onto 96-well plates at a density of 1,000 cells per well. After 24 hours, the media was replaced with fresh media containing the respective oligonucleotides. Twenty-four hours following transfection, the media was again replaced with fresh media supplemented with 10% DFBS.

[00151] Cytotoxicity Analysis: Cytotoxicity of all the miRNAs were assessed without using any delivery vehicle. For this, cells were seeded and transfected as described above. Briefly, cells were directly seeded onto 96-well plates at a density of 1,000 cells/well. After 24 hours, cells were transfected at varying concentrations of oligonucleotides. Cell viability was measured 6 days post-transfection using WST-1 Cell Proliferation Reagent (MilliporeSigma, St. Louis, MO, USA) according to the manufacturer's protocol. In brief, cells were incubated with 10 μ L of WST-1 per 100 μ L of media for 1 h at 37°C. After incubation, absorbance was measured at 450 and 630 nm using a SpectraMax i3 (Molecular Devices, San Jose, CA, USA) plate reader. The O.D. was calculated by subtracting the absorbance at 630 nm from that at 450 nm. Relative proliferation was calculated by normalizing the O.D. to cells only transfected with negative control. Absolute IC50 values were calculated using GraphPad Prism 9 (GraphPad Software, San Diego, CA, USA) using the following equation, where "Y" = 50.

$$Y = \text{Bottom} + (\text{Top} - \text{Bottom}) / (1 + (\text{IC}_{50} / X)^{\text{HillSlope}})$$

[00152] Cell Cycle Analysis: OC cells were seeded onto 6-well plates at a density of 100,000 cells/well. The cells were then transfected with their respective oligonucleotides and concentrations as described above or treated with 300 nM 5-FU and 150 nM Gem (the concentration equivalent to 5-FU and Gem in 5-FU-Gem-miR-15a). 48h post-transfection, cells were resuspended in Krishan modified buffer supplemented with 0.02 mg/mL RNase H (Thermo Fisher Scientific, Waltham, MA, USA) and 0.05 mg/mL propidium iodide (MilliporeSigma, St. Louis, MO, USA). Cells were then analyzed by flow cytometry via CytoFLEX Flow Cytometer (Beckman Coulter, Brea, CA, USA) and results were analyzed by Modfit LT Software (BD Biosciences, Sparks, MD, USA).

[00153] Apoptosis Assay: The OC cells were plated and transfected with their respective treatment conditions via vehicle-mediated transfection as described above. 72 h post-transfection, the cells were stained with Annexin V (Thermo Fisher Scientific, Waltham, MA, USA) and propidium iodide (MilliporeSigma, St. Louis, MO, USA). Apoptotic cells were quantified by flow cytometric analysis on a CytoFLEX Flow Cytometer (Beckman Coulter, Brea, CA, USA) with Annexin V-positive cells categorized as “apoptotic”. Fold change in apoptotic cells was calculated in relation to the number of apoptotic cells observed in the negative control.

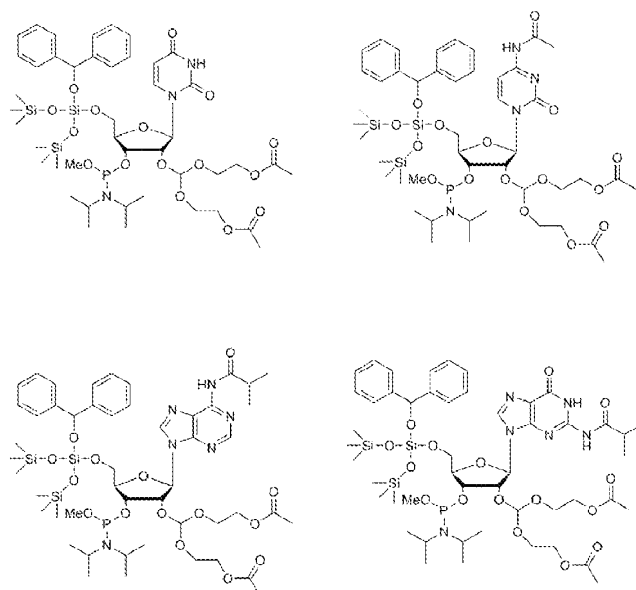
[00154] Cell Proliferation Assay: Cells were seeded onto 96 well plates at 1000 cells per well. 24 hours after seeding, MTX-5-FU-GEM-miR-15a was diluted in normal culturing media, the media was removed from the cells on the 96 well plates, and then the MTX-5-FU-GEM-miR-15a-containing media was added to the cells. These cells were incubated for 24 hours and then the media was changed to fresh media supplemented with 10% dialyzed FBS. Cell number was measured 6 days post transfection using WST-1 dye (Roche). Cells were incubated with 10µl of WST-1 dye per 100µl of media for 1 hour and absorbance was read at 450 and 630 nm. The O.D. was calculated by subtracting the absorbance at 630 nm from that at 450 nm and the relative proliferation was calculated by normalizing the O.D. to negative control.

[00155] Western Immunoblot Analysis: Ovarian cancer cells were seeded and transfected with or without a transfection vehicle as described above. Cells were transfected

with their respective oligonucleotides and concentrations as described above. 72 h post-transfection, cells were lysed with a mixture of RIPA buffer (MilliporeSigma, St. Louis, MO, USA) and protease inhibitor cocktail (MilliporeSigma, St. Louis, MO, USA), and the protein samples were then collected and used for western immunoblot analysis. Proteins were probed with rabbit anti-BMI1 antibody (Cell Signaling, 6964, 1:1000), rabbit anti-WEE1 antibody (Cell Signaling, 13084, 1:1000), rabbit anti-BCL2 antibody (Cell Signaling, 3498, 1:1000), rabbit anti-DCAMLK1 antibody (Abcam, Ab31704, 1:500), mouse anti-GAPDH antibody (Santa Cruz, sc47724, 1:100,000). Primary antibodies were diluted in 5% milk (Bio-Rad, Hercules, CA, USA) in TBST. After staining with primary antibodies, proteins were probed with either secondary antibodies goat anti-mouse-HRP (Bio-Rad, 1706516, 1:5000) or goat anti-rabbit-HRP (Bio-Rad, 1721019, 1:5000) depending on the used primary antibody. Protein bands were visualized using an LI-COR Biosciences Odyssey FC imaging system after the addition of SuperSignal West Pico PLUS Chemiluminescent Substrate (Thermo Fisher Scientific). Proteins were then quantified with Image Studio Version 5.2.4 (LI-COR Biosciences, Lincoln, NE, USA).

[00156] 5-FU-modified miR-15a: The 5-FU modified miR-15a molecules were synthesized by an automated oligonucleotide synthesis process and purified by HPLC. The two strands were annealed to make the mature modified 5-FU-miR-15a. More specifically, a process referred to as "2'-ACE RNA synthesis" was used. The 2'-ACE RNA synthesis was based on a protecting group scheme in which a silylether was employed to protect the 5'-hydroxyl group in combination with an acid-labile orthoester protecting group on the 2'-hydroxy (2'-ACE). This combination of protecting groups was then used with standard phosphoramidite solid-phase synthesis technology. See, for example, S.A. Scaringe, F.E. Wincott, and M.H. Caruthers, *J. Am. Chem. Soc.*, 120 (45), 11820-11821 (1998); International PCT Application WO/1996/041809; M.D. Matteucci, M.H. Caruthers, *J. Am. Chem. Soc.*, 103, 3185-3191 (1981); S.L. Beaucage, M.H. Caruthers, *Tetrahedron Lett.* 22, 1859-1862 (1981), the entire contents of each of which were expressly incorporated herein.

[00157] Some exemplary structures of the protected and functionalized ribonucleoside phosphoramidites currently in use were shown below:



Example 4: MTX-5-FU-GEM-miR-15a and a dose-dependent inhibition of cancer cell growth without the use of delivery vehicle thereof.

[00158] MTX-5-FU-GEM-miR-15a was able to inhibit proliferation in MD-MBA-231 cells ($IC_{50} = 4.6$ nM) without use of delivery vehicle, whereas miR-15a was unable to enter the cell and inhibit proliferation without delivery vehicle. Furthermore, MTX-5-FU-GEM-miR-15a inhibited cancer cell growth without the use of delivery vehicle in A549 non-small cell lung cancer cells, MD-MBA-231 triple-negative breast cancer cells, and in MIA PaCa-2 and PANC-1 pancreatic cancer cells. A549 had a non-detectable level of folate receptor while MD-MBA-231 cells displayed a relatively high level of folate receptor. Compared to each other, MD-MBA-231 cells were 22.2-fold more sensitive than A549 cells. Similarly, in PDAC, PANC-1 had a very low expression of folate receptor, while MIA PaCa-2 had a higher expression of folate receptor and their relative sensitivities appeared to be correlated with folate receptor status, as MIA PaCa-2 cells were 1.8-fold more sensitive (FIG. 2).

[00159] Table 1 as shown below depicted IC_{50} Values of 5-FU-miR-15a, MTX-5-FU-GEM-miR-15a, and gemcitabine in hT89 human PDAC organoids.

miRNA	IC ₅₀ (nM)
5-FU-miR-15a	24.7 ± 3.4
MTX-5-FU-Gem-miR-15a	1.2 ± 0.2
Gemcitabine	1168.7 ± 221.3

[00160] While there have been shown and described what are at present considered the preferred embodiments of the disclosure, those skilled in the art may make various changes and modifications which remain within the scope of the disclosure defined by the appended claims.

WHAT IS CLAIMED IS:

1. A double-stranded nucleic acid composition comprising a modified double-stranded nucleic acid sequence that comprises uracil, gemcitabine, and methotrexate (MTX), wherein the uracil comprises 5-fluorouracil.
2. The double-stranded nucleic acid composition of claim 1, wherein said modified double-stranded nucleic acid sequence comprises a modified microRNA nucleotide sequence.
3. The double-stranded nucleic acid composition of claim 2, wherein said modified microRNA nucleotide sequence comprises a microRNA nucleotide sequence of miR-15a as set forth in SEQ ID NO. 1.
4. The double-stranded nucleic acid composition of claim 3, wherein said miR-15a microRNA nucleotide sequence comprises a guide strand and a passenger strand.
5. The double-stranded nucleic acid composition of claim 4, wherein cytidines are replaced with gemcitabines on the guide strand of the miR-15a microRNA nucleotide sequence.
6. The double-stranded nucleic acid composition of claim 4, wherein the uracils on the guide strand of the miR-15a microRNA nucleotide sequence are 5-fluorouracils.
7. The double-stranded nucleic acid composition of claim 4, wherein the methotrexate (MTX) is conjugated on the passenger strand of the miR-15a microRNA nucleotide sequence.
8. The double-stranded nucleic acid composition of claim 7, wherein the methotrexate (MTX) is conjugated onto the 5' end of the passenger strand of the miR-15a microRNA nucleotide sequence.

9. The double-stranded nucleic acid composition of claim 1, wherein dual modifications of miR-15a using 5-fluorouracil and gemcitabine enhance the therapeutic efficacy of tumor suppression compared to unmodified miR-15a.
10. The double-stranded nucleic acid composition of claim 1, wherein the methotrexate (MTX) conjugation of modified miR-15a improves tumor specificity compared to unconjugated modified miR-15a and adds additional tumor killing effect of the modified miR-15a miRNA tumor suppressor once the methotrexate (MTX) breaks down from the modified miR-15a miRNA.
11. A pharmaceutical composition comprising:
- (i) a double-stranded nucleic acid composition comprising a double-stranded nucleic acid sequence that comprises uracil, gemcitabine and methotrexate (MTX), wherein the uracil comprises 5-fluorouracil; and
- (ii) a pharmaceutically acceptable carrier.
12. The pharmaceutical composition of claim 11, wherein said modified double-stranded nucleic acid sequence comprises a modified microRNA nucleotide sequence.
13. The pharmaceutical composition of claim 12, wherein said modified microRNA nucleotide sequence comprises a microRNA nucleotide sequence of miR-15a as set forth in SEQ ID NO. 1.
14. The pharmaceutical composition of claim 13, wherein said miR-15a microRNA nucleotide sequence comprises a guide strand and a passenger strand.
15. The pharmaceutical composition of claim 14, wherein cytidines are replaced with gemcitabines on the guide strand of the miR-15a microRNA nucleotide sequence.
16. The pharmaceutical composition of claim 14, wherein the uracils on the guide strand of the miR-15a microRNA nucleotide sequence are 5-fluorouracils.

17. The pharmaceutical composition of claim 14, wherein the methotrexate (MTX) is conjugated on the passenger strand of the miR-15a microRNA nucleotide sequence.
18. The pharmaceutical composition of claim 17, wherein the methotrexate (MTX) is conjugated onto the 5' end of the passenger strand of miR-15a microRNA nucleotide sequence.
19. A method for treating cancer comprising:

administering to a subject an effective amount of a double-stranded nucleic acid composition comprising a modified double-stranded nucleic acid sequence that comprises uracil, gemcitabine and methotrexate (MTX), wherein the uracil comprises 5-fluorouracil.
20. The method of claim 19, wherein the subject is a mammal.
21. The method of claim 20, wherein the mammal is a human.
22. The method of claim 19, wherein said subject has a cancer selected from the group consisting of colorectal, stomach, esophageal, breast, lung, prostate, ovarian, uterine, pancreatic, liver, skin, blood or cervical cancer.
23. The method of claim 19, wherein said subject has pancreatitis or fibrosis.
24. The method of claim 22, wherein said subject has pancreatic cancer.
25. The method of claim 24, wherein said pancreatic cancer is pancreatic ductal adenocarcinoma cancer (PDAC).
26. The method of claim 22, wherein said subject has ovarian cancer.
27. The method of claim 19, wherein said double-stranded nucleic acid composition is administered to the subject by injection.
28. The method of claim 19, wherein said modified double-stranded nucleic acid sequence comprises a modified microRNA nucleotide sequence.

29. The method of claim 28, wherein said modified microRNA nucleotide sequence comprises a microRNA nucleotide sequence of miR-15a as set forth in SEQ ID NO. 1.
30. The method of claim 29, wherein said miR-15a microRNA nucleotide sequence comprises a guide strand and a passenger strand.
31. The method of claim 30, wherein cytidines are replaced with gemcitabines on the guide strand of the miR-15a microRNA nucleotide sequence.
32. The method of claim 30, wherein the uracils on the guide strand of the miR-15a microRNA nucleotide sequence are 5-fluorouracils.
33. The method of claim 30, wherein the methotrexate (MTX) is conjugated on the passenger strand of the miR-15a microRNA nucleotide sequence.
34. The method of claim 33, wherein the methotrexate (MTX) is conjugated onto the 5' end of the passenger strand of the miR-15a microRNA nucleotide sequence.
35. The method of claim 19, wherein the modified double-stranded nucleic acid sequence induces apoptosis.
36. The method of claim 35, wherein the modified double-stranded nucleic acid sequence comprises MTX-5FU-Gem-miR-15a.
37. The method of claim 36, wherein the modified miR-15a exhibits an enhanced potency in inducing apoptosis when compared to both unmodified miR-15a and co-treatment of 5-FU and gemcitabine.