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(54) **RNA INTERFERENCE AGENTS FOR GST-PI GENE MODULATION**

RNA-INTERFERENZ-WIRKSTOFFE ZUR GST-PI-GEN-MODULIERUNG

AGENTS D'INTERFÉRENCE ARN POUR LA MODULATION DU GÈNE GST-PI

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EP 3 236 974 B9

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Description

TECHNICAL FIELD OF THE INVENTION

5 **[0001]** This invention relates to the fields of biopharmaceuticals and therapeutics composed of nucleic acid based molecules. More particularly, this invention relates to compounds and compositions utilizing RNA interference (RNAi) for modulating the expression of human GST- π , and uses thereof.

BACKGROUND OF THE INVENTION

10 **[0002]** Various human cancer tissues have been found to correlate with the appearance of mutated KRAS gene. In some cases, the tissues also present an elevated level of Glutathione S-Transferase Pi (GST- π) expression. (Miyanishi et al., Gastroenterology, 2001, Vol. 121:865-874, Abstract) For example, elevated serum GST- π levels were observed in patients with various gastrointestinal malignancies. (Niitsu et al., Cancer, 1989, Vol.63, No. 2, pp. 317-323, Abstract)

15 **[0003]** GST- π is a member of a GST family of enzymes that play a role in detoxification by catalyzing the conjugation of hydrophobic and electrophilic compounds with reduced glutathione. GST- π expression can be reduced in vitro with a siRNA. (Niitsu et al., US 2014/0315975 A1). Hokaiwado et al. (Carcinogenesis vol.29 no.6 pp. 1134-1138, 2008) discusses glutathione S-transferase Pi mediating proliferation of androgen-independent prostate cancer cells. Müller et al. (Int. J. Cancer 123, 1797-1806, 2008) describes thiazolides that inhibit growth and induce glutathione-S-transferase Pi (GSTP1)-dependent cell death in human colon cancer cells. Sawers et al. (British Journal of Cancer 111, 1150-1158, 2014) relates to glutathione S-transferase P1 (GSTP1) directly influencing platinum drug chemosensitivity in ovarian tumour cell lines. WO 2007/061922 A2 concerns methods to predict and prevent resistance to taxoid compounds. EP 2 724 729 A1 describes an apoptosis-inducing agent containing as active components a drug that suppresses GST- π and a drug that suppresses autophagy. However, there are many drawbacks of existing siRNA agents, such as insufficient activity, off target effects, lack of serum stability, and lack of in vivo potency or efficacy.

20 **[0004]** There is an urgent need for compositions and methods for modulating the expression of genes associated with cancer. In particular, therapeutics based on inhibition of GST- π expression will require highly potent and stable siRNA sequences and structures, which can reduce off target effects.

25 **[0005]** What is needed are siRNA sequences, compounds and structures for modulating GST- π expression, with uses for treating disease, such as malignant tumors.

BRIEF SUMMARY

30 **[0006]** This invention relates to compounds, compositions and their use in methods for modulating the expression of human GST- π using RNA interference.

35 **[0007]** In some embodiments, this invention provides molecules, comprising a sense strand of SEQ ID NO: 131 and an antisense strand of SEQ ID NO: 157 forming a duplex region, for RNA interference gene silencing of GST- π .

[0008] In further embodiments, the structures, molecules and compositions of this invention can be used in methods for preventing or treating diseases, or ameliorating symptoms of conditions or disorders associated with GST- π , including malignant tumor.

40 **[0009]** Embodiments of this invention include the following:

A nucleic acid molecule for inhibiting expression of GST- π comprising a sense strand of SEQ ID NO: 131 and an antisense strand of SEQ ID NO: 157, wherein the strands form a duplex region. The nucleic acid molecules can be siRNA molecules for inhibiting expression of GST- π , and may contain one or more nucleotides that are chemically-modified.

45 **[0010]** In some embodiments, the nucleic acid siRNA molecules for inhibiting expression of GST- π may include 2'-deoxy nucleotides, 2'-O-alkyl substituted nucleotides, 2'-deoxy-2'-fluoro substituted nucleotides, or any combination thereof. In certain embodiments, the 2'-deoxy nucleotides may be in the seed region of the siRNA molecules. In certain aspects, the siRNA molecules for inhibiting expression of GST- π may have deoxynucleotides in a plurality of positions in the antisense strand.

50 **[0011]** The nucleic acid molecules of this invention may advantageously inhibit expression of GST- π mRNA with an IC50 of less than 300 pM. In certain embodiments, the nucleic acid molecules may inhibit expression of GST- π mRNA levels by at least 25% in vivo, upon a single administration of the molecules. In some embodiments, the nucleic acid molecules may have passenger strand off target activity reduced, or reduced by at least 50-fold, or at least 100-fold.

55 **[0012]** Embodiments of this invention further provide pharmaceutical compositions containing the siRNA molecules and a pharmaceutically acceptable carrier. In some embodiments, the carrier may be a lipid molecule, or liposome. This disclosure includes vectors or cells comprising the nucleic acid molecules.

[0013] Also contemplated in this invention are compositions for use in methods for treating a disease associated with GST- π expression, by administering to a subject in need a composition containing an siRNA, where the disease is

malignant tumor, cancer, cancer caused by cells expressing mutated KRAS, sarcoma, or carcinoma.

BRIEF DESCRIPTION OF THE DRAWINGS

5 [0014]

FIG. 1: Fig. 1 shows the profound reduction of orthotopic lung cancer tumors in vivo by a siRNA of this invention targeted to GST- π . The GST- π siRNA was administered in a liposomal formulation at a dose of 2 mg/kg to athymic nude mice presenting A549 orthotopic lung cancer tumors. Final primary tumor weights were measured at necropsy for the treatment group and a vehicle control group. The GST- π siRNA showed significant efficacy for inhibition of lung cancer tumors in this six-week study. As shown in Fig. 1, after 43 days, the GST- π siRNA showed markedly advantageous tumor inhibition, with final primary tumor average weights significantly reduced by 2.8-fold, as compared to control.

FIG. 2: Fig. 2 shows tumor inhibition efficacy in vivo for a GST- π siRNA. A cancer xenograft model using A549 cells was utilized with a relatively low dose of siRNA at 0.75 mg/kg. The GST- π siRNA showed advantageous tumor inhibition within a few days. After 36 days, the GST- π siRNA showed markedly advantageous tumor inhibition, with final tumor average volumes significantly reduced by about 2-fold, as compared to control.

FIG. 3: Fig. 3 shows tumor inhibition efficacy in vivo for a GST- π siRNA at the endpoint of Fig. 2. The GST- π siRNA showed advantageous tumor inhibition with average tumor weights reduced by more than 2-fold.

FIG. 4: Fig. 4 shows that a GST- π siRNA of this invention greatly increased cancer cell death by apoptosis in vitro. The GST- π siRNA caused upregulation of PUMA, a biomarker for apoptosis, which is associated with loss in cell viability. In Fig. 4, the expression of PUMA was greatly increased from 2-6 days after transfection of the GST- π siRNA.

FIG. 5: Fig. 5 shows that a GST- π siRNA of this invention provided knockdown efficacy for A549 xenograft tumors in vivo. Dose dependent knockdown of GST- π mRNA was observed in athymic nude (nu/nu) female mice (Charles River) with the siRNA targeted to GST- π . As shown in Fig. 5, at a dose of 4mg/kg, significant reduction of about 40% in GST- π mRNA was detected 24 hours after injection.

FIG. 6: Fig. 6 shows that a GST- π siRNA of this invention inhibited pancreatic cancer xenograft tumors in vivo. The GST- π siRNA provided gene silencing potency in vivo when administered in a liposomal formulation to pancreatic cancer xenograft tumors in athymic nude female mice, 6 to 8 weeks old. As shown in Fig. 6, a dose response was obtained with doses ranging from 0.375 mg/kg to 3 mg/kg of siRNA targeted to GST- π . The GST- π siRNA showed advantageous tumor inhibition within a few days after administration, the tumor volume being reduced by about 2-fold at the endpoint.

FIG. 7: Fig. 7 shows that a GST- π siRNA of this invention exhibited increased serum stability. As shown in Fig. 7, the half-life ($t_{1/2}$) in serum for both the sense strand (Fig. 7, top) and antisense strand (Fig. 7, bottom) of a GST- π siRNA was about 100 minutes.

FIG. 8: Fig. 8 shows that a GST- π siRNA of this invention exhibited enhanced stability in formulation in plasma. Fig. 8 shows incubation of a liposomal formulation of a GST- π siRNA in 50% human serum in PBS, and detection of remaining siRNA at various time points. As shown in Fig. 8, the half-life ($t_{1/2}$) in plasma of the formulation of the GST- π siRNA was significantly longer than 100 hours.

FIG. 9: Fig. 9 shows in vitro knockdown for the guide strand of a GST- π siRNA. As shown in Fig. 9, the guide strand knockdown of the GST- π siRNA was approximately exponential, as compared to a control with scrambled sequence that exhibited no effect.

FIG. 10: Fig. 10 shows in vitro knockdown for the passenger strand of the GST- π siRNA of Fig. 9. As shown in Fig. 10, the passenger strand off target knockdown for the GST- π siRNA was greatly reduced, with essentially no effect.

FIG. 11: Fig. 11 shows in vitro knockdown for the guide strands of several highly active GST- π siRNAs. As shown in Fig. 11, the guide strand knockdown activities of the GST- π siRNAs were approximately exponential.

FIG. 12: Fig. 12 shows in vitro knockdown for the passenger strand of the GST- π siRNAs of Fig. 11. As shown in

Fig. 12, the passenger strand off target knockdown activities for the GST- π siRNAs were significantly reduced below about 500 pM.

FIG. 13: Fig. 13 shows in vitro knockdown for the guide strand of a highly active GST- π siRNA. As shown in Fig. 13, the guide strand knockdown activity of the GST- π siRNA was approximately exponential.

FIG. 14: Fig. 14 shows in vitro knockdown for the passenger strand of the GST- π siRNA of Fig. 13. As shown in Fig. 14, the passenger strand off target knockdown activity for the GST- π siRNA was significantly reduced.

DETAILED DESCRIPTION OF THE INVENTION

[0015] This invention relates to compounds, compositions and their use in methods for nucleic acid based therapeutics for modulating expression of GST- π .

[0016] In some embodiments, this invention provides molecules active in RNA interference, comprising a sense strand of SEQ ID NO: 131 and an antisense strand of SEQ ID NO: 157 forming a duplex, as well as structures and compositions that can silence expression of GST- π .

[0017] The structures and compositions of this disclosure can be used in preventing or treating various diseases such as malignant tumor.

[0018] In further embodiments, this disclosure provides compositions for delivery and uptake of one or more therapeutic RNAi molecules of this invention, as well as methods of use thereof. The RNA-based compositions of this invention can be used in methods for preventing or treating malignant tumors, such as cancers.

[0019] Therapeutic compositions of this invention include nucleic acid molecules, as defined in the claims, that are active in RNA interference. The therapeutic nucleic acid molecules can be targeted to GSTP1 (GST- π) for gene silencing.

[0020] In various embodiments, this invention provides a range of molecules, as defined in the claims, that can be active as a small interfering RNA (siRNA), and can regulate or silence GST- π gene expression.

[0021] The siRNAs of this invention can be used for preventing or treating malignant tumors.

[0022] Aspects of this disclosure further provide a vehicle, formulation, or lipid nanoparticle formulation for delivery of the inventive siRNAs to subjects in need of preventing or treating a malignant tumor. This disclosure further contemplates methods for administering siRNAs as therapeutics to mammals.

[0023] The therapeutic molecules and compositions of this invention can be used for RNA interference directed to preventing or treating a GST- π associated disease, by administering a compound or composition to a subject in need.

[0024] The methods of this disclosure can utilize the inventive compounds for preventing or treating malignant tumor.

[0025] In some aspects, the malignant tumor can be presented in various diseases, for example, cancers that highly expressing GST- π , cancers caused by cells expressing mutated KRAS, sarcomas, fibrosarcoma, malignant fibrous histiocytoma, liposarcoma, rhabdomyosarcoma, leiomyosarcoma, angiosarcoma, Kaposi's sarcoma, lymphangiosarcoma, synovial sarcoma, chondrosarcoma, osteosarcoma, and carcinomas.

[0026] In certain aspects, methods of this disclosure can utilize the inventive compounds for preventing or treating malignant tumors and cancers in any organ or tissue, including, for example, brain tumor, head and neck cancer, breast cancer, lung cancer, esophageal cancer, stomach cancer, duodenal cancer, colorectal cancer, liver cancer, pancreatic cancer, gallbladder cancer, bile duct cancer, kidney cancer, urethral cancer, bladder cancer, prostate cancer, testicular cancer, uterine cancer, ovary cancer, skin cancer, leukemia, malignant lymphoma, epithelial malignant tumors, and non-epithelial malignant tumors.

[0027] In certain cases a combination of therapeutic molecules of this disclosure can be used for silencing or inhibiting GST- π gene expression.

[0028] This disclosure provides a range of RNAi molecules, where each molecule has a polynucleotide sense strand and a polynucleotide antisense strand; each strand of the molecule is from 15 to 30 nucleotides in length; a contiguous region of from 15 to 30 nucleotides of the antisense strand is complementary to a sequence of an mRNA encoding GST- π ; and at least a portion of the sense strand is complementary to at least a portion of the antisense strand, and the molecule has a duplex region of from 15 to 30 nucleotides in length.

[0029] A RNAi molecule of this disclosure can have a contiguous region of from 15 to 30 nucleotides of the antisense strand that is complementary to a sequence of an mRNA encoding GST- π , which is located in the duplex region of the molecule.

[0030] In some cases a RNAi molecule can have a contiguous region of from 15 to 30 nucleotides of the antisense strand that is complementary to a sequence of an mRNA encoding GST- π .

[0031] Aspects of this disclosure may further provide methods for preventing, treating or ameliorating one or more symptoms of malignant tumor, or reducing the risk of developing malignant tumor, or delaying the onset of malignant tumor in a mammal in need thereof.

GST- π and RNAi molecules

[0032] The nucleic acid sequence of an example target human glutathione S-transferase pi (human GST- π) mRNA is disclosed in GenBank accession number NM_000852.3 (hGSTP1), and is 986 nucleotides in length.

[0033] One of ordinary skill in the art would understand that a reported sequence may change over time and to incorporate any changes needed in the nucleic acid molecules herein accordingly.

[0034] Embodiments of this invention can provide compositions and their use in methods for gene silencing of GST- π expression using small nucleic acid molecules as defined in the claims. Examples of nucleic acid molecules include molecules active in RNA interference (RNAi molecules), short interfering RNA (siRNA), double-stranded RNA (dsRNA), micro-RNA (miRNA), and short hairpin RNA (shRNA) molecules, as well as DNA-directed RNAs (ddRNA), Piwi-interacting RNAs (piRNA), and repeat associated siRNAs (rasiRNA). Such molecules are capable of mediating RNA interference against GST- π gene expression.

[0035] The composition and methods disclosed herein can also be used in treating various kinds of malignant tumors in a subject.

[0036] The nucleic acid molecules, as defined in the claims, and their use in methods of this invention may be used to down regulate the expression of genes that encode GST- π .

[0037] The compositions and methods of this disclosure can include one or more nucleic acid molecules, which, independently or in combination, can modulate or regulate the expression of GST- π protein and/or genes encoding GST- π proteins, proteins and/or genes encoding GST- π associated with the maintenance and/or development of diseases, conditions or disorders associated with GST- π , such as malignant tumor.

[0038] The compositions and their use in methods of this invention are described with reference to exemplary sequences of GST- π . A person of ordinary skill in the art would understand that various aspects and embodiments of the invention are directed to any related GST- π genes, sequences, or variants, such as homolog genes and transcript variants, and polymorphisms, including single nucleotide polymorphism (SNP) associated with any GST- π genes.

[0039] In some embodiments, the compositions, as defined in the claims, and their use in methods of this invention can provide a double-stranded short interfering nucleic acid (siRNA) molecule that downregulates the expression of a GST- π gene, for example human GST- π .

[0040] A RNAi molecule of this invention, as defined in the claims, can be targeted to GST- π and any homologous sequences, for example, using complementary sequences or by incorporating non-canonical base pairs, for example, mismatches and/or wobble base pairs, that can provide additional target sequences.

[0041] In instances where mismatches are identified, non-canonical base pairs, for example, mismatches and/or wobble bases can be used to generate nucleic acid molecules that target more than one gene sequence.

[0042] For example, non-canonical base pairs such as UU and CC base pairs can be used to generate nucleic acid molecules that are capable of targeting sequences for differing GST- π targets that share sequence homology. Thus, a RNAi molecule can be targeted to a nucleotide sequence that is conserved between homologous genes, and a single RNAi molecule can be used to inhibit expression of more than one gene.

[0043] In some aspects, the compositions, as defined in the claims, and their use in methods of this invention include RNAi molecules that are active against GST- π mRNA, where the RNAi molecule includes a sequence complementary to any mRNA encoding a GST- π sequence.

[0044] In some embodiments, a RNAi molecule of this disclosure can have activity against GST- π RNA, where the RNAi molecule includes a sequence complementary to an RNA having a variant GST- π encoding sequence, for example, a mutant GST- π gene known in the art to be associated with malignant tumor.

[0045] In further embodiments, a RNAi molecule of this invention, as defined in the claims, can include a nucleotide sequence that can interact with a nucleotide sequence of a GST- π gene and mediate silencing of GST- π gene expression.

[0046] The nucleic acid molecules for inhibiting expression of GST- π have a sense strand and an antisense strand, wherein the strands form a duplex region. The nucleic acid molecules may have one or more of the nucleotides in the duplex region being chemically-modified, including such modifications as are known in the art. Any nucleotide in an overhang of the siRNA may also be chemically-modified.

[0047] In some embodiments, the preferred chemically-modified nucleotides are 2'-deoxy nucleotides. In additional embodiments, the chemically-modified nucleotides can include 2'-O-alkyl substituted nucleotides, 2'-deoxy-2'-fluoro substituted nucleotides, phosphorothioate nucleotides, locked nucleotides, or any combination thereof.

[0048] In certain embodiments, a preferred structure can have an antisense strand containing deoxynucleotides in a plurality of positions, the plurality of positions being one of the following: each of positions 4, 6 and 8, from the 5' end of the antisense strand; each of positions 3, 5 and 7, from the 5' end of the antisense strand; each of positions 1, 3, 5 and 7, from the 5' end of the antisense strand; each of positions 3-8, from the 5' end of the antisense strand; and each of positions 5-8, from the 5' end of the antisense strand. Any of these structures can be combined with one or more 2'-deoxy-2'-fluoro substituted nucleotides in the duplex region.

[0049] The nucleic acid molecules of this invention can inhibit expression of GST- π mRNA with an advantageous IC50

of less than about 300 pM, or less than about 200 pM, or less than about 100 pM, or less than about 50 pM.

[0050] Further, the nucleic acid molecules can inhibit expression of GST- π mRNA levels by at least 25% in vivo, upon a single administration.

[0051] Pharmaceutical compositions are contemplated in this invention, which can contain siRNAs as described herein, in combination with a pharmaceutically acceptable carrier. Any suitable carrier may be used, including those known in the art, as well as lipid molecules, nanoparticles, or liposomes, any of which may encapsulate the siRNA molecules.

[0052] This invention discloses pharmaceutical compositions for use in methods for treating a disease associated with GST- π expression, which methods include administering to a subject in need a composition containing the siRNAs. Diseases to be treated may include malignant tumor, cancer, cancer caused by cells expressing mutated KRAS, sarcoma, and carcinoma, among others.

[0053] Examples of RNAi molecules of this disclosure targeted to GST- π mRNA are shown in Table 1.

Table 1: RNAi molecule sequences for GST- π

ID	Ref Pos	SEQ ID NO	SENSE STRAND (5'-->3') SEQ ID NOS: 1 to 65	SEQ ID NO	ANTISENSE STRAND (5'-->3') SEQ ID NOS:66 to 130
A1	652	1	UCCCAGAACCAGGGAGGCAtt	66	UGCCUCCCUGGUUCUGGGACa
A10	635	2	CUUUUGAGACCCUGCUGUCtt	67	GACAGCAGGGUCUCAAAAAGgc
A11	649	3	CUGUCCCAGAACCAGGGAGtt	68	CUCCCUGGUUCUGGGACAGca
A12	650	4	UGUCCCAGAACCAGGGAGGtt	69	CCUCCCUGGUUCUGGGACAGc
A13	631	5	AAGCCUUUUGAGACCCUGCtt	70	GCAGGGUCUCAAAAAGGCUuca
A14	638	6	UUGAGACCCUGCUGUCCAtt	71	UGGGACAGCAGGGUCUCAAAa
A15	636	7	UUUUGAGACCCUGCUGUCtt	72	GGACAGCAGGGUCUCAAAAagg
A16	640	8	GAGACCCUGCUGUCCAGAtt	73	UCUGGGACAGCAGGGUCUCaa
A17	332	9	GCUGGAAGGAGGAGGUGGUtt	74	ACCACCUCCUCCUCCAGCtc
A18	333	10	CUGGAAGGAGGAGGUGGUtt	75	CACCACCUCCUCCUCCAGct
A19	321	11	UCAGGGCCAGAGCUGGAAGtt	76	CUUCCAGCUCUGGCCCCUGAtc
A2	639	12	UGAGACCCUGCUGUCCAGtt	77	CUGGGACAGCAGGGUCUCAaa
A20	323	13	AGGGCCAGAGCUGGAAGAtt	78	UCCUCCAGCUCUGGCCCCUga
A21	331	14	AGCUGGAAGGAGGAGGUGGtt	79	CCACCUCCUCCUCCAGCUct
A22	641	15	AGACCCUGCUGUCCAGAAtt	80	UUCUGGGACAGCAGGGUCUca
A23	330	16	GAGCUGGAAGGAGGAGGUGtt	81	CACCUCUCCUCCUCCAGCUCtg
A25	647	17	UGCUGUCCAGAACCAGGGtt	82	CCUUGGUUCUGGGACAGCAgg
A26	653	18	CCCAGAACCAGGGAGGCAAtt	83	UUGCCUCCCUGGUUCUGGGac
A3	654	19	CCAGAACCAGGGAGGCAAGtt	84	CUUGCCUCCCUGGUUCUGGga
A4	637	20	UUUGAGACCCUGCUGUCCtt	85	GGGACAGCAGGGUCUCAAAag
A5	642	21	GACCCUGCUGUCCAGAAct	86	GUUCUGGGACAGCAGGGUCtc
A6	319	22	GAUCAGGGCCAGAGCUGGAtt	87	UCCAGCUCUGGCCCCUGAUtg
A7	632	23	AGCCUUUUGAGACCCUGCUtt	88	AGCAGGGUCUCAAAAAGGCUtc
A8	633	24	GCCUUUUGAGACCCUGCUGtt	89	CAGCAGGGUCUCAAAAAGGctt
A9	634	25	CCUUUUGAGACCCUGCUGUtt	90	ACAGCAGGGUCUCAAAAAGGct
AG7	632	26	CGCCUUUUGAGACCCUGCAtt	91	UGCAGGGUCUCAAAAAGGCGtc
AK1	257	27	CCUACACCGUGGUCUAUUUtt	92	AAAUAGACCACGGUGUAGGgc
AK10	681	28	UGUGGGAGACCAGAUCUCtt	93	GGAGAUCUGGUCUCCACAat

EP 3 236 974 B9

(continued)

ID	Ref Pos	SEQ ID NO	SENSE STRAND (5'-->3') SEQ ID NOS: 1 to 65	SEQ ID NO	ANTISENSE STRAND (5'-->3') SEQ ID NOS:66 to 130
AK11	901	29	GCGGGAGGCAGAGUUUGCct	94	GGCAAACUCUGCCUCCCGctc
AK12	922	30	CCUUUCUCCAGGACCAAUAtt	95	UAUUGGUCCUGGAGAAAGGaa
AK13/A24	643	31	ACCCUGCUGUCCAGAACct	96	GGUUCUGGGACAGCAGGGUct
AK2	267	32	GGUCUAUUUCCCAGUUCGAtt	97	UCGAACUGGAAAUAGACCac
AK3	512	33	CCCUGGUGGACAUGGUGAAtt	98	UUCACCAUGUCCACCAGGGct
AK4	560	34	ACAUCUCCCUCAUCUACACTt	99	GUGUAGAUGAGGGAGAUGUat
AK5	593	35	GCAAGGAUGACUAUGUGAAtt	100	UUCACAUAGUCAUCCUUGCcc
AK6	698	36	CCUUCGCUGACUACAACCUtt	101	AGGUUGUAGUCAGCGAAGGag
AK7	313	37	CUGGCAGAUCAGGGCCAGAtt	102	UCUGGCCCUGAUCUGCCAGca
AK8	421	38	GACGGAGACCUACCCUGUtt	103	ACAGGGUGAGGUCUCCGUCct
AK9	590	39	CGGGCAAGGAUGACUAUGUtt	104	ACAUAGUCAUCCUUGCCCgcc
AU10	635	40	CUUUUGAGACCCUGCUGUAtt	105	UACAGCAGGGUCUCAAAAGgc
AU23	330	41	GAGCUGGAAGGAGGAGGUAtt	106	UACCUCUCCUUCUCCAGCUCtg
AU24	643	42	ACCCUGCUGUCCAGAACAtt	107	UGUUCUGGGACAGCAGGGUct
AU25	648	43	UGCUGUCCAGAACCAGGAtt	108	UCCUGGUUCUGGGACAGCAgg
AU7	632	44	AGCCUUUUGAGACCCUGCAtt	109	UGCAGGGUCUCAAAAGGCUtc
AU9	634	45	CCUUUUGAGACCCUGCUGAtt	110	UCAGCAGGGUCUCAAAAGGct
B1	629	46	UGAAGCCUUUUGAGACCCUtt	111	AGGGUCUCAAAAGGCUUCAgt
B10	627	47	ACUGAAGCCUUUUGAGACct	112	GGUCUCAAAAGGCUUCAGUtg
B11	596	48	AGGAUGACUAUGUGAAGGct	113	GCCUUCACAUAGUCAUCCUtg
B12	597	49	GGAUGACUAUGUGAAGGcAtt	114	UGCCUUCACAUAGUCAUCct
B13	598	50	GAUGACUAUGUGAAGGcAtt	115	GUGCCUUCACAUAGUCAUCct
B14	564	51	CUCCCUCAUCUACACCAACTt	116	GUUGGUGUAGAUGAGGGAGat
B2	630	52	GAAGCCUUUUGAGACCCUGtt	117	CAGGGUCUCAAAAGGCUUCag
B3	563	53	UCUCCCUCAUCUACACCAAtt	118	UUGGUGUAGAUGAGGGAGatg
B4	567	54	CCUCAUCUACACCAACUAUtt	119	AUAGUUGGUGUAGAUGAGGga
B5	566	55	CCCUCAUCUACACCAACUAtt	120	UAGUUGGUGUAGAUGAGGGag
B6	625	56	CAACUGAAGCCUUUUGAGAtt	121	UCUCAAAAGGCUUCAGUUGcc
B7	626	57	AACUGAAGCCUUUUGAGACTt	122	GUCUCAAAAGGCUUCAGUUgc
B8	628	58	CUGAAGCCUUUUGAGACCCct	123	GGGUCUCAAAAGGCUUCAGtt
B9	565	59	UCCCUCAUCUACACCAACUtt	124	AGUUGGUGUAGAUGAGGGAgga
BG3	563	60	GCUCCCUCAUCUACACCAAtt	125	UUGGUGUAGAUGAGGGAGctg
BU2	630	61	GAAGCCUUUUGAGACCCUAtt	126	UAGGGUCUCAAAAGGCUUCag
BU10	627	62	ACUGAAGCCUUUUGAGACAtt	127	UGUCUCAAAAGGCUUCAGUtg
BU14	565	63	CUCCCUCAUCUACACCAAAAtt	128	UUUGGUGUAGAUGAGGGAGat
BU4	567	64	CCUCAUCUACACCAACUAtt	129	UUAGUUGGUGUAGAUGAGGga

EP 3 236 974 B9

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ID	Ref Pos	SEQ ID NO	SENSE STRAND (5'-->3') SEQ ID NOS: 1 to 65	SEQ ID NO	ANTISENSE STRAND (5'-->3') SEQ ID NOS:66 to 130
c1-934	934	65	ACCAAUAAAUUUCUAAGAtt	130	UCUUAGAAAUUUUUUAUUGGUcc

[0054] Key for Table 1: Upper case A, G, C and U refer to ribo-A, ribo-G, ribo-C and ribo-U, respectively. The lower case letters a, u, g, c, t refer to 2'-deoxy-A, 2'-deoxy-U, 2'-deoxy-G, 2'-deoxy-C, and deoxythymidine respectively.

[0055] Examples of RNAi molecules of this disclosure and the invention targeted to GST- π mRNA are shown in Table 2.

Table 2: RNAi molecule sequences for GST- π

ID	SEQ ID NO	SENSE STRAND (5'-->3') SEQ ID NOS:131 to 156	SEQ ID NO	ANTISENSE STRAND (5'-->3') SEQ ID NOS:157 to 182
BU2'	131	GAAGCCUUUUGAGACCCUANN	157	UAGGGUCUCAAAAGGCUUCNN
14	132	GAAGCCUUUUGAGACCCUAUU	158	UAGGGUCUCAAAAGGCUUCUU
15	133	GAAGCCUUUUGAGACCCUAUU	159	uagggucuCAAAGGCUUCUU
16	134	GAAGCCUUUUGAGACCCUAUU	160	UagggucuCAAAGGCUUCUU
17	135	GAAGCCUUUUGAGACCCUAUU	161	UAgggucuCAAAGGCUUCUU
18	136	GAAGCCUUUUGAGACCCUAUU	162	UAGggucuCAAAGGCUUCUU
19	137	GAAGCCUUUUGAGACCCUAUU	163	UAGGgucuCAAAGGCUUCUU
20	138	GAAGCCUUUUGAGACCCUAUU	164	uAgGgUcUCAAAAGGCUUCUU
21	139	GAAGCCUUUUGAGACCCUAUU	165	UAgGgUcUCAAAAGGCUUCUU
ID	SEQ ID NO	SENSE STRAND (5'-->3') SEQ ID NOS: 131 to 156	SEQ NO	ANTISENSE STRAND (5'-->3') SEQ ID NOS:157 to 182
22	140	GAAGCCUUUUGAGACCCUAUU	166	UaGgGuCuCAAAGGCUUCUU
23	141	GAAGCCUUUUGAGACCCUAUU	167	UAGgGuCuCAAAGGCUUCUU
24	142	GAAGCCUUUUGAGACCCUAtt	168	UagggucuCAAAGGCUUCUU
25	143	GAAGCLUUUUGAGACCCUAUU	169	UAGGGUCUCAAAAGGCUUCUU
26	144	GAAGCCUUUUGAGACCCUAUU	170	fUAGGGUCUCAAAAGGCUUCUU
27	145	GAAGCCUUUUGAGACCCUAUU	171	uAGGGUCUCAAAAGGCUUCUU
28	146	GAAGCCUUUUGAGACCCUAUU	172	UsAGGGUCUCAAAAGGCUUCUU
29	147	GAAGCCUUUUGAGACCCUfAUU	173	fUAGGGUCUfCAAAGGCFUUCUU
30	148	GAAGCCUUUUGAGfACCCUfAUU	174	fUAGGGUCUfCAfAFAAGGCFUUCUU
31	149	GAAGCCUUUUGAGACCCUAUU	175	UAGGGUCUCAAAAGGCUUCUU
31'	150	GAAGCCUUUUGAGACCCUAUU	176	fUAGGGUCUCfCAAAGGCUUCUU
32	151	GAAGCCUUUUGAGACCCUAUU	177	UAGGGUCUCAAAAGGCUUCUU
39	152	GAAGCCUUUUUGAGACCCUAUU	178	UAGgGuCuCAAAGGCUUCUU
45	153	GAAGCCUUUUUGAGACCCUAUU	179	UAGgGuCuCAAAGGCUUCUU
46	154	GAAGCCUUUUUGAGACCCUAUU	180	UAGgGuCuCAAAGGCUUCUU
47	155	GAAGCCUUUUUGAGACCCUAUU	181	UAGgGuCuCAAAGGCUUCUU
48	156	GAAGCCUUUUUGAGACCCUAUU	182	fUAGgGuCuCAAAGGCUUCUU

[0056] Key for Table 2: Upper case A, G, C and U refer to ribo-A, ribo-G, ribo-C and ribo-U, respectively. The lower case letters a, u, g, c, t refer to 2'-deoxy-A, 2'-deoxy-U, 2'-deoxy-G, 2'-deoxy-C, and deoxythymidine (dT = T = t) respectively. Underlining refers to 2'-OMe-substituted, e.g., U. The lower case letter f refers to 2'-deoxy-2'-fluoro substitution, e.g. fU is 2'-deoxy-2'-fluoro-U. N is A, C, G, U, U, a, c, g, u, t, or a modified, inverted, or chemically modified nucleotide. An "s" character represents a phosphorothioate linkage.

[0057] Examples of RNAi molecules of this disclosure targeted to GST- π mRNA are shown in Table 3.

Table 3: RNAi molecule sequences for GST- π

ID	SEQ ID NO	SENSE STRAND (5'-->3') SEQ ID NOS: 183 to 194	SEQ ID NO	ANTISENSE STRAND (5'-->3') SEQ ID NOS:195 to 206
A9'	183	CCUUUUGAGACCCUGCUGUNN	195	ACAGCAGGGUCUCAAAAGGNN
1	184	CCUCAUCUACACCAACU <u>UUU</u>	196	AUAGUUGGUGUAGAUGAG <u>GUU</u>
2	185	CCUCAUCUACACCAACU <u>UUU</u>	197	auaguuggUGUAGAUGAG <u>GUU</u>
3	186	CCUCAUCUACACCAACU <u>UUU</u>	198	AuaguuggUGUAGAUGAG <u>GUU</u>
4	187	CCUCAUCUACACCAACU <u>UUU</u>	199	AUaguuggUGUAGAUGAG <u>GUU</u>
5	188	CCUCAUCUACACCAACU <u>UUU</u>	200	AUAguuggUGUAGAUGAG <u>GUU</u>
6	189	CCUCAUCUACACCAACU <u>UUU</u>	201	AUAGuuggUGUAGAUGAG <u>GUU</u>
7	190	CCUCAUCUACACCAACU <u>UUU</u>	202	auaGuUgGUGUAGAUGAG <u>GUU</u>
8	191	CCUCAUCUACACCAACU <u>UUU</u>	203	AUaGuUgGUGUAGAUGAG <u>GUU</u>
9	192	CCUCAUCUACACCAACU <u>UUU</u>	204	AuAgUuGgUGUAGAUGAG <u>GUU</u>
10	193	CCUCAUCUACACCAACU <u>UUU</u>	205	AUAgUuGgUGUAGAUGAG <u>GUU</u>
11	194	<u>CCUCAUCUACACCAACU<u>UUU</u></u>	206	AuaguuggUGUAGA <u>UAGAG<u>GUU</u></u>

[0058] Key for Table 3: Upper case A, G, C and U refer to ribo-A, ribo-G, ribo-C and ribo-U, respectively. The lower case letters a, u, g, c, t refer to 2'-deoxy-A, 2'-deoxy-U, 2'-deoxy-G, 2'-deoxy-C, and deoxythymidine (dT = T = t) respectively. Underlining refers to 2'-OMe-substituted, e.g., U. The lower case letter f refers to 2'-deoxy-2'-fluoro substitution, e.g. fU is 2'-deoxy-2'-fluoro-U. N is A, C, G, U, U, a, c, g, u, t, or a modified, inverted, or chemically modified nucleotide.

[0059] Examples of RNAi molecules of this disclosure targeted to GST- π mRNA are shown in Table 4.

Table 4: RNAi molecule sequences for GST- π

ID	SEQ ID NO	SENSE STRAND (5'-->3') SEQ ID NOS:207 to 221	SEQ ID NO	ANTISENSE STRAND (5'-->3') SEQ ID NOS:222 to 236
B13'	207	GAUGACUAUGUGAAGGCACNN	222	GUGCCUUCACAUAGUCAUCNN
4	208	GGAUGACUAUGUGAAGGCA <u>UU</u>	223	UGCCUUCACAUAGUCAUCC <u>UU</u>
5	209	GGAUGACUAUGUGAAGGCA <u>UU</u>	224	ugccuUCAUAGUCAUCC <u>UU</u>
6	210	GGAUGACUAUGUGAAGGCA <u>UU</u>	225	UgccuUCAUAGUCAUCC <u>UU</u>
7	211	GGAUGACUAUGUGAAGGCA <u>UU</u>	226	UGccuUCAUAGUCAUCC <u>UU</u>
8	212	GGAUGACUAUGUGAAGGCA <u>UU</u>	227	UGCcuUCAUAGUCAUCC <u>UU</u>
9	213	GGAUGACUAUGUGAAGGCA <u>UU</u>	228	UGCCcuUCAUAGUCAUCC <u>UU</u>
10	214	GGAUGACUAUGUGAAGGCA <u>UU</u>	229	uGcCuUcACAUAGUCAUCC <u>UU</u>
11	215	GGAUGACUAUGUGAAGGCA <u>UU</u>	230	UGcCuUcACAUAGUCAUCC <u>UU</u>
12	216	GGAUGACUAUGUGAAGGCA <u>UU</u>	231	UgCcUuCaCAUAGUCAUCC <u>UU</u>
13	217	GGAUGACUAUGUGAAGGCA <u>UU</u>	232	UGCcUuCaCAUAGUCAUCC <u>UU</u>

(continued)

ID	SEQ ID NO	SENSE STRAND (5'-->3') SEQ ID NOS:207 to 221	SEQ ID NO	ANTISENSE STRAND (5'-->3') SEQ ID NOS:222 to 236
14	218	<u>GGAUGACUAUGUGAAGGCAUU</u>	233	UgccuUCAUAGUCAU <u>CCUU</u>
15	219	<u>GGAUGACUAUfGUfGAAGGCAUU</u>	234	UGCfCUUCACAUAGUCAU <u>CCUU</u>
17	220	<u>GGAUGACUAUGUGAAGGCAUU</u>	235	UGCCUUCACAUAGUCAU <u>CCUU</u>
18	221	<u>GGAUGACUAUGUGAAGGCAUU</u>	236	UGCCUUCACAUAGUCAU <u>CCUU</u>

[0060] Key for Table 4: Upper case A, G, C and U refer to ribo-A, ribo-G, ribo-C and ribo-U, respectively. The lower case letters a, u, g, c, t refer to 2'-deoxy-A, 2'-deoxy-U, 2'-deoxy-G, 2'-deoxy-C, and deoxythymidine (dT = T = t) respectively. Underlining refers to 2'-OMe-substituted, e.g., U. The lower case letter f refers to 2'-deoxy-2'-fluoro substitution, e.g. fU is 2'-deoxy-2'-fluoro-U. N is A, C, G, U, U, a, c, g, u, t, or a modified, inverted, or chemically modified nucleotide.

[0061] Examples of RNAi molecules of this disclosure targeted to GST- π mRNA are shown in Table 5.

Table 5: RNAi molecule sequences for GST- π

ID	SEQ ID NO	SENSE STRAND (5'-->3') SEQ ID NOS:237 to 248	SEQ ID NO	ANTISENSE STRAND (5'-->3') SEQ ID NOS:249 to 260
B2'	237	GAAGCCUUUUGAGACCCUGNN	249	CAGGGUCUCAAAAAGGCUUCNN
1	238	GAAGCCUUUUGAGACCCUG <u>UU</u>	250	CAGGGUCUCAAAAAGGCUUC <u>UU</u>
2	239	GAAGCCUUUUGAGACCCUG <u>UU</u>	251	cagggucuCAAAGGCUUC <u>UU</u>
3	240	GAAGCCUUUUGAGACCCUG <u>UU</u>	252	CagggucuCAAAGGCUUC <u>UU</u>
4	241	GAAGCCUUUUGAGACCCUG <u>UU</u>	253	CAGggucuCAAAGGCUUC <u>UU</u>
5	242	GAAGCCUUUUGAGACCCUG <u>UU</u>	254	CAGggucuCAAAGGCUUC <u>UU</u>
6	243	GAAGCCUUUUGAGACCCUG <u>UU</u>	255	CAGGgucuCAAAGGCUUC <u>UU</u>
7	244	GAAGCCUUUUGAGACCCUG <u>UU</u>	256	cAgGgUcUCAAAGGCUUC <u>UU</u>
8	245	GAAGCCUUUUGAGACCCUG <u>UU</u>	257	CAGgGucUCAAAGGCUUC <u>UU</u>
9	246	GAAGCCUUUUGAGACCCUG <u>UU</u>	258	CaGgGuCuCAAAGGCUUC <u>UU</u>
10	247	GAAGCCUUUUGAGACCCUG <u>UU</u>	259	CAGgGuCuCAAAGGCUUC <u>UU</u>
11	248	<u>GAAGCCUUUUGAGACCCUGUU</u>	260	CagggucuCAA <u>AAUGGCUUCUU</u>

[0062] Key for Table 5: Upper case A, G, C and U refer to ribo-A, ribo-G, ribo-C and ribo-U, respectively. The lower case letters a, u, g, c, t refer to 2'-deoxy-A, 2'-deoxy-U, 2'-deoxy-G, 2'-deoxy-C, and deoxythymidine (dT = T = t) respectively. Underlining refers to 2'-OMe-substituted, e.g., U. The lower case letter f refers to 2'-deoxy-2'-fluoro substitution, e.g. fU is 2'-deoxy-2'-fluoro-U. N is A, C, G, U, U, a, c, g, u, t, or a modified, inverted, or chemically modified nucleotide.

[0063] Examples of RNAi molecules of this disclosure targeted to GST- π mRNA are shown in Table 6.

Table 6: RNAi molecule sequences for GST- π

ID	SEQ ID NO	SENSE STRAND (5'-->3') SEQ ID NOS:261 to 272	SEQ ID NO	ANTISENSE STRAND (5'-->3') SEQ ID NOS:273 to 284
B4'	261	CCUCAUCUACACCAACUAUNN	273	AUAGUUGGUGUAGAUGAGGNN
1	262	CCUCAUCUACACCAACUA <u>UUU</u>	274	AUAGUUGGUGUAGAUGAG <u>GUU</u>

(continued)

ID	SEQ ID NO	SENSE STRAND (5'-->3') SEQ ID NOS:261 to 272	SEQ ID NO	ANTISENSE STRAND (5'-->3') SEQ ID NOS:273 to 284
2	263	CCUCAUCUACACCAACUAUUU	275	auaguuggUGUAGAUGAGGUU
3	264	CCUCAUCUACACCAACUAUUU	276	AuaguuggUGUAGAUGAGGUU
4	265	CCUCAUCUACACCAACUAUUU	277	AUaguuggUGUAGAUGAGGUU
5	266	CCUCAUCUACACCAACUAUUU	278	AUAguuggUGUAGAUGAGGUU
6	267	CCUCAUCUACACCAACUAUUU	279	AUAGuuggUGUAGAUGAGGUU
7	268	CCUCAUCUACACCAACUAUUU	280	aUaGuUgGUGUAGAUGAGGUU
8	269	CCUCAUCUACACCAACUAUUU	281	AUaGuUgGUGUAGAUGAGGUU
9	270	CCUCAUCUACACCAACUAUUU	282	AuAgUuGgUGUAGAUGAGGUU
10	271	CCUCAUCUACACCAACUAUUU	283	AUAgUuGgUGUAGAUGAGGUU
11	272	<u>CCUCAUCUACACCAACUAUUU</u>	284	AuaguuggUGUAGA <u>UGAGGUU</u>

[0064] Key for Table 6: Upper case A, G, C and U refer to ribo-A, ribo-G, ribo-C and ribo-U, respectively. The lower case letters a, u, g, c, t refer to 2'-deoxy-A, 2'-deoxy-U, 2'-deoxy-G, 2'-deoxy-C, and deoxythymidine (dT = T = t) respectively. Underlining refers to 2'-OMe-substituted, e.g., U. The lower case letter f refers to 2'-deoxy-2'-fluoro substitution, e.g. fU is 2'-deoxy-2'-fluoro-U. N is A, C, G, U, U, a, c, g, u, t, or a modified, inverted, or chemically modified nucleotide.

[0065] In some aspects, this disclosure provides a range of nucleic acid molecules, wherein: a) the molecule has a polynucleotide sense strand and a polynucleotide antisense strand; b) each strand of the molecule is from 15 to 30 nucleotides in length; c) a contiguous region of from 15 to 30 nucleotides of the antisense strand is complementary to a sequence of an mRNA encoding GST- π ; d) at least a portion of the sense strand is complementary to at least a portion of the antisense strand, and the molecule has a duplex region of from 15 to 30 nucleotides in length.

[0066] In some cases, the nucleic acid molecule can have contiguous region of from 15 to 30 nucleotides of the antisense strand that is complementary to a sequence of an mRNA encoding GST- π is located in the duplex region of the molecule.

[0067] In additional cases, the nucleic acid molecule can have a contiguous region of from 15 to 30 nucleotides of the antisense strand that is complementary to a sequence of an mRNA encoding GST- π .

[0068] In certain cases, each strand of the nucleic acid molecule can be from 18 to 22 nucleotides in length. The duplex region of the nucleic acid molecule can be 19 nucleotides in length.

[0069] In alternative forms, the nucleic acid molecule can have a polynucleotide sense strand and a polynucleotide antisense strand that are connected as a single strand, and form a duplex region connected at one end by a loop.

[0070] Some aspects of a nucleic acid molecule of this disclosure can have a blunt end. In certain cases, a nucleic acid molecule can have one or more 3' overhangs.

[0071] This disclosure provides a range of nucleic acid molecules that are RNAi molecules active for gene silencing. The disclosed nucleic acid molecules can be a dsRNA, a siRNA, a micro-RNA, or a shRNA active for gene silencing, as well as a DNA-directed RNA (ddRNA), Piwi-interacting RNA (piRNA), or a repeat associated siRNA (rasiRNA). The nucleic acid molecules can be active for inhibiting expression of GST- π .

[0072] Embodiments of this invention further provide nucleic acid molecules, as defined in the claims, having an IC50 for knockdown of GST- π of less than 100 pM.

[0073] Additional embodiments of this invention provide nucleic acid molecules, as defined in the claims, having an IC50 for knockdown of GST- π of less than 50 pM.

[0074] This invention further contemplates compositions containing one or more of the inventive nucleic acid molecules, as defined in the claims along with a pharmaceutically acceptable carrier. In certain embodiments, the carrier can be a lipid molecule or liposome.

[0075] The compounds and compositions of this invention, as defined in the claims, are useful in methods for preventing or treating a GST- π associated disease, by administering a compound or composition to a subject in need.

[0076] The methods of this disclosure can utilize the inventive compounds for preventing or treating malignant tumor. The malignant tumor can be presented in various diseases, for example, cancers associated with GST- π expression, cancers caused by cells expressing mutated KRAS, sarcomas, fibrosarcoma, malignant fibrous histiocytoma, liposar-

coma, rhabdomyosarcoma, leiomyosarcoma, angiosarcoma, Kaposi's sarcoma, lymphangiosarcoma, synovial sarcoma, chondrosarcoma, osteosarcoma, carcinomas, brain tumor, head and neck cancer, breast cancer, lung cancer, esophageal cancer, stomach cancer, duodenal cancer, appendix cancer, colorectal cancer, rectal cancer, liver cancer, pancreatic cancer, gallbladder cancer, bile duct cancer, anus cancer, kidney cancer, urethral cancer, urinary bladder cancer, prostate cancer, testicular cancer, uterine cancer, ovary cancer, skin cancer, leukemia, malignant lymphoma, epithelial malignant tumors, and non-epithelial malignant tumors.

Chemically modified siRNAs

[0077] Embodiments of this invention encompass siRNA molecules, as defined in the claims, that are chemically-modified to provide enhanced properties for therapeutic use, such as increased activity and potency for gene silencing. This invention provides chemically-modified siRNA molecules, as defined in the claims, that can have increased serum stability, as well as reduced off target effects, without loss of activity and potency of the siRNA molecules for gene modulation and gene silencing. In some aspects, this invention provides siRNAs, as defined in the claims, having chemical modifications in various combinations, which enhance the stability and efficacy of the siRNA.

[0078] In some embodiments, the siRNA molecules of this invention, as defined in the claims, can have passenger strand off target activity reduced by at least 10-fold, or at least 20-fold, or at least 30-fold, or at least 50-fold, or at least 100-fold.

[0079] As used herein, the terms modified and chemically-modified refer to changes made in the structure of a naturally-occurring nucleotide or nucleic acid structure of an siRNA, which encompasses siRNAs having one or more nucleotide analogs, altered nucleotides, non-standard nucleotides, non-naturally occurring nucleotides, and combinations thereof.

[0080] In some embodiments, the number of chemically-modified structures in an siRNA can include all of the structural components, and/or all of the nucleotides of the siRNA molecule.

[0081] Examples of modified and chemically-modified siRNAs include siRNAs having modification of the sugar group of a nucleotide, modification of a nucleobase of a nucleotide, modification of a nucleic acid backbone or linkage, modification of the structure of a nucleotide or nucleotides at the terminus of a siRNA strand, and combinations thereof.

[0082] Examples of modified and chemically-modified siRNAs include siRNAs having modification of the substituent at the 2' carbon of the sugar.

[0083] Examples of modified and chemically-modified siRNAs include siRNAs having modification at the 5' end, the 3' end, or at both ends of a strand.

[0084] Examples of modified and chemically-modified siRNAs include siRNAs having modifications that produce complementarity mismatches between the strands.

[0085] Examples of modified and chemically-modified siRNAs include siRNAs having a 5'-propylamine end, a 5'-phosphorylated end, a 5'-puromycin end, or a 3'-biotin end group.

[0086] Examples of modified and chemically-modified siRNAs include siRNAs having a 2'-fluoro substituted ribonucleotide, a 2'-OMe substituted ribonucleotide, a 2'-deoxy ribonucleotide, a 2'-amino substituted ribonucleotide, a 2'-thio substituted ribonucleotide.

[0087] Examples of modified and chemically-modified siRNAs include siRNAs having one or more 5-halouridines, 5-halocytidines, 5-methylcytidines, ribothymidines, 2-aminopurines, 2,6-diaminopurines, 4-thiouridines, or 5-aminoallyluridines.

[0088] Examples of modified and chemically-modified siRNAs include siRNAs having one or more phosphorothioate groups.

[0089] Examples of modified and chemically-modified siRNAs include siRNAs having one or more 2'-fluoro substituted ribonucleotides, 2'-fluorouridines, 2'-fluorocytidines, 2'-deoxyribonucleotides, 2'-deoxyadenosines, or 2'-deoxyguanosines.

[0090] Examples of modified and chemically-modified siRNAs include siRNAs having one or more phosphorothioate linkages.

[0091] Examples of modified and chemically-modified siRNAs include siRNAs having one or more alkylene diol linkages, oxy-alkylthio linkages, or oxycarbonyloxy linkages.

[0092] Examples of modified and chemically-modified siRNAs include siRNAs having one or more deoxyabasic groups, inosines, N3-methyl-uridines, N6,N6-dimethyl-adenosines, pseudouridines, purine ribonucleosides, and ribavirins.

[0093] Examples of modified and chemically-modified siRNAs include siRNAs having one or more 3' or 5' inverted terminal groups.

[0094] Examples of modified and chemically-modified siRNAs include siRNAs having one or more 5-(2-amino)propyluridines, 5-bromouridines, adenosines, 8-bromo guanosines, 7-deaza-adenosines, or N6-methyl adenosine.

Methods for modulating GST- π and treating malignant tumor

[0095] Embodiments of this invention can provide RNAi molecules, as defined in the claims, that can be used to down regulate or inhibit the expression of GST- π and/or GST- π proteins.

[0096] In some embodiments, a RNAi molecule of this invention, as defined in the claims, can be used to down regulate or inhibit the expression of GST- π and/or GST- π proteins arising from GST- π haplotype polymorphisms that may be associated with a disease or condition such as malignant tumor.

[0097] Monitoring of GST- π protein or mRNA levels can be used to characterize gene silencing, and to determine the efficacy of compounds and compositions of this invention.

[0098] The RNAi molecules of this disclosure can be used individually, or in combination with other siRNAs for modulating the expression of one or more genes.

[0099] The RNAi molecules of this disclosure can be used individually, or in combination, or in conjunction with other known drugs for preventing or treating diseases, or ameliorating symptoms of conditions or disorders associated with GST- π , including malignant tumor.

[0100] The RNAi molecules of this invention, as defined in the claims, can be used to modulate or inhibit the expression of GST- π in a sequence-specific manner.

[0101] The RNAi molecules of this disclosure can include a guide strand for which a series of contiguous nucleotides are at least partially complementary to a GST- π mRNA.

[0102] In certain aspects, malignant tumor may be treated by RNA interference using a RNAi molecule of this inventions, as defined in the claims

[0103] Treatment of malignant tumor may be characterized in suitable cell-based models, as well as ex vivo or in vivo animal models.

[0104] Treatment of malignant tumor may be characterized by determining the level of GST- π mRNA or the level of GST- π protein in cells of affected tissue.

[0105] Treatment of malignant tumor may be characterized by non-invasive medical scanning of an affected organ or tissue.

[0106] Embodiments of this invention may include pharmaceutical compositions for use in methods for preventing, treating, or ameliorating the symptoms of a GST- π associated disease or condition in a subject in need thereof.

[0107] In some cases methods for preventing, treating, or ameliorating the symptoms of malignant tumor in a subject can include administering to the subject a RNAi molecule of this invention to modulate the expression of a GST- π gene in the subject or organism.

[0108] In some aspects this disclosure contemplates methods for down regulating the expression of a GST- π gene in a cell or organism, by contacting the cell or organism with a RNAi molecule of this invention.

[0109] Aspects of this disclosure encompass siRNA molecules of Tables 1-6 that are modified or chemically-modified according to the examples above.

RNA Interference

[0110] RNA interference (RNAi) refers to sequence-specific post-transcriptional gene silencing in animals mediated by short interfering RNAs (siRNAs). See, e.g., Zamore et al., *Cell*, 2000, Vol. 101, pp. 25-33; Fire et al., *Nature*, 1998, Vol. 391, pp. 806811; Sharp, *Genes & Development*, 1999, Vol. 13, pp. 139-141.

[0111] An RNAi response in cells can be triggered by a double stranded RNA (dsRNA), although the mechanism is not yet fully understood. Certain dsRNAs in cells can undergo the action of Dicer enzyme, a ribonuclease III enzyme. See, e.g., Zamore et al., *Cell*, 2000, Vol. 101, pp. 25-33; Hammond et al., *Nature*, 2000, Vol. 404, pp. 293-296. Dicer can process the dsRNA into shorter pieces of dsRNA, which are siRNAs.

[0112] In general, siRNAs can be from about 21 to about 23 nucleotides in length and include a base pair duplex region about 19 nucleotides in length.

[0113] RNAi involves an endonuclease complex known as the RNA induced silencing complex (RISC). An siRNA has an antisense or guide strand which enters the RISC complex and mediates cleavage of a single stranded RNA target having a sequence complementary to the antisense strand of the siRNA duplex. The other strand of the siRNA is the passenger strand. Cleavage of the target RNA takes place in the middle of the region complementary to the antisense strand of the siRNA duplex See, e.g., Elbashir et al., *Genes & Development*, 2001, Vol 15, pp. 188-200.

[0114] As used herein, the term "sense strand" refers to a nucleotide sequence of a siRNA molecule that is partially or fully complementary to at least a portion of a corresponding antisense strand of the siRNA molecule. The sense strand of a siRNA molecule can include a nucleic acid sequence having homology with a target nucleic acid sequence.

[0115] As used herein, the term "antisense strand" refers to a nucleotide sequence of a siRNA molecule that is partially or fully complementary to at least a portion of a target nucleic acid sequence. The antisense strand of a siRNA molecule can include a nucleic acid sequence that is complementary to at least a portion of a corresponding sense strand of the

siRNA molecule.

[0116] RNAi molecules can down regulate or knock down gene expression by mediating RNA interference in a sequence-specific manner. See, e.g., Zamore et al., *Cell*, 2000, Vol. 101, pp. 25-33; Elbashir et al, *Nature*, 2001, Vol. 411, pp. 494-498; Kreutzer et al., WO2000/044895; Zemicka-Goetz et al., WO2001/36646; Fire et al., WO1999/032619; Plaetinck et al., WO2000/01846; Mello et al., WO2001/029058.

[0117] As used herein, the terms "inhibit," "down-regulate," or "reduce" with respect to gene expression means that the expression of the gene, or the level of mRNA molecules encoding one or more proteins, or the activity of one or more of the encoded proteins is reduced below that observed in the absence of a RNAi molecule or siRNA of this invention. For example, the level of expression, level of mRNA, or level of encoded protein activity may be reduced by at least 1%, or at least 10%, or at least 20%, or at least 50%, or at least 90%, or more from that observed in the absence of a RNAi molecule or siRNA of this invention.

[0118] RNAi molecules can also be used to knock down viral gene expression, and therefore affect viral replication.

[0119] RNAi molecules can be made from separate polynucleotide strands: a sense strand or passenger strand, and an antisense strand or guide strand. The guide and passenger strands are at least partially complementary. The guide strand and passenger strand can form a duplex region having from about 15 to about 49 base pairs.

[0120] In some cases the duplex region of a siRNA can have 17, 18, 19, 20, 21, 22, 23, 24, 25, 26, 27, 28, 29, 30, 31, 32, 33, 34, 35, 36, 37, 38, 39, 40, 41, 42, 43, 44, 45, 46, 47, 48, or 49 base pairs.

[0121] In certain embodiments, a RNAi molecule can be active in a RISC complex, with a length of duplex region active for RISC.

[0122] In additional embodiments, a RNAi molecule can be active as a Dicer substrate, to be converted to a RNAi molecule that can be active in a RISC complex.

[0123] In some aspects, a RNAi molecule can have complementary guide and passenger sequence portions at opposing ends of a long molecule, so that the molecule can form a duplex region with the complementary sequence portions, and the strands are linked at one end of the duplex region by either nucleotide or non-nucleotide linkers. For example, a hairpin arrangement, or a stem and loop arrangement. The linker interactions with the strands can be covalent bonds or non-covalent interactions.

[0124] A RNAi molecule of this disclosure may include a nucleotide, non-nucleotide, or mixed nucleotide/non-nucleotide linker that joins the sense region of the nucleic acid to the antisense region of the nucleic acid. A nucleotide linker can be a linker of ≥ 2 nucleotides in length, for example about 3, 4, 5, 6, 7, 8, 9, or 10 nucleotides in length. The nucleotide linker can be a nucleic acid aptamer. By "aptamer" or "nucleic acid aptamer" as used herein refers to a nucleic acid molecule that binds specifically to a target molecule wherein the nucleic acid molecule has sequence that includes a sequence recognized by the target molecule in its natural setting. Alternately, an aptamer can be a nucleic acid molecule that binds to a target molecule, where the target molecule does not naturally bind to a nucleic acid. For example, the aptamer can be used to bind to a ligand-binding domain of a protein, thereby preventing interaction of the naturally occurring ligand with the protein. See, e.g., Gold et al., *Annu Rev Biochem*, 1995, Vol. 64, pp. 763-797; Brody et al., *J. Biotechnol.*, 2000, Vol. 74, pp. 5-13; Hermann et al., *Science*, 2000, Vol. 287, pp. 820-825.

[0125] Examples of a non-nucleotide linker include an abasic nucleotide, polyether, polyamine, polyamide, peptide, carbohydrate, lipid, polyhydrocarbon, or other polymeric compounds, for example polyethylene glycols such as those having from 2 to 100 ethylene glycol units. Some examples are described in Seela et al., *Nucleic Acids Research*, 1987, Vol. 15, pp. 3113-3129; Cload et al., *J. Am. Chem. Soc.*, 1991, Vol. 113, pp. 6324-6326; Jaeschke et al., *Tetrahedron Lett.*, 1993, Vol. 34, pp. 301; Arnold et al., WO1989/002439; Usman et al., WO1995/006731; Dudycz et al., WO1995/011910, and Ferentz et al., *J. Am. Chem. Soc.*, 1991, Vol. 113, pp. 4000-4002.

[0126] A RNAi molecule can have one or more overhangs from the duplex region. The overhangs, which are non-base-paired, single strand regions, can be from one to eight nucleotides in length, or longer. An overhang can be a 3'-end overhang, wherein the 3'-end of a strand has a single strand region of from one to eight nucleotides. An overhang can be a 5'-end overhang, wherein the 5'-end of a strand has a single strand region of from one to eight nucleotides.

[0127] The overhangs of a RNAi molecule can have the same length, or can be different lengths.

[0128] A RNAi molecule can have one or more blunt ends, in which the duplex region ends with no overhang, and the strands are base paired to the end of the duplex region.

[0129] A RNAi molecule of this disclosure can have one or more blunt ends, or can have one or more overhangs, or can have a combination of a blunt end and an overhang end.

[0130] A 5'-end of a strand of a RNAi molecule may be in a blunt end, or can be in an overhang. A 3'-end of a strand of a RNAi molecule may be in a blunt end, or can be in an overhang.

[0131] A 5'-end of a strand of a RNAi molecule may be in a blunt end, while the 3'-end is in an overhang. A 3'-end of a strand of a RNAi molecule may be in a blunt end, while the 5'-end is in an overhang.

[0132] In some cases both ends of a RNAi molecule are blunt ends.

[0133] In an embodiment, both ends of a RNAi molecule have an overhang.

[0134] The overhangs at the 5'- and 3'-ends may be of different lengths.

[0135] In certain cases, a RNAi molecule may have a blunt end where the 5'-end of the anti sense strand and the 3'-end of the sense strand do not have any overhanging nucleotides.

[0136] In further cases, a RNAi molecule may have a blunt end where the 3'-end of the antisense strand and the 5'-end of the sense strand do not have any overhanging nucleotides.

[0137] A RNAi molecule may have mismatches in base pairing in the duplex region.

[0138] Any nucleotide in an overhang of a RNAi molecule can be a deoxyribonucleotide, or a ribonucleotide.

[0139] One or more deoxyribonucleotides may be at the 5'-end, where the 3'-end of the other strand of the RNAi molecule may not have an overhang, or may not have a deoxyribonucleotide overhang.

[0140] One or more deoxyribonucleotides may be at the 3'-end, where the 5'-end of the other strand of the RNAi molecule may not have an overhang, or may not have a deoxyribonucleotide overhang.

[0141] In some embodiments, one or more, or all of the overhang nucleotides of a RNAi molecule, as defined in these claims, may be 2'-deoxyribonucleotides.

Dicer Substrate RNAi Molecules

[0142] In some aspects, a RNAi molecule can be of a length suitable as a Dicer substrate, which can be processed to produce a RISC active RNAi molecule. See, e.g., Rossi et al., US2005/0244858.

[0143] A double stranded RNA (dsRNA) that is a Dicer substrate can be of a length sufficient such that it is processed by Dicer to produce an active RNAi molecule, and may further include one or more of the following properties: (i) the Dicer substrate dsRNA can be asymmetric, for example, having a 3' overhang on the antisense strand, and (ii) the Dicer substrate dsRNA can have a modified 3' end on the sense strand to direct orientation of Dicer binding and processing of the dsRNA to an active RNAi molecule.

[0144] In certain cases, the longest strand in a Dicer substrate dsRNA may be 24-30 nucleotides in length.

[0145] A Dicer substrate dsRNA can be symmetric or asymmetric.

[0146] In some cases, a Dicer substrate dsRNA can have a sense strand of 22-28 nucleotides and an antisense strand of 24-30 nucleotides.

[0147] In certain embodiments, a Dicer substrate dsRNA may have an overhang on the 3' end of the antisense strand.

[0148] In further cases, a Dicer substrate dsRNA may have a sense strand 25 nucleotides in length, and an antisense strand 27 nucleotides in length, with a 2 base 3'-overhang. The overhang may be 1, 2 or 3 nucleotides in length. The sense strand may also have a 5' phosphate.

[0149] An asymmetric Dicer substrate dsRNA may have two deoxyribonucleotides at the 3'-end of the sense strand in place of two of the ribonucleotides.

[0150] The sense strand of a Dicer substrate dsRNA may be from about 22 to about 30, or from about 22 to about 28; or from about 24 to about 30; or from about 25 to about 30; or from about 26 to about 30; or from about 26 and 29; or from about 27 to about 28 nucleotides in length.

[0151] The sense strand of a Dicer substrate dsRNA may be 22, 23, 24, 25, 26, 27, 28, 29 or 30 nucleotides in length.

[0152] In certain cases, a Dicer substrate dsRNA may have sense and antisense strands that are at least about 25 nucleotides in length, and no longer than about 30 nucleotides in length.

[0153] In certain cases, a Dicer substrate dsRNA may have sense and antisense strands that are 26 to 29 nucleotides in length.

[0154] In certain cases, a Dicer substrate dsRNA may have sense and antisense strands that are 27 nucleotides in length.

[0155] The sense and antisense strands of a Dicer substrate dsRNA may be the same length as in being blunt ended, or different lengths as in having overhangs, or may have a blunt end and an overhang.

[0156] A Dicer substrate dsRNA may have a duplex region of 19, 20, 21, 22, 23, 24, 25, 26 or 27 nucleotides in length.

[0157] The antisense strand of a Dicer substrate dsRNA may have any sequence that anneals to at least a portion of the sequence of the sense strand under biological conditions, such as within the cytoplasm of a eukaryotic cell.

[0158] A Dicer substrate with a sense and an antisense strand can be linked by a third structure, such as a linker group or a linker oligonucleotide. The linker connects the two strands of the dsRNA, for example, so that a hairpin is formed upon annealing.

[0159] The sense and antisense strands of a Dicer substrate are in general complementary, but may have mismatches in base pairing.

[0160] In some cases, a Dicer substrate dsRNA can be asymmetric such that the sense strand has 22-28 nucleotides and the antisense strand has 24-30 nucleotides.

[0161] A region of one of the strands, particularly the antisense strand, of the Dicer substrate dsRNA may have a sequence length of at least 19 nucleotides, wherein these nucleotides are in the 21-nucleotide region adjacent to the 3' end of the antisense strand and are sufficiently complementary to a nucleotide sequence of the RNA produced from the target gene.

[0162] An antisense strand of a Dicer substrate dsRNA can have from 1 to 9 ribonucleotides on the 5'-end, to give a length of 22-28 nucleotides. When the antisense strand has a length of 21 nucleotides, then 1-7 ribonucleotides, or 2-5 ribonucleotides, or 4 ribonucleotides may be added on the 3'-end. The added ribonucleotides may have any sequence.

[0163] A sense strand of a Dicer substrate dsRNA may have 24-30 nucleotides. The sense strand may be substantially complementary with the antisense strand to anneal to the antisense strand under biological conditions.

Methods for using RNAi molecules

[0164] The nucleic acid molecules and RNAi molecules of this disclosure may be delivered to a cell or tissue by direct application of the molecules, or with the molecules combined with a carrier or a diluent.

[0165] The nucleic acid molecules and RNAi molecules of this invention, as defined in these claims, can be delivered or administered to a cell, tissue, organ, or subject by direct application of the molecules with a carrier or diluent, or any other delivery vehicle that acts to assist, promote or facilitate entry into a cell, for example, viral sequences, viral material, or lipid or liposome formulations.

[0166] The nucleic acid molecules and RNAi molecules of this invention, as defined in the claims, can be complexed with cationic lipids, packaged within liposomes, or otherwise delivered to target cells or tissues. The nucleic acid or nucleic acid complexes can be locally administered to relevant tissues *ex vivo*, or *in vivo* through direct dermal application, transdermal application, or injection.

[0167] Delivery systems may include, for example, aqueous and nonaqueous gels, creams, emulsions, microemulsions, liposomes, ointments, aqueous and nonaqueous solutions, lotions, aerosols, hydrocarbon bases and powders, and can contain excipients such as solubilizers and permeation enhancers.

[0168] Compositions and methods of this disclosure can include an expression vector that includes a nucleic acid sequence encoding at least one RNAi molecule of this invention in a manner that allows expression of the nucleic acid molecule.

[0169] The nucleic acid molecules and RNAi molecules of this invention, as defined in the claims, can be expressed from transcription units inserted into DNA or RNA vectors. Recombinant vectors can be DNA plasmids or viral vectors. Viral vectors can be used that provide for transient expression of nucleic acid molecules.

[0170] For example, the vector may contain sequences encoding both strands of a RNAi molecule of a duplex, or a single nucleic acid molecule that is self-complementary and thus forms a RNAi molecule. An expression vector may include a nucleic acid sequence encoding two or more nucleic acid molecules.

[0171] A nucleic acid molecule may be expressed within cells from eukaryotic promoters. Those skilled in the art realize that any nucleic acid can be expressed in eukaryotic cells from the appropriate DNA/RNA vector.

[0172] In some aspects, a viral construct can be used to introduce an expression construct into a cell, for transcription of a dsRNA construct encoded by the expression construct.

[0173] Lipid formulations can be administered to animals by intravenous, intramuscular, or intraperitoneal injection, or orally or by inhalation or other methods as are known in the art.

[0174] Pharmaceutically acceptable formulations for administering oligonucleotides are known and can be used.

Example protocol for *in vitro* knockdown

[0175] One day before the transfection, cells were plated in a 96-well plate at 2×10^3 cells per well with 100 μ l of DMEM (HyClone Cat. # SH30243.01) containing 10% FBS and culture in a 37°C incubator containing a humidified atmosphere of 5% CO₂ in air. Before transfection, medium was changed to 90 μ l of Opti-MEM I Reduced Serum Medium (Life Technologies Cat. # 31985-070) containing 2% FBS. Then, 0.2 μ l of Lipofectamine RNAiMax (Life Technologies Cat. # 13778-100) was mixed with 4.8 μ l of Opti-MEM I for 5 minutes at room temperature. Next, 1 μ l of siRNA was mixed with 4 μ l of Opti-MEM I and combined with the LF2000 solution, and mixed gently, without vortex. After 5 minutes at room temperature, the mixture was incubated for an additional 10 minutes at room temperature to allow the RNA-RNAiMax complexes to form. Further, the 10 μ l of RNA-RNAiMax complexes was added to a well, and the plate was shaken gently by hand. The cells were incubated in a 37°C incubator containing a humidified atmosphere of 5% CO₂ in air for 2 hours. The medium was changed to fresh Opti-MEM I Reduced Serum Medium containing 2% FBS. 24 hours after transfection, the cells were washed with ice-cold PBS once. The cells were lysed with 50 μ l of Cell-to-Ct Lysis Buffer (Life Technologies Cat. # 4391851 C) for 5-30 minutes at room temperature. 5 μ l of Stop Solution was added, and it was incubated for 2 minutes at room temperature. The mRNA level was measured by RT-qPCR with TAQMAN immediately. Samples could be frozen at -80 °C and assayed at a later time.

Example protocol for serum stability

[0176] 0.2 mg/ml siRNA was incubated with 10% human serum at 37 °C. At certain time points (0, 5, 15 and 30 min),

EP 3 236 974 B9

200 μ l of sample was aliquoted and extracted with 200 μ l extraction solvent (Chloroform: phenol: Isoamyl alcohol =24:25:1). The sample was vortexed and centrifuged at 13,000 rpm for 10 min at RT, then the top layer solution was transferred and filtered it with 0.45 μ m filter. The filtrate was transferred into a 300 μ l HPLC injection vial. For LCMS, the Mobile phase was MPA: 100 mM HFIP + 7 mM TEA in H₂O, MPB: 50 % Methanol + 50% Acetonitrile. The Column: Waters Acquity OST 2.1 x 50 mm, 1.7 μ m.

EXAMPLES

[0177] Example 1: siRNAs of this disclosure targeted to GST- π were found to be active for gene silencing in vitro. The dose-dependent activities of GST- π siRNAs for gene knockdown were found to exhibit an IC₅₀ below about 250 picomolar (pM), and as low as 1 pM.

[0178] In vitro transfection was performed in an A549 cell line to determine siRNA knockdown efficacy. Dose dependent knockdown for GST- π mRNA was observed with siRNAs of Table 1, as shown in Table 7.

Table 7: Dose dependent knockdown for GST- π mRNA in an A549 cell line

siRNA structure	IC ₅₀ (pM)
A9 (SEQ ID NOs:25 and 90)	24
B2 (SEQ ID NOs:52 and 117)	121
B3 (SEQ ID NOs:53 and 118)	235
B4 (SEQ ID NOs:54 and 119)	229
B13 (SEQ ID NOs:50 and 115)	17
BU2 (SEQ ID NOs:61 and 126)	31

[0179] As shown in Table 7, the activities of GST- π siRNAs of Table 1 were in the range 17-235 pM, which is suitable for many uses, including as a drug agent to be used in vivo.

[0180] Example 2: The structure of GST- π siRNAs of this disclosure having deoxynucleotides located in the seed region of the antisense strand of the siRNA provided unexpectedly and advantageously increased gene knockdown activity in vitro.

[0181] In vitro transfection was performed in an A549 cell line to determine knockdown efficacy for GST- π siRNAs based on structure BU2' (SEQ ID NOs: 131 and 157). Dose dependent knockdown of GST- π mRNA was observed with GST- π siRNAs based on structure BU2' as shown in Table 8.

Table 8: Dose dependent knockdown of GST- π mRNA in an A549 cell line for GST- π siRNAs based on structure BU2'

GST- π siRNA structure	IC ₅₀ (pM)
BU2 with no deoxynucleotides in the duplex region (SEQ ID NOs:61 and 126)	31
BU2 with deoxynucleotides in positions 3, 5, and 7 of the seed region antisense strand (SEQ ID NOs:139 and 165)	5
BU2 with deoxynucleotides in positions 4, 6, and 8 of the seed region antisense strand (SEQ ID NOs: 141 and 167)	8
BU2 with deoxynucleotides in positions 4, 6, and 8 of the seed region antisense strand (SEQ ID NOs:156 and 182)	5

[0182] As shown in Table 8, the activities of GST- π siRNAs based on structure BU2' having three deoxynucleotides in the seed region of the antisense strand were surprisingly and unexpectedly increased by up to 6-fold, as compared to a GST- π siRNA without deoxynucleotides in the duplex region.

[0183] These data show that GST- π siRNAs having a structure with three deoxynucleotides located at positions 3, 5 and 7, or at positions 4, 6 and 8 in the seed region of the antisense strand provided surprisingly increased gene knockdown activity as compared to a GST- π siRNA without deoxynucleotides in the duplex region.

[0184] The activities shown in Table 8 for GST- π siRNAs having three deoxynucleotides in the seed region of the antisense strand were in the range 5 to 8 pM, which is exceptionally suitable for many uses, including as a drug agent to be used in vivo.

[0185] Example 3: The structure of GST- π siRNAs of this disclosure having deoxynucleotides located in the seed region of the antisense strand of the siRNA provided unexpectedly and advantageously increased gene knockdown activity in vitro.

[0186] In vitro transfection was performed in an A549 cell line to determine knockdown efficacy for GST- π siRNAs based on structure A9' (SEQ ID NOs:183 and 195). Dose dependent knockdown of GST- π mRNA was observed with the GST- π siRNAs based on structure A9', as shown in Table 9.

Table 9: Dose dependent knockdown of GST- π mRNA in an A549 cell line for GST- π siRNAs based on structure A9'

GST- π siRNA structure	IC50 (pM)
A9 with no deoxynucleotides in the duplex region (SEQ ID NOs:25 and 90)	24
A9 with deoxynucleotides in positions 4, 6, and 8 of the seed region antisense strand (SEQ ID NOs:193 and 205)	1
A9 with deoxynucleotides in positions 1, 3, 5, and 7 of the seed region antisense strand (SEQ ID NOs:190 and 202)	5
A9 with deoxynucleotides in positions 3-8 of the seed region antisense strand (SEQ ID NOs:187 and 199)	6
A9 with deoxynucleotides in positions 5-8 of the seed region antisense strand (SEQ ID NOs:189 and 201)	7
A9 with deoxynucleotides in positions 3, 5, and 7 of the seed region antisense strand (SEQ ID NOs: 191 and 203)	15

[0187] As shown in Table 9, the activities of GST- π siRNAs based on structure A9' having three to six deoxynucleotides in the seed region of the antisense strand were surprisingly increased by up to 24-fold, as compared to a GST- π siRNA without deoxynucleotides in the duplex region.

[0188] These data show that GST- π siRNAs having a structure with three to six deoxynucleotides located at positions 4, 6 and 8, or at positions 1, 3, 5 and 7, or at positions 3-8, or at positions 5-8, or at positions 3, 5 and 7 in the seed region of the antisense strand provided unexpectedly increased gene knockdown activity as compared to a GST- π siRNA without deoxynucleotides in the duplex region.

[0189] The activity shown in Table 9 for GST- π siRNAs having three to six deoxynucleotides in the seed region of the antisense strand was in the range 1 to 15 pM, which is exceptionally suitable for many uses, including as a drug agent to be used in vivo.

[0190] Example 4: The structure of GST- π siRNAs having deoxynucleotides located in the seed region of the antisense strand of the siRNA provided unexpectedly and advantageously increased gene knockdown activity in vitro.

[0191] In vitro transfection was performed in an A549 cell line to determine knockdown efficacy for GST- π siRNAs based on structure B13' (SEQ ID NOs:207 and 222). Dose dependent knockdown of GST- π mRNA was observed with the GST- π siRNAs based on structure B13', as shown in Table 10.

Table 10: Dose dependent knockdown of GST- π mRNA in an A549 cell line for GST- π siRNAs based on structure B13'

GST- π siRNA structure	IC50 (pM)
B13 with no deoxynucleotides in the duplex region (SEQ ID NOs:50 and 115)	17
B13 with deoxynucleotides in positions 4, 6, and 8 of the seed region antisense strand (SEQ ID NOs:217 and 232)	11

[0192] As shown in Table 10, the activity of a GST- π siRNA based on structure B13' having three deoxynucleotides in the seed region of the antisense strand was unexpectedly increased, as compared to a GST- π siRNA without deoxynucleotides in the duplex region.

[0193] These data show that GST- π siRNAs having a structure with three deoxynucleotides located at positions 4, 6 and 8 in the seed region of the antisense strand provided unexpectedly increased gene knockdown activity as compared to a GST- π siRNA without deoxynucleotides in the duplex region.

[0194] The activity shown in Table 10 for GST- π siRNAs having three deoxynucleotides in the seed region of the antisense strand was in the picomolar range at 11 pM, which is exceptionally suitable for many uses, including as a drug

agent to be used in vivo.

[0195] Example 5: The structure of GST- π siRNAs having deoxynucleotides located in the seed region of the antisense strand of the siRNA provided unexpectedly and advantageously increased gene knockdown activity in vitro.

[0196] In vitro transfection was performed in an A549 cell line to determine knockdown efficacy for GST- π siRNAs based on structure B4' (SEQ ID NOs:261 and 273). Dose dependent knockdown of GST- π mRNA was observed with the GST- π siRNAs based on structure B4', as shown in Table 11.

Table 11: Dose dependent knockdown of GST- π mRNA in an A549 cell line for GST- π siRNAs based on structure B4'

GST- π siRNA structure	IC50 (pM)
B4 with no deoxynucleotides in the duplex region (SEQ ID NOs:54 and 119)	229
B4 with deoxynucleotides in positions 3-8 of the seed region antisense strand (SEQ ID NOs:265 and 277)	113

[0197] As shown in Table 11, the activities of GST- π siRNAs based on structure B4' having six deoxynucleotides in the seed region of the antisense strand were unexpectedly increased by more than two-fold, as compared to a GST- π siRNA without deoxynucleotides in the duplex region.

[0198] These data show that GST- π siRNAs having a structure with six deoxynucleotides located at positions 3-8 in the seed region of the antisense strand provided surprisingly increased gene knockdown activity as compared to a GST- π siRNA without deoxynucleotides in the duplex region.

[0199] The activity shown in Table 11 for a GST- π siRNA having six deoxynucleotides in the seed region of the antisense strand was in the picomolar range at 113 pM, which is exceptionally suitable for many uses, including as a drug agent to be used in vivo.

[0200] Example 6: The structure of GST- π siRNAs having deoxynucleotides located in the seed region of the antisense strand of the siRNA provided unexpectedly and advantageously increased gene knockdown activity in vitro.

[0201] In vitro transfection was performed in an A549 cell line to determine knockdown efficacy for GST- π siRNAs based on structure B2' (SEQ ID NOs:237 and 249). Dose dependent knockdown of GST- π mRNA was observed with the GST- π siRNAs based on structure B2', as shown in Table 12.

Table 12: Dose dependent knockdown of GST- π mRNA in an A549 cell line for GST- π siRNAs based on structure B2'

GST- π siRNA structure	IC50 (pM)
B2 with no deoxynucleotides in the duplex region (SEQ ID NOs:52 and 117)	121
B2 with deoxynucleotides in positions 5-8 of the seed region antisense strand (SEQ ID NOs:243 and 255)	30
B2 with deoxynucleotides in positions 1, 3, 5, and 7 of the seed region antisense strand (SEQ ID NOs:244 and 256)	50
B2 with deoxynucleotides in positions 3, 5, and 7 of the seed region antisense strand (SEQ ID NOs:245 and 257)	100

[0202] As shown in Table 12, the activities of GST- π siRNAs based on structure B2' having three to four deoxynucleotides in the seed region of the antisense strand were surprisingly increased by up to 4-fold, as compared to a GST- π siRNA without deoxynucleotides in the duplex region.

[0203] These data show that GST- π siRNAs having a structure with three to four deoxynucleotides located at positions 5-8, or at positions 1, 3, 5 and 7, or at positions 3, 5 and 7 in the seed region of the antisense strand provided unexpectedly increased gene knockdown activity as compared to a GST- π siRNA without deoxynucleotides in the duplex region.

[0204] The activities shown in Table 12 for GST- π siRNAs having three to four deoxynucleotides in the seed region of the antisense strand were in the range 30-100 pM, which is exceptionally suitable for many uses, including as a drug agent to be used in vivo.

[0205] Example 7: The structure of GST- π siRNAs containing one or more 2'-deoxy-2'-fluoro substituted nucleotides provided unexpectedly increased gene knockdown activity in vitro.

[0206] In vitro transfection was performed in an A549 cell line to determine knockdown efficacy for GST- π siRNAs based on structure BU2' (SEQ ID NOs:131 and 157). Dose dependent knockdown of GST- π mRNA was observed with the GST- π siRNAs based on structure BU2', as shown in Table 13.

Table 13: Dose dependent knockdown of GST- π mRNA in an A549 cell line for GST- π siRNAs based on structure BU2'

GST- π siRNA structure	IC50 (pM)
BU2 with no 2'-F deoxynucleotides (SEQ ID NOs:61 and 126)	31
BU2 with seven 2'-F deoxynucleotides, one in position 1 at the 3' end of the antisense strand (SEQ ID NOs:148 and 174)	3
BU2 with four 2'-F deoxynucleotides, one in position 1 at the 3' end of the antisense strand (SEQ ID NOs:147 and 173)	11
BU2 with one 2'-F deoxynucleotide in position 1 at the 3' end of the antisense strand (SEQ ID NOs:144 and 170)	13

[0207] As shown in Table 13, the activities of GST- π siRNAs based on structure BU2' having one or more 2'-F deoxynucleotides were surprisingly increased by up to 10-fold, as compared to a GST- π siRNA without 2'-F deoxynucleotides.

[0208] These data show that GST- π siRNAs having a structure with one or more 2'-F deoxynucleotides provided unexpectedly increased gene knockdown activity as compared to a GST- π siRNA without a 2'-F deoxynucleotide.

[0209] The activities shown in Table 13 for GST- π siRNAs having one or more 2'-F deoxynucleotides were in the range 3 to 13 pM, which is exceptionally suitable for many uses, including as a drug agent to be used in vivo.

[0210] **Example 8:** The structure of GST- π siRNAs containing one or more 2'-deoxy-2'-fluoro substituted nucleotides provided unexpectedly increased gene knockdown activity in vitro.

[0211] In vitro transfection was performed in an A549 cell line to determine knockdown efficacy for GST- π siRNAs based on structure B13' (SEQ ID NOs:207 and 222). Dose dependent knockdown of GST- π mRNA was observed with the GST- π siRNAs based on structure B13', as shown in Table 14.

Table 14: Dose dependent knockdown of GST- π mRNA in an A549 cell line for GST- π siRNAs based on structure B13'

GST- π siRNA structure	IC50 (pM)
B13 with no 2'-F deoxynucleotides (SEQ ID NOs:50 and 115)	17
B13 with three 2'-F deoxynucleotides located in non-overhang positions (SEQ ID NOs:219 and 234)	6

[0212] As shown in Table 14, the activity of a GST- π siRNA based on structure B13' having three 2'-F deoxynucleotides located in non-overhang positions was surprisingly increased by about 3-fold, as compared to a GST- π siRNA without 2'-F deoxynucleotides.

[0213] These data show that GST- π siRNAs having a structure with one or more 2'-F deoxynucleotides provided unexpectedly increased gene knockdown activity as compared to a GST- π siRNA without a 2'-F deoxynucleotide.

[0214] The activity shown in Table 14 for GST- π siRNAs having one or more 2'-F deoxynucleotides was in the picomolar range at 6 pM, which is exceptionally suitable for many uses, including as a drug agent to be used in vivo.

[0215] **Example 9: Orthotopic A549 lung cancer mouse model.** The GST- π siRNAs of this disclosure can exhibit profound reduction of orthotopic lung cancer tumors in vivo. In this example, a GST- π siRNA provided gene knockdown potency in vivo when administered in a liposomal formulation to the orthotopic lung cancer tumors in athymic nude mice.

[0216] In general, an orthotopic tumor model can exhibit direct clinical relevance for drug efficacy and potency, as well as improved predictive ability. In the orthotopic tumor model, tumor cells are implanted directly into the same kind of organ from which the cells originated.

[0217] The anti-tumor efficacy of the siRNA formulation against human lung cancer A549 was evaluated by comparing the final primary tumor weights measured at necropsy for the treatment group and the vehicle control group.

[0218] Fig. 1 shows orthotopic lung cancer tumor inhibition in vivo for a GST- π siRNA based on structure BU2 (SEQ ID NOs:61 and 126). An orthotopic A549 lung cancer mouse model was utilized with a relatively low dose at 2 mg/kg of the siRNA targeted to GST- π .

[0219] The GST- π siRNA showed significant and unexpectedly advantageous lung tumor inhibition efficacy in this six-week study. As shown in Fig. 1, after 43 days, the GST- π siRNA showed markedly advantageous tumor inhibition efficacy, with final tumor average weights significantly reduced by 2.8-fold as compared to control.

[0220] For this study, male NCr nu/nu mice, 5-6 weeks old, were used. The experimental animals were maintained in a HEPA filtered environment during the experimental period. The siRNA formulations were stored at 4° C before use, and warmed to room temperature 10 minutes prior to injection in mouse.

[0221] For this A549 human lung cancer orthotopic model, on the day of surgical orthotopic implantation (SOI), the stock tumors were harvested from the subcutaneous site of animals bearing A549 tumor xenograft and placed in RPMI-1640 medium. Necrotic tissues were removed and viable tissues were cut into 1.5-2 mm³ pieces. The animals were anesthetized with isoflurane inhalation and the surgical area was sterilized with iodine and alcohol. A transverse incision approximately 1.5 cm long was made in the left chest wall of the mouse using a pair of surgical scissors. An intercostal incision was made between the third and the fourth rib and the left lung was exposed. One A549 tumor fragment was transplanted to the surface of the lung with an 8-0 surgical suture (nylon). The chest wall was closed with a 6-0 surgical suture (silk). The lung was re-inflated by intrathoracic puncture using a 3 cc syringe with a 25 G X 1 ½ needle to draw out the remaining air in the chest cavity. The chest wall was closed with a 6-0 surgical silk suture. All procedures of the operation described above were performed with a 7 x magnification microscope under HEPA filtered laminar flow hoods.

[0222] Three days after tumor implantation, the model tumor-bearing mice were randomly divided into groups of ten mice per group. For the group of interest, treatment of the ten mice was initiated three days after tumor implantation.

[0223] For the group of interest, the formulation was (Ionizable lipid:cholesterol:DOPE:DOPC:DPPE-PEG-2K:DSPE-PEG-2K), a liposomal composition. The liposomes encapsulated the GST- π siRNA.

[0224] For the study endpoint, the experimental mice were sacrificed forty-two days after treatment initiation. Primary tumors were excised and weighed on an electronic balance for subsequent analysis.

[0225] For an estimation of compound toxicity, the mean body weight of the mice in the treated and control groups was maintained within the normal range during the entire experimental period. Other symptoms of toxicity were not observed in the mice.

[0226] Example 10: The GST- π siRNAs of this disclosure exhibited profound reduction of cancer xenograft tumors in vivo. The GST- π siRNAs provided gene knockdown potency in vivo when administered in a liposomal formulation to the cancer xenograft tumors.

[0227] Fig. 2 shows tumor inhibition efficacy for a GST- π siRNA (SEQ ID Nos:156 and 182). A cancer xenograft model was utilized with a relatively low dose at 0.75 mg/kg of siRNA targeted to GST- π .

[0228] The GST- π siRNA showed significant and unexpectedly advantageous tumor inhibition efficacy within a few days after administration. After 36 days, the GST- π siRNA showed markedly advantageous tumor inhibition efficacy, with tumor volume reduced by 2-fold as compared to control.

[0229] As shown in Fig. 3, the GST- π siRNA demonstrated significant and unexpectedly advantageous tumor inhibition efficacy at the endpoint day. In particular, tumor weight was reduced by more than 2-fold.

[0230] The GST- π siRNA was administered in two injections (day 1 and 15) of a liposomal formulation having the composition (Ionizable lipid: Cholesterol: DOPE: DOPC: DPPE-PEG-2K) (25:30:20:20:5).

[0231] For the cancer xenograft model, an A549 cell line was obtained from ATCC. The cells were maintained in culture medium supplemented with 10% Fetal Bovine Serum and 100 U/ml penicillin and 100 μ g/ml streptomycin. Cells were split 48 hrs before inoculation so that cells were in log phase growth when harvested. Cells were lightly trypsinized with trypsin-EDTA and harvested from tissue culture. The number of viable cells was counted and determined in a hemocytometer in the presence of trypan blue (only viable cells are counted). The cells were resuspended to a concentration of 5×10^7 /ml in media without serum. Then the cell suspension was mixed well with ice thawed BD matrigel at 1:1 ratio for injection.

[0232] Mice were Charles River Laboratory Athymic Nude (nu/nu) Female Mice, immuno-compromised, 6-8 weeks old, 7-8 mice per group.

[0233] For tumor model preparation, each mouse was inoculated subcutaneously in the right flank with 0.1 ml an inoculum of 2.5×10^6 of A549 cells using a 25 G needle and syringe, one inoculum per mouse. Mice were not anesthetized for inoculation.

[0234] For tumor volume measurements and randomization, tumor size was measured to the nearest 0.1 mm. Tumor volumes were calculated using the formula: Tumor volume =length x width²/2. Once the established tumors reached approximately 120 - 175 mm³, average tumor volume was about 150 mm³, the mice were assigned into the various vehicle control and treatment groups such that the mean tumor volumes in the treated groups were within 10% of the mean tumor volume in the vehicle control group, ideally, the CV% of tumor volume was less than 25%. On the same day, test articles and control vehicle were administered according to the dosing regimen. Tumor volumes were monitored three times for week 1, twice for the rest of weeks, including the day of study termination.

[0235] For dosage administration, on the dosing day, the test articles were taken out from -80 °C freezer and thawed on ice. Before applied to syringes, the bottle containing formulation was reverted by hands for a few times. All test articles were dosed at 0.75 mg/kg by IV, q2w X 2, at 10 ml/kg.

[0236] For body weight, mice were weighed to the nearest 0.1 g Body weights were monitored and recorded daily within 7 days post dosing for first dose. Body weights were monitored and recorded twice for weeks, for the rest of weeks, including the day of study termination.

[0237] For tumors collection, on 28 days post first dosing, tumor volume was measured, and tumor was dissected for weight measurement, and stored for PD biomarker study. Tumor weight was recorded.

[0238] Example 11: The GST- π siRNAs of this disclosure demonstrated increased cancer cell death by apoptosis of cancer cells in vitro. The GST- π siRNAs provided GST- π knockdown, which resulted in upregulation of PUMA, a biomarker for apoptosis and associated with loss in cell viability.

[0239] GST- π siRNA SEQ ID NOs:156 and 182, which contained a combination of deoxynucleotides in the seed region, a 2'-F substituted deoxynucleotide, and 2'-OMe substituted ribonucleotides, provided unexpectedly increased apoptosis of cancer cells.

[0240] The level of expression of PUMA for GST- π siRNA SEQ ID NOs:156 and 182 was measured as shown in Fig. 4. In Fig. 4, the expression of PUMA was greatly increased from 2-4 days after transfection of the GST- π siRNA.

[0241] These data show that the structure of GST- π siRNAs containing a combination of deoxynucleotides in the seed region, a 2'-F substituted deoxynucleotide, and 2'-OMe substituted ribonucleotides provided unexpectedly increased apoptosis of cancer cells.

[0242] The protocol for the PUMA biomarker was as follows. One day before transfection, cells were plated in a 96-well plate at 2×10^3 cells per well with 100 μ l of DMEM (HyClone Cat. # SH30243.01) containing 10% FBS and cultured in a 37°C incubator containing a humidified atmosphere of 5% CO₂ in air. Next day, before transfection the medium was replaced with 90 μ l of Opti-MEM I Reduced Serum Medium (Life Technologies Cat. # 31985-070) containing 2% FBS. Then, 0.2 μ l of Lipofectamine RNAiMAX (Life Technologies Cat. #13778-100) were mixed with 4.8 μ l of Opti-MEM I for 5 minutes at room temperature. 1 μ l of the GST- π siRNA (stock conc. 1 μ M) was mixed with 4 μ l of Opti-MEM I and combined with the RNAiMAX solution and then mixed gently. The mixture was incubated for 10 minutes at room temperature to allow the RNA-RNAiMAX complexes to form. 10 μ l of RNA-RNAiMAX complexes were added per well, to final concentration of the siRNA 10 nM. The cells were incubated for 2 hours and medium changed to fresh Opti-MEM I Reduced Serum Medium containing 2% FBS. For 1, 2, 3, 4, and 6 days post transfection, the cells were washed with ice-cold PBS once and then lysed with 50 μ l of Cell-to-Ct Lysis Buffer (Life Technologies Cat. # 4391851 C) for 5-30 minutes at room temperature. 5 μ l of Stop Solution was added and incubated for 2 minutes at room temperature. PUMA (BBC3, Cat# Hs00248075, Life Technologies) mRNA levels were measured by qPCR with TAQMAN.

[0243] Example 12: The GST- π siRNAs of this disclosure can exhibit profound reduction of cancer xenograft tumors in vivo. The GST- π siRNAs can provide gene knockdown potency in vivo when administered in a liposomal formulation to the cancer xenograft tumors.

[0244] Fig. 5 shows tumor inhibition efficacy for a GST- π siRNA (SEQ ID NOs:61 and 126). Dose dependent knockdown of GST- π mRNA was observed in vivo with the siRNA targeted to GST- π . A cancer xenograft model was utilized with a siRNA targeted to GST- π .

[0245] The GST- π siRNA showed significant and unexpectedly advantageous tumor inhibition efficacy within a few days after administration. As shown in Fig. 5, treatment with a GST- π siRNA resulted in significant reduction of GST- π mRNA expression 4 days after injection in a lipid formulation. At the higher dose of 4mg/kg, significant reduction of about 40% was detected 24 hours after injection.

[0246] The GST- π siRNA was administered in a single injection of 10 mL/kg of a liposomal formulation having the composition (Ionizable lipid: Cholesterol: DOPE: DOPC: DPPE-PEG-2K) (25:30:20:20:5).

[0247] For the cancer xenograft model, an A549 cell line was obtained from ATCC. The cells were maintained in RPMI-1640 supplemented with 10% Fetal Bovine Serum and 100 U/ml penicillin and 100 μ g/ml streptomycin. Cells were split 48 hrs before inoculation so that cells were in log phase growth when harvested. Cells were lightly trypsinized with trypsin-EDTA and harvested from tissue culture. The number of viable cells was counted and determined in a hemocytometer in the presence of trypan blue (only viable cells are counted). The cells were resuspended to a concentration of 4×10^7 /ml in RPMI media without serum. Then the cell suspension was mixed well with ice thawed BD matrigel at 1:1 ratio for injection.

[0248] Mice were Charles River Laboratory Athymic Nude (nu/nu) Female Mice, immuno-compromised, 6-8 weeks old, 3 mice per group.

[0249] For tumor model preparation, each mouse was inoculated subcutaneously in the right flank with 0.1 ml an inoculum of 2×10^6 of A549 cells using a 25 G needle and syringe, one inoculum per mouse. Mice were not anesthetized for inoculation.

[0250] For tumor volume measurements and randomization, tumor size was measured to the nearest 0.1 mm. Tumor volumes were calculated using the formula: Tumor volume =length x width²/2. Tumor volumes were monitored twice a week. Once the established tumors reached approximately 350 - 600 mm³, the mice were assigned into groups with varied time points. On the same day, test articles were administered according to the dosing regimen.

[0251] For dosage administration, on the day when the established tumors reached approximately 350 - 600 mm³, the test articles were taken out from 4°C fridge. Before being applied to syringes, the bottle containing formulation was reverted by hand for a few times to make a homogeneous solution.

[0252] For body weight, mice were weighed to the nearest 0.1 g. Body weights were monitored and recorded twice for weeks, for the rest of weeks, including the day of study termination.

[0253] For tumors collection, animals were sacrificed by overdosed CO₂ and tumors were dissected at 0, 24, 48, 72,

96(optional), and 168 hours following the dosing. Tumors were first wet weighted, and then separated into three parts for KD, distribution and biomarker analysis. The samples were snap frozen in liquid nitrogen and stored at - 80°C until ready to be processed.

[0254] Example 13: The GST- π siRNAs of this disclosure inhibited pancreatic cancer xenograft tumors in vivo. The GST- π siRNAs provided gene knockdown potency in vivo when administered in a liposomal formulation to the pancreatic cancer xenograft tumors.

[0255] In this xenograft model, each mouse was inoculated subcutaneously in the right flank with 0.1 ml an inoculum of 2.5×10^6 of PANC-1 cells. Athymic nude female mice, 6 to 8 weeks, Charles River, were used. Tumor size was measured to the nearest 0.1 mm. Once the established tumors reached approximately 150 - 250 mm³ (average tumor volume at about 200 mm³), the mice were assigned into the various vehicle control and treatment groups such that the mean tumor volumes in the treated groups were within 10% of the mean tumor volume in the vehicle control group. On the same day, test articles and control vehicle were administered according to the dosing regimen. Tumor volumes were monitored three times for week 1, twice for the rest of weeks, including the day of study termination.

[0256] Fig. 6 shows tumor inhibition efficacy for a GST- π siRNA (SEQ ID Nos:61 and 126). As shown in Fig. 6, a dose response was obtained with doses ranging from 0.375 mg/kg to 3 mg/kg of siRNA targeted to GST- π . The GST- π siRNA showed significant and unexpectedly advantageous tumor inhibition efficacy within a few days after administration. Thus, the GST- π siRNA demonstrated significant and unexpectedly advantageous tumor inhibition efficacy at the endpoint.

[0257] The GST- π siRNAs were administered in a liposomal formulation having the composition (Ionizable lipid: cholesterol: DOPE: DOPC: DPPE-PEG-2K) (25:30:20:20:5).

[0258] Example 14: The GST- π siRNAs of this disclosure exhibited increased serum stability.

[0259] Fig. 7 shows incubation in human serum and detection of remaining siRNA at various time points by HPLS/LCMS. As shown in Fig. 7, the half-life ($t_{1/2}$) in serum for both the sense strand (Fig. 7, top) and antisense strand (Fig. 7, bottom) of a GST- π siRNA (SEQ ID Nos:61 and 126) was about 100 minutes.

[0260] Example 15: The GST- π siRNAs of this disclosure exhibited enhanced stability in formulation in plasma.

[0261] Fig. 8 shows incubation of formulation in plasma and detection of remaining siRNA at various time points. As shown in Fig. 8, the half-life ($t_{1/2}$) in plasma of a formulation of GST- π siRNA (SEQ ID Nos:61 and 126) was significantly longer than 100 hours.

[0262] The GST- π siRNA was prepared in a liposomal formulation having the composition (Ionizing lipid: cholesterol: DOPE: DOPC: DPPE-PEG-2K) (25:30:20:20:5). The z-average size for the liposomal nanoparticles was 40.0 nm, and the siRNA was 91% encapsulated.

[0263] The formulation was incubated in 50% human serum in PBS for 40min, 1.5h, 3h, 24h, and 96h. The amount of the GST- π siRNA was determined by an ELISA-based assay.

[0264] Example 16: The GST- π siRNAs of this disclosure exhibited reduced off target effects by the passenger strand.

[0265] For the GST- π siRNA (SEQ ID Nos: 156 and 182), Fig. 9 shows that in vitro knockdown for the guide strand was approximately exponential, as compared to a control with scrambled sequence that exhibited no effect. The IC₅₀ of this siRNA was measured at 5 pM. Fig. 10 shows in vitro knockdown for the passenger strand of the same GST- π siRNA. As shown in Fig. 10, the passenger strand off target knockdown for the GST- π siRNA was greatly reduced, by more than 100-fold.

[0266] For the GST- π siRNAs (SEQ ID Nos: 187 and 199), (SEQ ID Nos:189 and 201), and (SEQ ID Nos:190 and 202), Fig. 11 shows that the in vitro knockdowns for the guide strands were approximately exponential. The IC₅₀s of these siRNAs were measured at 6, 7, and 5 pM, respectively. As shown in Fig. 12, the in vitro knockdowns for the passenger strands of these GST- π siRNAs were significantly reduced by at least 10-fold. All of these GST- π siRNAs had deoxynucleotides in the seed region of the duplex region, with no other modifications in the duplex region.

[0267] For the GST- π siRNAs (SEQ ID Nos:217 and 232), Fig. 13 shows that the in vitro knockdown for the guide strand of this highly active GST- π siRNA was approximately exponential. The IC₅₀ of this siRNA was measured at 11 pM. As shown in Fig. 14, the in vitro knockdown for the passenger strand of this GST- π siRNA was significantly reduced by more than 100-fold. This GST- π siRNA had deoxynucleotides in the seed region of the duplex region, with no other modifications in the duplex region.

[0268] Off-target effects were determined using the expression reporter plasmid psiCHECK-2, which encodes the *Renilla luciferase* gene. (Dual-Luciferase Reporter Assay System, Promega, Cat#:E1960). The siRNA concentration was typically 50 pM. Protocol: Day 1, HeLa cell seeded at 5 to 7.5 x 10³/100ul/well. Day 2, co-transfection with cell confluence about 80%. Day 3, cells harvested for luciferase activity measurement. Luciferase activity was measured using Promega's Luciferase Assay System (E4550), according to manufacturer's protocol.

[0269] The psiCHECK-2 vector enabled monitoring of changes in expression of a target gene fused to the reporter gene of *Renilla luciferase*. The siRNA constructs were cloned into the multiple cloning region, and the vector was cotransfected with the siRNA into HeLa cells. If a specific siRNA binds to the target mRNA and initiates the RNAi process, the fused *Renilla luciferase*: construct mRNA will be cleaved and subsequently degraded, decreasing the *Renilla luciferase* signal.

[0270] For example, the plasmid inserts for siRNAs with the BU2' structure were as follows:

PsiCHECK-2 (F) plasmid insert:

SEQ ID NO.: 285

ctcgag gggcaacTGAAGCCTTTTGAGACCCTGcTgTcccag gcggccgc

PsiCHECK-2 (R) plasmid insert:

SEQ ID NO.: 286

ctcgag cTgggacagCAGGGTCTCAAAGGCTTCagTTgccc gcggccgc

[0271] Example 17: The GST- π siRNAs of this disclosure exhibited advantageously reduced miRNA-like off target effects, which are seed-dependent unintended off-target gene silencing.

[0272] For the GST- π siRNAs (SEQ ID Nos: 156 and 182), (SEQ ID Nos: 187 and 199), (SEQ ID Nos:189 and 201), (SEQ ID Nos:190 and 202), and (SEQ ID Nos:217 and 232), off target activity mimicking miRNA was found to be essentially negligible. The seed-dependent unintended off-target gene silencing for these GST- π siRNAs was at least 10-fold to 100-fold less than the on-target activity of the guide strand.

[0273] For testing miRNA-related off target effects, one to four repeats of seed-matched target sequences complementary to the entire seed-containing region, positions 1-8 of the 5' end of the antisense strand, but not to the remaining non-seed region, positions 9-21, were introduced into the region corresponding to the 3'UTR of the luciferase mRNA, to determine the efficiency of the seed-dependent unintended off-target effects. Plasmid inserts were used to mimic a miRNA with complete matching in the seed region and mismatches (bulges) in the non-seed region.

[0274] For example, the plasmid inserts for siRNAs with the BU2' structure were as follows:

PsiCHECK-2 (Fmi1) plasmid insert:

SEQ ID NO.: 287

ctcgag gggcaacTCTACGCAAACAGACCCTGcTgTcccag gcggccgc

PsiCHECK-2 (Fmi2) plasmid insert:

SEQ ID NO.: 288

ctcgag gggcaacTCTACGCAAACAGACCCTGcT CTACGCAAACAGACCCTGcT
gTcccag gcggccgc

PsiCHECK-2 (Fmi3) plasmid insert:

SEQ ID NO.: 289

ctcgag gggcaacTCTACGCAAACAGACCCTGcT CTACGCAAACAGACCCTGcT
CTACGCAAACAGACCCTGcT gTcccag gcggccgc

PsiCHECK-2 (Fmi4) plasmid insert:

SEQ ID NO.: 290

ctcgag gggcaacTCTACGCAAACAGACCCTGcT CTACGCAAACAGACCCTGcT
CTACGCAAACAGACCCTGcT CTACGCAAACAGACCCTGcT gTcccag
gcggccgc

[0275] The embodiments described herein are not limiting and one skilled in the art can readily appreciate that specific combinations of the modifications described herein can be tested without undue experimentation toward identifying

nucleic acid molecules with improved RNAi activity.

[0276] It is understood that this invention is not limited to the particular methodology, protocols, materials, and reagents described, as these may vary. It is also to be understood that the terminology used herein is for the purpose of describing particular embodiments only, and is not intended to limit the scope of the present invention. It will be readily apparent to one skilled in the art that varying substitutions and modifications can be made to the description disclosed herein.

[0277] It must be noted that as used herein and in the appended claims, the singular forms "a", "an", and "the" include plural reference unless the context clearly dictates otherwise. As well, the terms "a" (or "an"), "one or more" and "at least one" can be used interchangeably herein. It is also to be noted that the terms "comprises," "comprising", "containing," "including", and "having" can be used interchangeably, and shall be read expansively and without limitation.

[0278] Recitation of ranges of values herein are merely intended to serve as a shorthand method of referring individually to each separate value falling within the range, unless otherwise indicated herein, and each separate value is incorporated into the specification as if it were individually recited herein. For Markush groups, those skilled in the art will recognize that this description includes the individual members, as well as subgroups of the members of the Markush group.

[0279] Without further elaboration, it is believed that one skilled in the art can, based on the above description, utilize the present invention to its fullest extent. The following specific embodiments are, therefore, to be construed as merely illustrative, and not limitative of the remainder of the disclosure in any way whatsoever.

[0280] All of the features disclosed in this specification may be combined in any combination. Each feature disclosed in this specification may be replaced by an alternative feature serving the same, equivalent, or similar purpose.

Claims

1. A nucleic acid molecule for inhibiting expression of GST- π comprising a sense strand and an antisense strand, wherein the strands form a duplex region, and wherein the antisense strand is SEQ ID NOs: 157 and the sense strand is SEQ ID NOs: 131.
2. The nucleic acid molecule of claim 1, wherein one or more of the nucleotides in the duplex region is chemically-modified, wherein the chemically-modified nucleotides are 2'-deoxy nucleotides, 2'-O-alkyl substituted nucleotides, 2'-deoxy-2'-fluoro substituted nucleotides, phosphorothioate nucleotides, locked nucleotides, or any combination thereof.
3. The nucleic acid molecule of claim 2, wherein the antisense strand has deoxynucleotides in a plurality of positions, the plurality of positions being one of the following:
 - each of positions 4, 6 and 8, from the 5' end of the antisense strand;
 - each of positions 3, 5 and 7, from the 5' end of the antisense strand;
 - each of positions 1, 3, 5 and 7, from the 5' end of the antisense strand;
 - each of positions 3-8, from the 5' end of the antisense strand; or
 - each of positions 5-8, from the 5' end of the antisense strand.
4. The nucleic acid molecule of claim 3, wherein the molecule has one or more 2'-deoxy-2'-fluoro substituted nucleotides in the duplex region.
5. The nucleic acid molecule of claim 2, wherein the antisense strand is SEQ ID NOs: 182 and the sense strand is SEQ ID NOs: 156.
6. The nucleic acid molecule of claim 2, wherein the antisense strand is SEQ ID NOs: 180 and the sense strand is SEQ ID NOs: 154.
7. The nucleic acid molecule of claim 2, wherein the antisense strand is SEQ ID NOs: 181 and the sense strand is SEQ ID NOs: 155.
8. The nucleic acid molecule of any one of claims 1 to 7, wherein the molecule inhibits expression of GST- π mRNA with an IC50 of less than 50 pM.
9. The nucleic acid molecule of any one of claims 1 to 7, wherein a single administration of the molecule inhibits expression of GST- π mRNA levels by at least 25% in vivo.

10. The nucleic acid molecule of any one of claims 1 to 7, wherein the passenger strand has off target activity reduced by at least 50-fold.

5 11. A pharmaceutical composition comprising the nucleic acid molecule of any one of claims 1 to 10 and a pharmaceutically acceptable carrier.

12. The pharmaceutical composition of claim 11, wherein the carrier comprises a lipid molecule or liposome.

10 13. The composition of any one of claims 11 or 12 for the use in a method for treating a disease associated with GST- π expression, the method comprising administering to a subject in need said composition, wherein the disease is malignant tumor, cancer, cancer caused by cells expressing mutated KRAS, sarcoma, or carcinoma.

Patentansprüche

15 1. Nukleinsäuremolekül zum Inhibieren der Expression von GST- π , umfassend einen Sense-Strang und einen Antisense-Strang, wobei die Stränge eine Duplexregion bilden, und wobei der Antisense-Strang SEQ ID NO: 157 ist und der Sense-Strang SEQ ID NO: 131 ist.

20 2. Nukleinsäuremolekül gemäß Anspruch 1, wobei eines oder mehrere der Nukleotide in der Duplexregion chemisch modifiziert ist/sind, wobei die chemisch modifizierten Nukleotide 2'-Desoxy-Nukleotide, 2'-O-Alkyl-substituierte Nukleotide, 2'-Desoxy-2'-Fluoro-substituierte Nukleotide, Phosphorothioat-Nukleotide, Locked-Nukleotide oder irgendeine Kombination davon sind.

25 3. Nukleinsäuremolekül gemäß Anspruch 2, wobei der Antisense-Strang Desoxynukleotide in einer Mehrzahl von Positionen aufweist, wobei die Mehrzahl der Position eine der folgenden ist:

jede der Positionen 4, 6 und 8, vom 5'-Ende des Antisense-Strangs;
 jede der Positionen 3, 5 und 7, vom 5'-Ende des Antisense-Strangs;
 30 jede der Positionen 1, 3, 5 und 7, vom 5'-Ende des Antisense-Strangs;
 jede der Positionen 3-8, vom 5'-Ende des Antisense-Strangs; oder
 jede der Positionen 5-8, vom 5'-Ende des Antisense-Strangs.

35 4. Nukleinsäuremolekül gemäß Anspruch 3, wobei das Molekül ein oder mehrere 2'-Desoxy-2'-Fluoro-substituierte Nukleotid(e) in der Duplexregion aufweist.

5. Nukleinsäuremolekül gemäß Anspruch 2, wobei der Antisense-Strang SEQ ID NO: 182 ist und der Sense-Strang SEQ ID NO: 156 ist.

40 6. Nukleinsäuremolekül gemäß Anspruch 2, wobei der Antisense-Strang SEQ ID NO: 180 ist und der Sense-Strang SEQ ID NO: 154 ist.

7. Nukleinsäuremolekül gemäß Anspruch 2, wobei der Antisense-Strang SEQ ID NO: 181 ist und der Sense-Strang SEQ ID NO: 155 ist.

45 8. Nukleinsäuremolekül gemäß irgendeinem der Ansprüche 1 bis 7, wobei das Molekül die Expression von GST- π -mRNA mit einem IC50-Wert von weniger als 50 pM inhibiert.

50 9. Nukleinsäuremolekül gemäß irgendeinem der Ansprüche 1 bis 7, wobei eine einzelne Verabreichung des Moleküls die Expression des GST- π -mRNA-Niveaus in vivo zu mindestens 25% inhibiert.

10. Nukleinsäuremolekül gemäß irgendeinem der Ansprüche 1 bis 7, wobei der Passenger-Strang mindestens 50-fach reduzierte Off-Target-Aktivität aufweist.

55 11. Pharmazeutische Zusammensetzung, umfassend das Nukleinsäuremolekül gemäß irgendeinem der Ansprüche 1 bis 10 und einen pharmazeutisch akzeptablen Trägerstoff.

12. Pharmazeutische Zusammensetzung gemäß Anspruch 11, wobei der Trägerstoff ein Lipidmolekül oder ein Liposom

umfasst.

- 5 13. Zusammensetzung gemäß irgendeinem der Ansprüche 11 oder 12, zur Verwendung in einem Verfahren zum Behandeln einer mit GST- π -Expression assoziierten Krankheit, das Verfahren umfassend das Verabreichen der Zusammensetzung an ein Subjekt, das dessen bedarf, wobei die Krankheit ein bösartiger Tumor, Krebs, Krebs, welcher durch Zellen, welche mutiertes KRAS exprimieren, verursacht wird, Sarkom oder Karzinom ist.

10 Revendications

1. Molécule d'acide nucléique pour inhiber l'expression de GST- π comprenant un brin sens et un brin antisens, dans laquelle les brins forment une région de duplex, et dans laquelle le brin antisens est la SEQ ID N° : 157 et le brin sens est la SEQ ID N° : 131.

- 15 2. Molécule d'acide nucléique selon la revendication 1, dans laquelle un ou plusieurs des nucléotides dans la région de duplex sont chimiquement modifiés, dans laquelle les nucléotides chimiquement modifiés sont des nucléotides 2'-déoxy, des nucléotides substitués par 2'-O-alkyle, des nucléotides substitués par 2'-déoxy-2'-fluoro, des nucléotides phosphorothioates, des nucléotides bloqués, ou toute combinaison de ceux-ci.

- 20 3. Molécule d'acide nucléique selon la revendication 2, dans laquelle le brin antisens présente des désoxynucléotides dans une pluralité de positions, la pluralité de positions étant une des suivantes :

chacune des positions 4, 6 et 8, depuis l'extrémité 5' du brin antisens ;
chacune des positions 3, 5 et 7, depuis l'extrémité 5' du brin antisens ;
25 chacune des positions 1, 3, 5 et 7, depuis l'extrémité 5' du brin antisens ;
chacune des positions 3 à 8, depuis l'extrémité 5' du brin antisens ; ou
chacune des positions 5 à 8, depuis l'extrémité 5' du brin antisens.

- 30 4. Molécule d'acide nucléique selon la revendication 3, dans laquelle la molécule présente un ou plusieurs nucléotides substitués par 2'-déoxy-2'-fluoro dans la région de duplex.

5. Molécule d'acide nucléique selon la revendication 2, dans laquelle le brin antisens est la SEQ ID N° : 182 et le brin sens est la SEQ ID N° : 156.

- 35 6. Molécule d'acide nucléique selon la revendication 2, dans laquelle le brin antisens est la SEQ ID N° : 180 et le brin sens est la SEQ ID N° : 154.

7. Molécule d'acide nucléique selon la revendication 2, dans laquelle le brin antisens est la SEQ ID N° : 181 et le brin sens est la SEQ ID N° : 155.

- 40 8. Molécule d'acide nucléique selon l'une quelconque des revendications 1 à 7, dans laquelle la molécule inhibe l'expression de GST- π d'ARNm avec un IC50 de moins de 50 pM.

- 45 9. Molécule d'acide nucléique selon l'une quelconque des revendications 1 à 7, dans laquelle une seule administration de la molécule inhibe l'expression de niveaux de GST- π d'ARNm d'au moins 25 % in vivo.

10. Molécule d'acide nucléique selon l'une quelconque des revendications 1 à 7, dans laquelle le brin passager présente une activité hors cible divisée au moins par 50.

- 50 11. Composition pharmaceutique comprenant la molécule d'acide nucléique selon l'une quelconque des revendications 1 à 10 et un support pharmaceutiquement acceptable.

12. Composition pharmaceutique selon la revendication 11, dans laquelle le support comprend une molécule lipidique ou un liposome.

- 55 13. Composition selon l'une quelconque des revendications 11 ou 12 pour l'utilisation dans un procédé pour le traitement d'une maladie associée à l'expression de GST- π , le procédé comprenant l'administration à un sujet dans le besoin de ladite composition, dans laquelle la maladie est une tumeur maligne, un cancer, un cancer provoqué par des

EP 3 236 974 B9

cellules exprimant le KRAS muté, un sarcome, ou un carcinome.

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FIG. 1

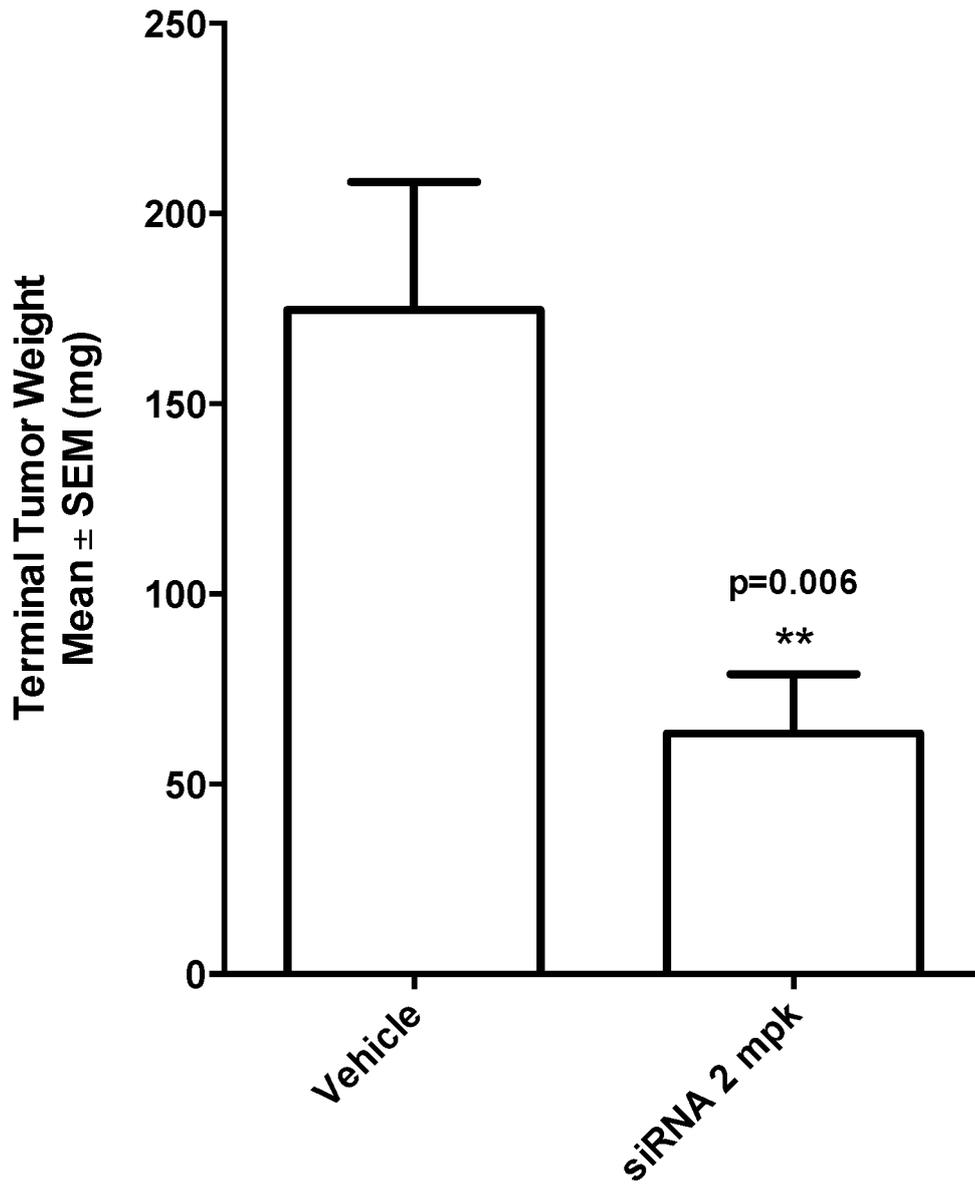


FIG. 2

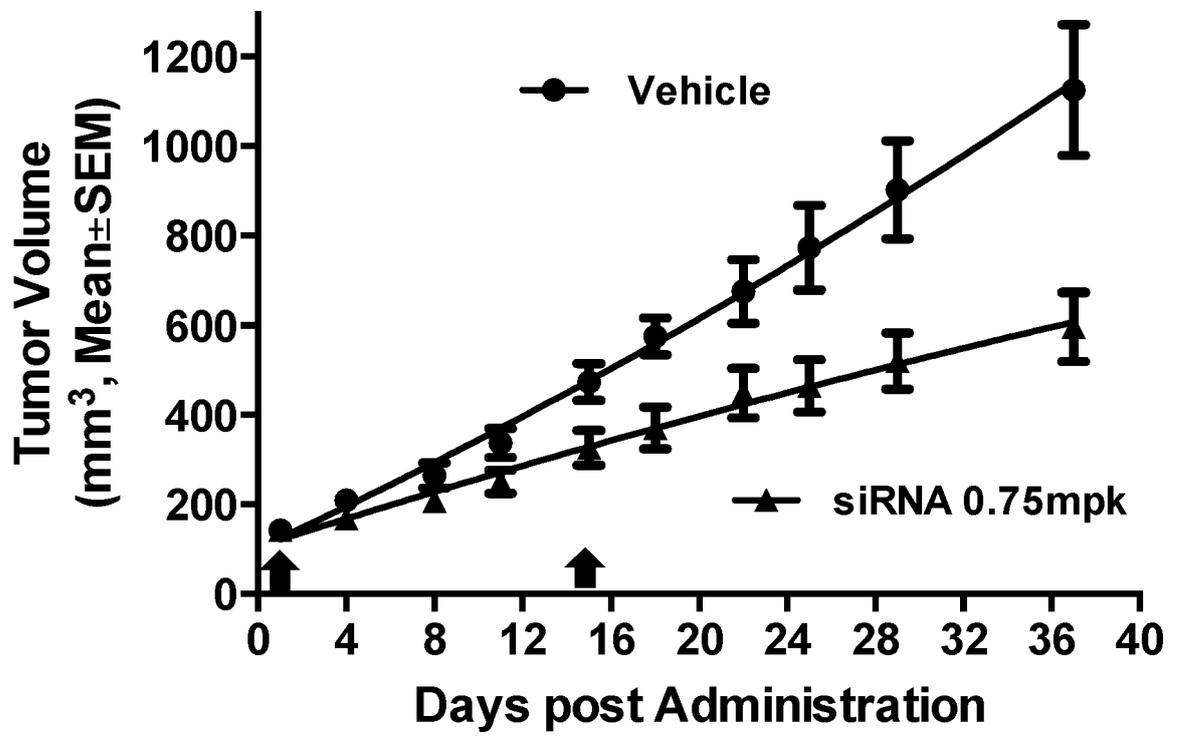


FIG. 3

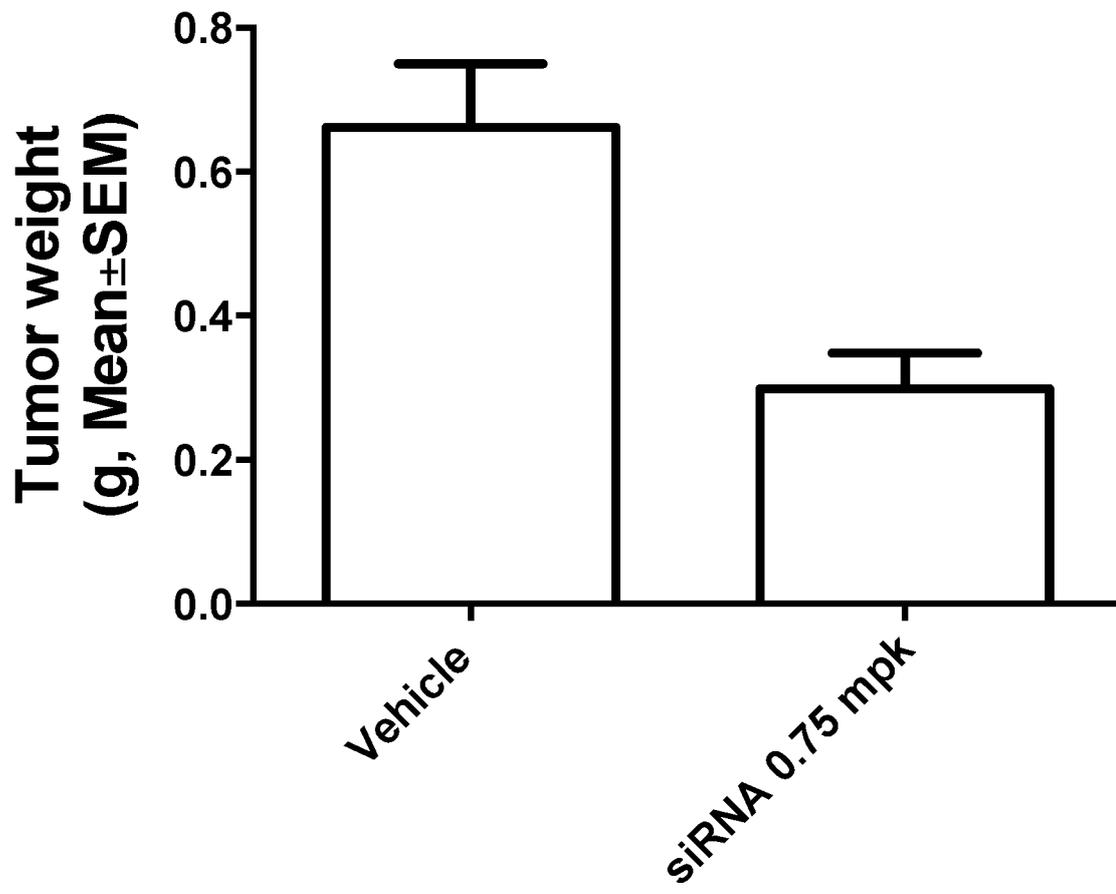


FIG. 4

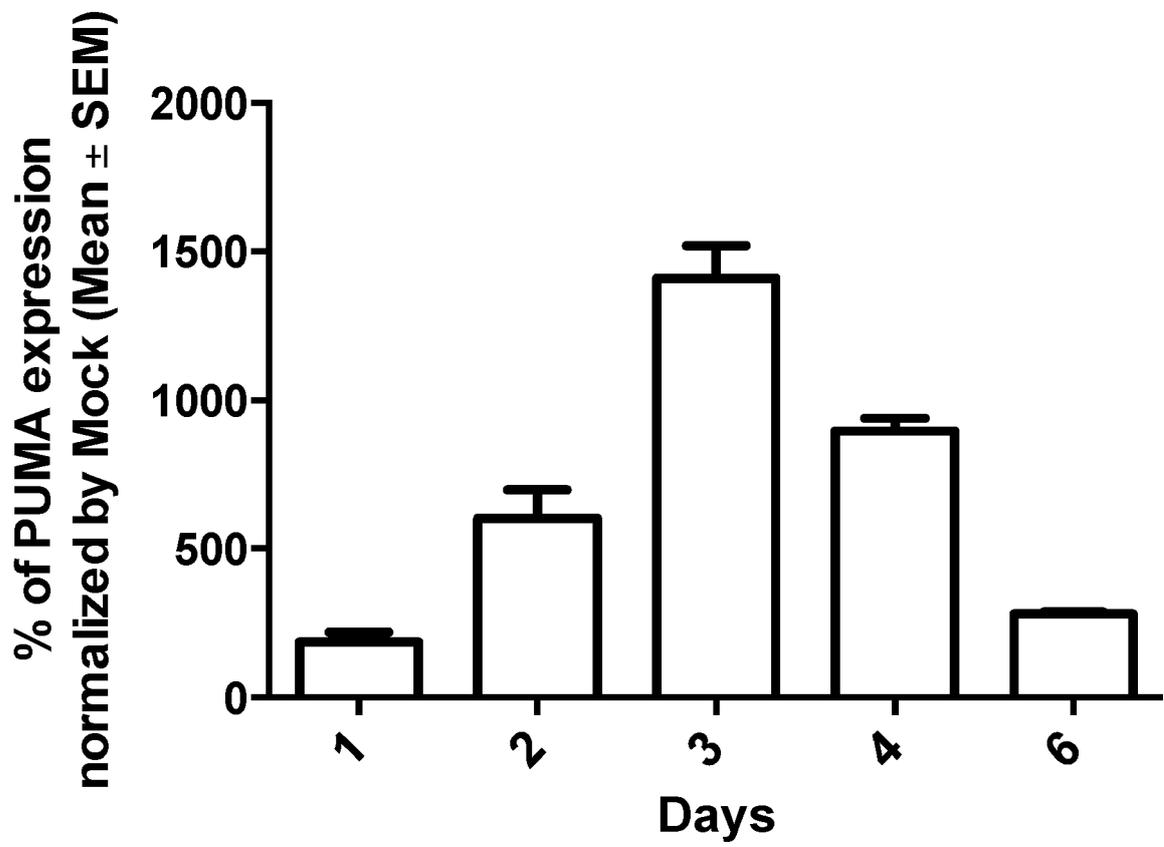


FIG. 5

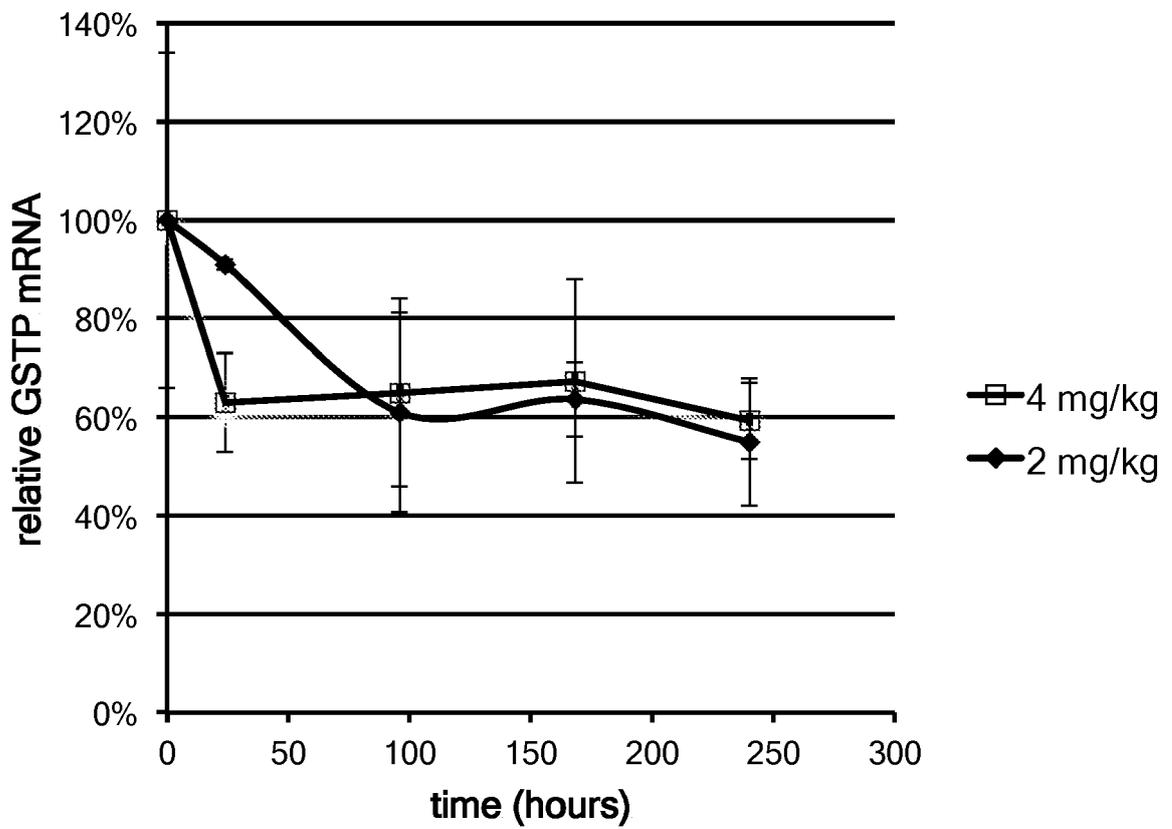


FIG. 6

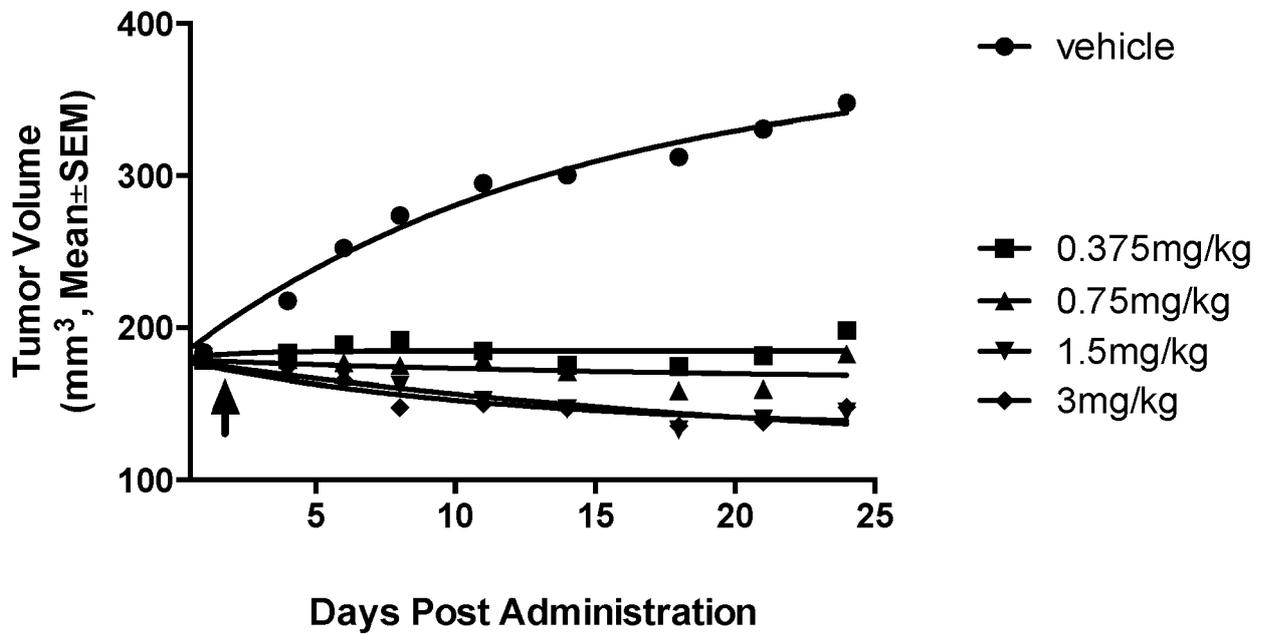


FIG. 7

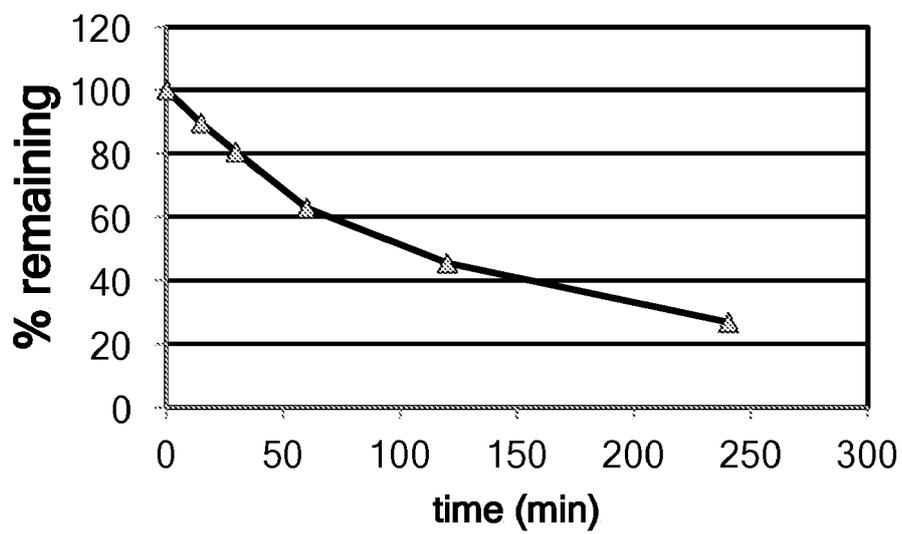
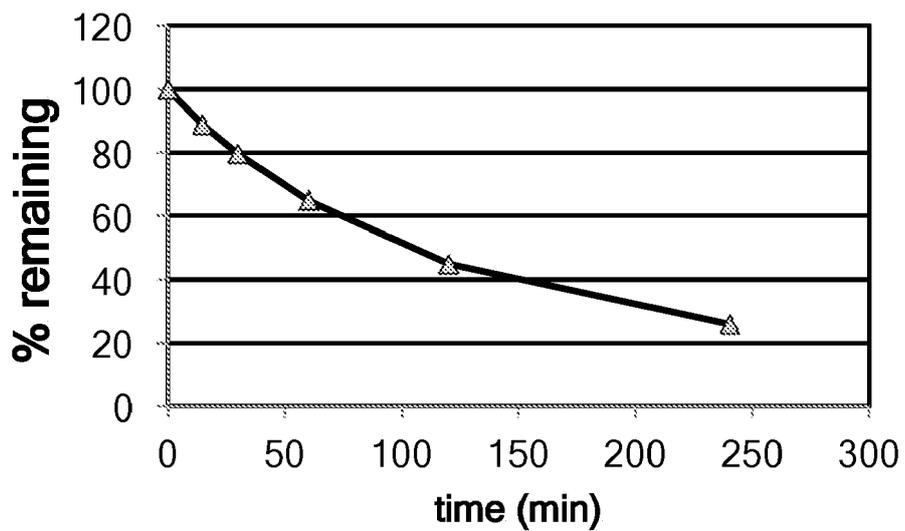


FIG. 8

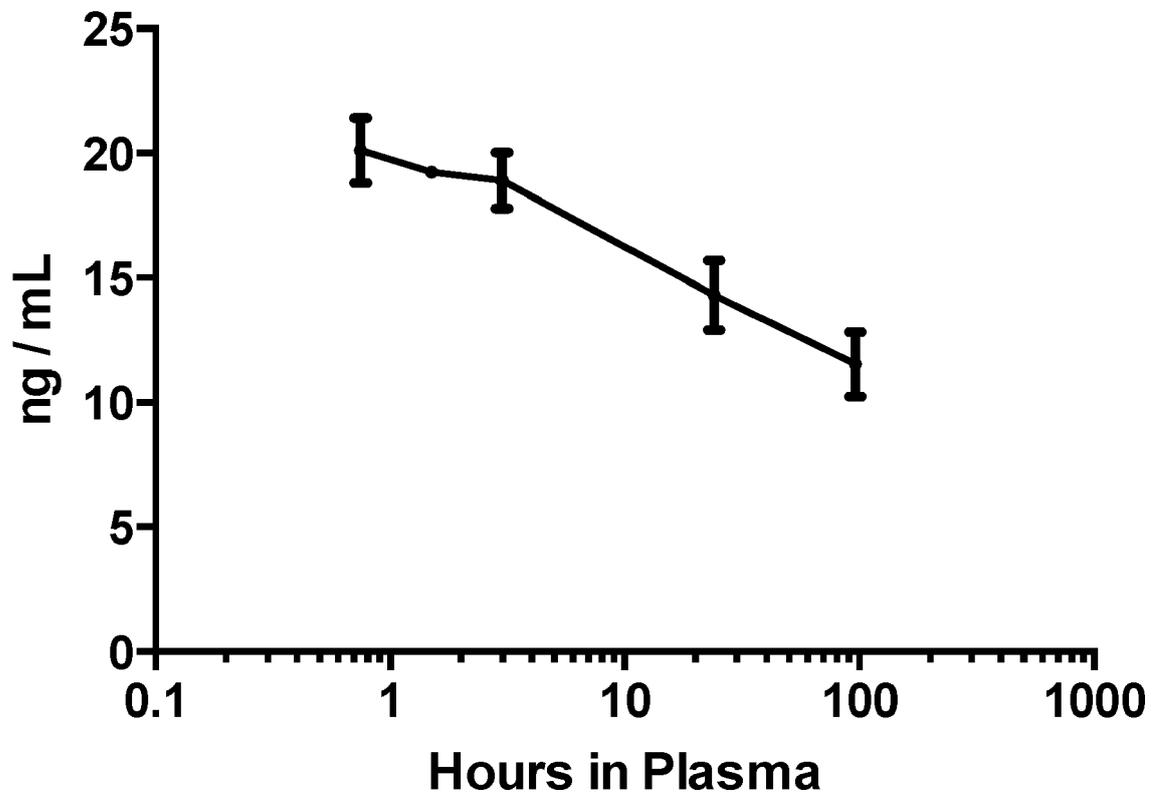


FIG. 9

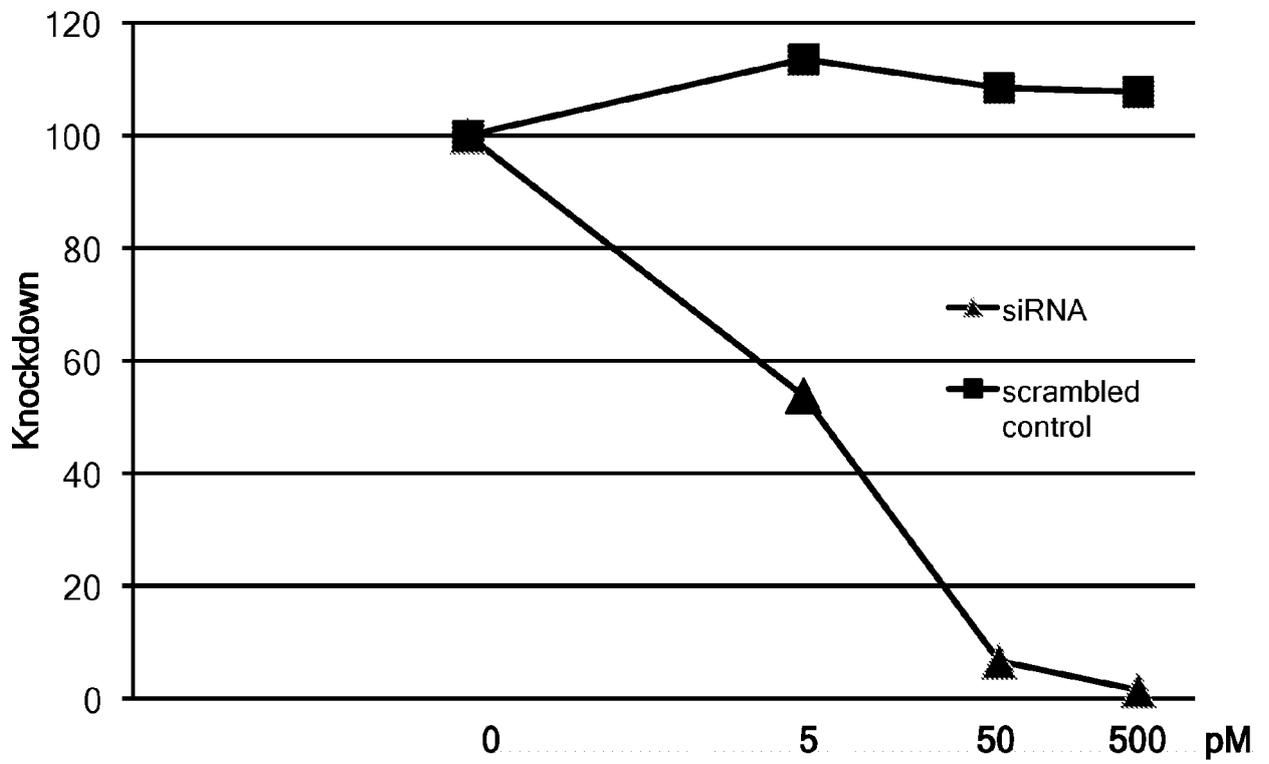


FIG. 10

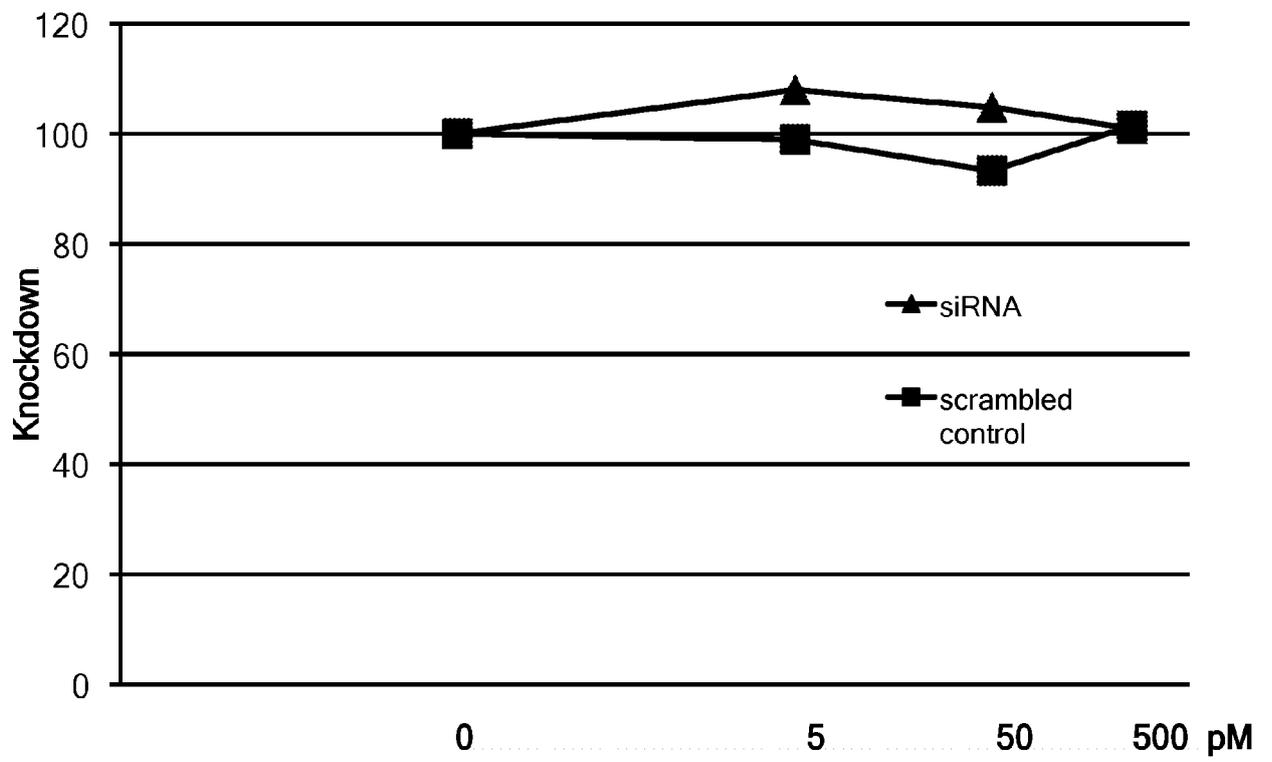


FIG. 11

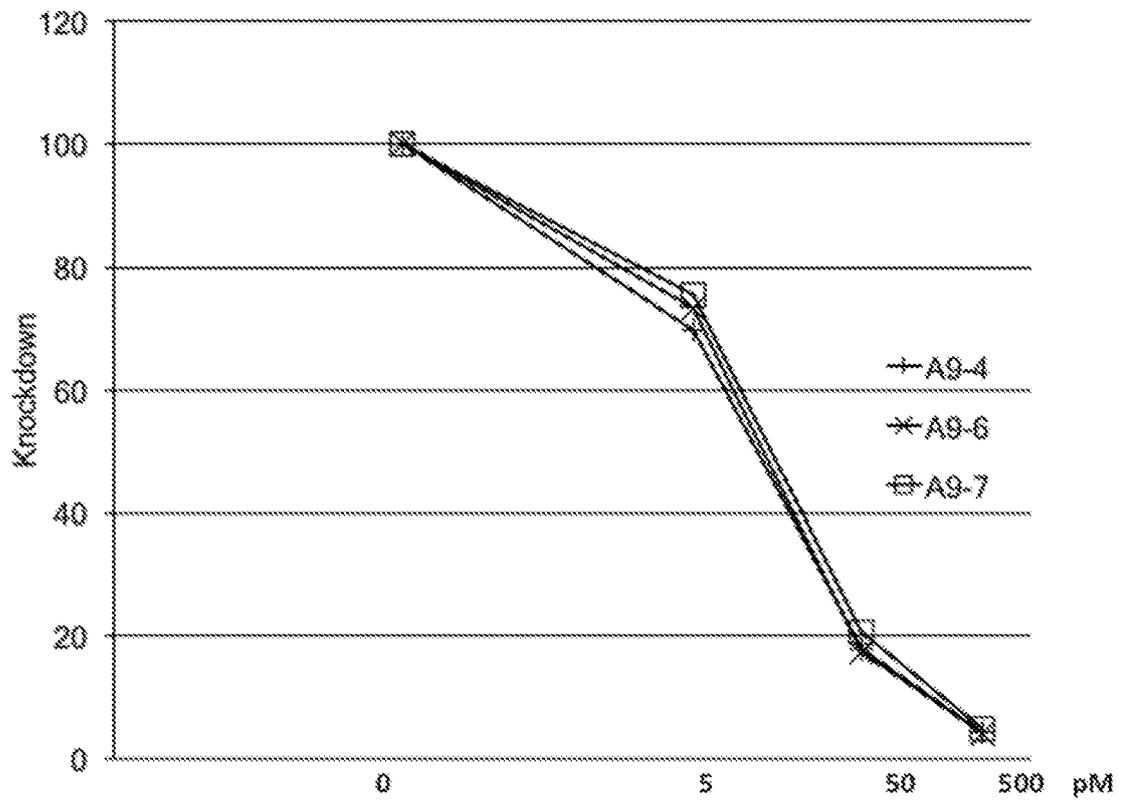


FIG. 12

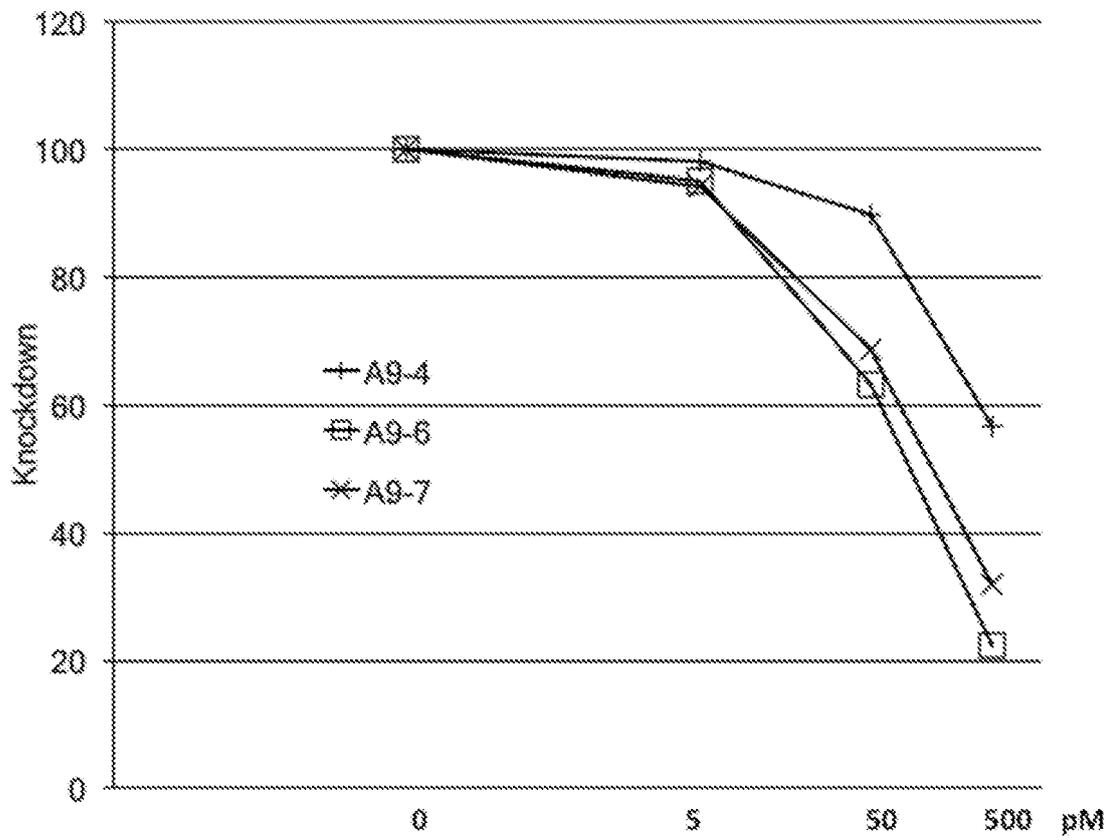


FIG. 13

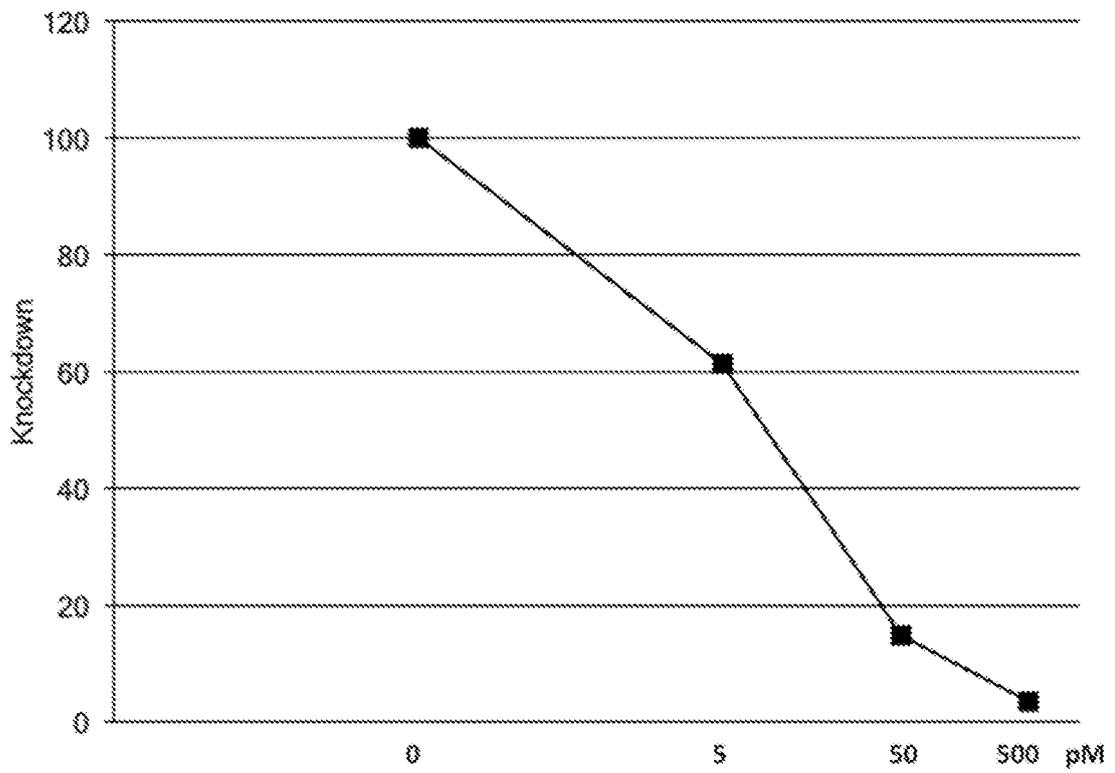
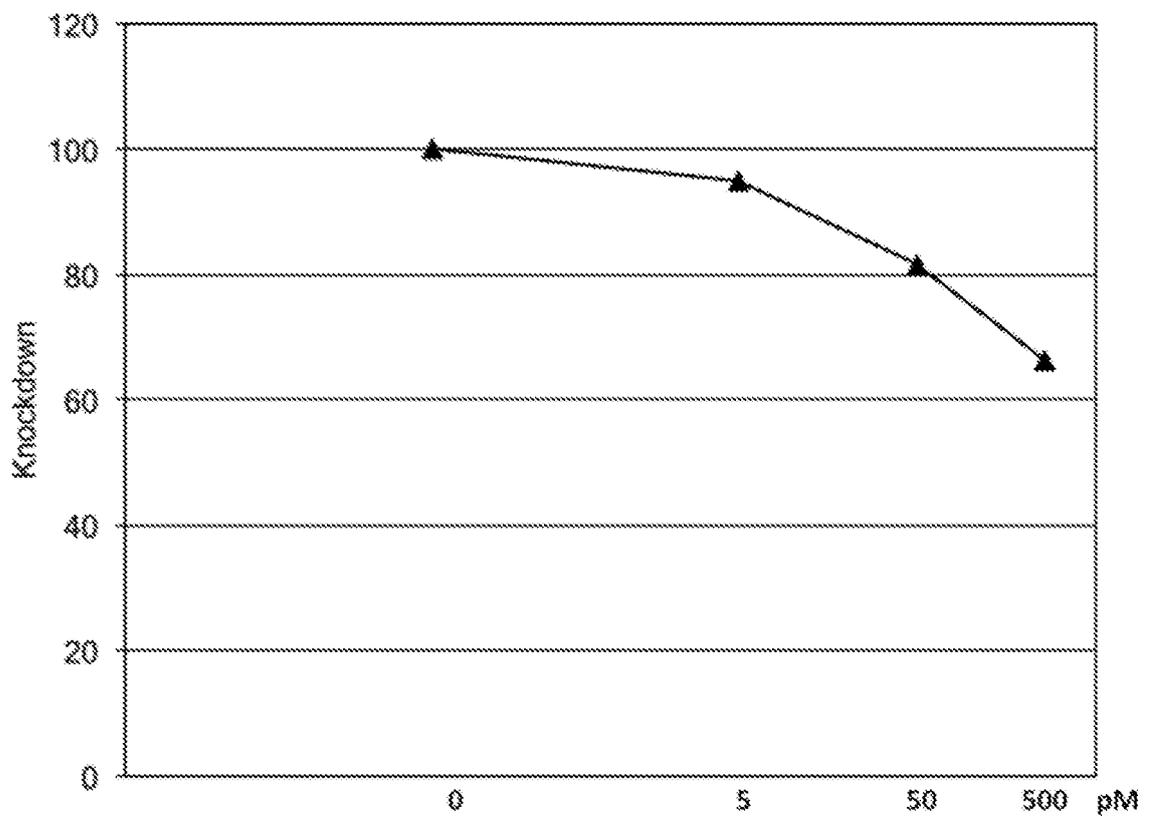


FIG. 14



REFERENCES CITED IN THE DESCRIPTION

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