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(54) Title: PLANT-DERIVED POLYSACCHARIDES FOR DELIVERY OF RNA-BASED THERAPIES

(57) Abstract: Compositions and methods are provided involving polysaccharides and nucleic acids. In particular embodiments, the nucleic acid is an siRNA directed to a mammalian heat shock protein coding sequence. In additional embodiments, the polysaccharide comprises galactomannan or galacturonic acid.
DESCRIPTION

PLANT-DERIVED POLYSACCHARIDES FOR DELIVERY OF RNA-BASED THERAPIES

BACKGROUND OF THE INVENTION

This application claims the benefit of priority to U.S. Provisional Patent Application Serial No. 61/367,507, filed July 25, 2010, and U.S. Provisional Patent Application Serial No. 61/424,434, filed December 17, 2010, both of which are hereby incorporated by reference in their entirety.

STATEMENT REGARDING FEDERALLY-SPONSORED RESEARCH OR DEVELOPMENT

This invention was made in part with government support under Grant No. ROI CA91889 awarded by the National Institutes of Health. The United States Government has certain rights in the invention.

BACKGROUND OF THE INVENTION

A. Field of the Invention

The invention relates to the fields of biology and medicine. More particularly, the invention provides compositions and methods for the delivery of RNAs and other therapeutics using plant derived polysaccharide compositions.

B. Related Art

The twenty-five kilo Dalton heat shock protein (Hsp25) belongs to the family of small HSP and is the murine homologue of human Hsp27, which was originally identified as an estrogen responsive gene in breast cancer cells (Oesterreich et al., 1996). Unlike the large HSP, which function through ATP-dependent mechanisms, Hsp25/27 operates through ATP-independent mechanisms (Egeblad and Werb, 2002; Soldes et al., 1999). Importantly, elevated Hsp27 levels have been found in various tumors, including breast, prostate, gastric, uterine, ovarian, head and neck, and tumor arising from the nervous system and urinary system (Budhram-Mahadeo and Heads, 2007). In ER-a positive benign neoplasia, elevated levels of Hsp27 have been shown to promote the progression to more malignant phenotypes.
(O’Neill et al., 2004). These studies were supported by findings that demonstrate enhanced Hsp27 protein in breast cancer cells correlated well with increased anchorage independent tumor growth (Rust et al., 1999), increased resistance to chemotherapeutic drugs (including cisplatin and doxorubicin) and increased metastatic potential in vitro (Ciocca et al., 1992; Oesterreich et al., 1993; Yamamoto et al., 2001). Together, these studies predict that elevated Hsp27 in breast cancer will give rise to aggressive disease that is refractory to treatment and so have poor prognosis (Budhram-Mahadeo and Heads, 2007). Indeed, elevated Hsp27 expression in tumors correlated with shorter disease free survival and recurrence in node-negative breast cancer (Storm et al., 1996; Thor et al., 1991), whereas its induction following chemotherapy also predicted poor prognosis and shorter disease free survival (Vargas-Roig et al., 1998). With all the background to date, no selective Hsp27 inhibitors have reached clinical trials.

The inability of CD8+ T lymphocytes to recognize tumor-associated antigenic (TAA) peptides presented on MHC class I molecules remains a formidable barrier limiting the success of immunotherapy (Sondel et al., 2001). In normal cells the proteasome system efficiently generates peptides from intracellular antigens, which are loaded onto MHC class I molecules for presentation to T lymphocytes (Kloetzel, 2004). Within the proteasome system, the proteasome activator, PA28, is a modulator of the proteasome-catalyzed generation of peptides presented via MHC class I molecules and the selective increase in cellular levels of PA28α results in improved antigen presentation (Groettrup et al., 1996; Dick et al., 1996). In addition, PA28 is essential for the recognition of epitope on melanoma cells by specific CTL (Sun et al., 2002) and may alter the quality of products generated by proteasome cleavage (Stohwasser et al., 2000; Whitby et al., 2000). The overexpression of the PA28αβ subunit enhanced MHC class I-restricted presentation of two viral epitopes and purified PA28α and β subunit accelerated T cell epitope generation by the 20S proteasome in vitro Groettrup et al., 1996). It is presumed that PA28α facilitates a transfer of antigenic peptides into the endoplasmic reticulum (Groettrup et al., 1995). These data suggest that an efficient, well functioning proteasome system is beneficial for specific CD8+ T lymphocyte recognition of tumors and ultimate cytolysis. However, to date there are no clinical trials of anti-cancer drugs based on an increase in proteasome activity.
**SUMMARY OF THE INVENTION**

Thus, in accordance with the present invention, there is provided a pharmaceutical composition comprising (a) an active therapeutic agent; (b) a purified water soluble polysaccharide comprising galactomannan or galacturonic acid; and, (c) a pharmaceutically acceptable formulation. The active agent may be a chemotherapeutic agent, a nucleic acid, or a protein.

The nucleic acid may be a double-stranded RNA 10-130 nucleotides in length comprising one strand with a sequence that is at least 85% complementary to a mammalian heat shock protein coding sequence, such as one that is 17-30 nucleotides in length. The heat shock protein may be human heat shock factor 27, and the RNA may have at least 85%, 90% or 95% sequence identity to SEQ ID NO:1 or SEQ ID NO:2. The RNA may be identical to SEQ ID NO:1 or SEQ ID NO:2, and/or may comprises the sequence of SEQ ID NO:1 or SEQ ID NO:2. The RNA may comprise two separate but hybridized strands, may be blunt-ended, may be a hairpin, may have a 5' and/or 3' overhang, and may comprise one or more modified nucleotides. The RNA and polysaccharides may be directly attached, such as with chemically conjugation. More than one RNA molecule may be conjugated to a polysaccharide. The ratio of RNA: polysaccharide may be from about 1:20 to about 10:1.

In certain embodiments, the nucleic acid is isolated or purified away from cellular components or from individual nucleic acid residues or truncated nucleic acids. In specific embodiments, the nucleic acid is synthetic. In other embodiments, the nucleic acid is recombinant.

The chemotherapeutic agent may be a cancer therapeutic. The active therapeutic agent may enhance the stability of microtubules. The therapeutic active agent may be a taxane, docetaxel, paclitaxel, a DNA intercalator, or selected from the group consisting of doxorubicin, berberine, ethidium bromide, proflavine, daunomycin, and thalidomide.

The polysaccharide may comprises a galactomannan moiety, such as the following structure:
wherein R1 or R2 is an OH, a sugar (such as glucose, mannose, etc), a substituted sugar, -OR (R is alkyl, substituted alkyl, alkene, substituted alkene, alkyne, or substituted alkyne and x + y is a number from 50 to 150 and the ratio of x:y is about 1:1 to about 1:5. In particular embodiments, x + y is a number that ranges from 85 to 100, and in more particular embodiments is a number that ranges from 90 to 95. In particular, R1 may be OH and R2 may be OH. The ratio of x:y may be about 1:1. The polysaccharide may have the structure of a polysaccharide obtained from Trigonella foenum-graecum, or obtained from the seeds of fenugreek. The polysaccharide may comprises a galacturonic acid moiety and/or a glucose moiety, and/or a glucopyranose moiety or a galactopyranose moiety. The polysaccharide may have the structure of a polysaccharide obtained from psyllium (Plantago psyllium), okra (Hibiscus esculentus), prickly pear cactus (Opuntia Ficus -Indica) and Aloe vera (Aloe barbadensis).

The pharmaceutical composition may comprise multiple different polysaccharides, such as wherein at least two of the different polysaccharides comprise a galactomannan or a galacturonic acid moiety. The at least two polysaccharides can be obtained from psyllium (Plantago psyllium), okra (Hibiscus esculentus), fenugreek (Trigonella foenum-graecum), prickly pear cactus (Opuntia Ficus -Indica), or Aloe vera (Aloe barbadensis).

The pharmaceutical composition may be in the form of nanoparticles comprising RNA and polysaccharides. The nucleic acid may be a double-stranded RNA 10-130 nucleotides in length comprising one strand with a sequence that is at least 85%
complementary to a mammalian heat shock protein coding sequence, such as one that is 17-30 nucleotides in length. The heat shock protein may be human heat shock factor 27.

In certain embodiments, the target is set forth in SEQ ID NO:1, so the RNA is partially or fully complementary to a portion of SEQ ID NO:1. In some embodiments, the RNA is 17, 18, 19, 20, 21, 22, 23, 24, 25, 26, 27, 28, 29, or 30 nucleotides in length (or any range derivable therein) and is or is at least 85, 86, 87, 88, 89, 90, 91, 92, 93, 94, 95, 96, 97, 98, 99, or 100% complementary, or any range derivable therein, to SEQ ID NO:1.

In specific embodiments, the RNA may have or have at least 90, 91, 92, 93, 94, 95, 96, 97, 98, 99, or 100% sequence identity with SEQ ID NO:2, SEQ ID NO:3, SEQ ID NO:4, or SEQ ID NO:5. The RNA may be identical to SEQ ID NO:2, SEQ ID NO:3, SEQ ID NO:4, or SEQ ID NO:5 or may comprise the sequence of SEQ ID NO:2, SEQ ID NO:3, SEQ ID NO:4, or SEQ ID NO:5. In certain embodiments, the RNA comprises or consists essentially of the sequence of SEQ ID NO:2. The RNA may comprise two separate but hybridized strands, may be blunt-ended, may be a hairpin, may have a 5' and/or 3' overhang, and may comprise one or more modified nucleotides.

In certain embodiments, there is a pharmaceutical composition consisting essentially of (a) an active therapeutic compound, such as a nucleic acid, (b) a purified water soluble polysaccharide comprising galactomannan or galacturonic acid; and, (c) a pharmaceutically acceptable formulation. It is specifically contemplated that in some embodiments, the only active therapeutic compound is a nucleic acid molecule. In other embodiments, the composition does not contain any other active compounds (the polysaccharide will not have any detectable therapeutic activity relative to the active therapeutic compound or to the therapeutic goal to be achieved).

In another embodiment, there is provided a method for treating a patient for a disease or condition comprising administering to the patient an effective amount of the pharmaceutical formulation as described above. The disease or condition may be cancer, a bacterial or viral infection, inflammation, or toxin exposure. In particular, the cancer may be breast cancer, lung cancer, head & neck cancer, esophageal cancer, prostate cancer, ovarian cancer, uterine cancer, cervical cancer, colon cancer, stomach cancer, liver cancer, testicular cancer, skin cancer, pancreatic cancer, rectal cancer, melanoma, glioma, neuroblastoma or sarcoma. The method may further comprise treating the patient with an additional cancer therapy, wherein the additional therapy is tumor resection, chemotherapy, or radiation. The patient may be treated with the additional cancer therapy prior to treatment with the
pharmaceutical composition, concurrently with the pharmaceutical composition or after treatment with the pharmaceutical composition. The method may further comprise identifying a patient in need of the pharmaceutical composition. The pharmaceutical composition may be administered intravenously, intratumorally, intraperitoneally, intrathecally, intraarterially, subcutaneously, orally, topically, by lavage, or by direct injection.

In still another embodiment, there is provided a method for administering a therapeutic agent to a cell comprising contacting the cell with the pharmaceutical composition as described above. The cell may be a cancer cell, such as one that overexpresses a heat shock protein, and the pharmaceutical composition inhibits expression of the heat shock protein.

In yet a further embodiment, there is provided a method for preparing a pharmaceutical composition comprising combining a reactive polysaccharide comprising galactomannan or galacturonic acid with a nucleic acid. The reactive polysaccharide may be a free radical formed by contacting the polysaccharide with a composition comprising eerie ion, nitric acid, and/or potassium persulfate. The nucleic acid may be an RNA molecule, such as one that is fully or partially double-stranded, and such as one that is 17-30 nucleotides in length. The RNA comprise one strand with a sequence that is at least 85%, 90% or 95% complementary to a mammalian heat shock protein coding sequence.

Another embodiment comprises a composition comprising (1) a water-soluble polysaccharide comprising galactomannan or galacturonic acid and (2) a nucleic acid made by a method comprising (a) obtaining purified polysaccharide; (b) creating a reactive polysaccharide through free radical polymerization; and (c) contacting the reactive polysaccharide with the nucleic acid to form a polysaccharide grafted with the nucleic acid.

It is contemplated that any method or composition described herein can be implemented with respect to any other method or composition described herein.

The use of the word "a" or "an" when used in conjunction with the term "comprising" in the claims and/or the specification may mean "one," but it is also consistent with the meaning of "one or more," "at least one," and "one or more than one."

These, and other, embodiments of the invention will be better appreciated and understood when considered in conjunction with the following description and the accompanying drawings. It should be understood, however, that the following description, while indicating various embodiments of the invention and numerous specific details thereof,
is given by way of illustration and not of limitation. Many substitutions, modifications, additions and/or rearrangements may be made within the scope of the invention without departing from the spirit thereof, and the invention includes all such substitutions, modifications, additions and/or rearrangements.
BRIEF DESCRIPTION OF THE DRAWINGS

The following drawings form part of the present specification and are included to further demonstrate certain aspects of the present invention. The invention may be better understood by reference to these drawings and the detailed description presented below.

FIG. 1A-C - Permanent gene silencing and expression of Hsp25shRNA in 4T1 breast adenocarcinoma cells using a lentiviral vector. (FIG. 1A) HIV-based lentivirus construct pLVTHM was employed to infect 4T1 cells. Construct contains a 5′-long terminal repeats (LTR), gene encoding GFP as reporter and woodchuck hepatitis virus response element (WPRE) as enhancer of gene expression, placed under the tight control of elongation factor alpha (EF-1 α) promoter. The Hsp25shRNA stem loop was placed downstream of the H1 promoter, and the self inactivating (SIN) element was placed downstream of the HIV-Hsp25shRNA sequence (top panel). Schematic representation of 4T1-Hsp25shRNA and 4T1-ControlshRNA hairpin sequences (bottom panel) (SEQ ID NO:2 and NO:6). (FIG. 1B) Sorted 4T1-Hsp25shRNA cells were imaged using a digital inverted fluorescent microscope. Micropictograms are phase contrast (left panel) and fluorescence images (right panel) and was obtained under 40X magnification. (FIG. 1C) Western blot analysis of freshly sorted protein lysates from 4T1-controlshRNA (left lane) and 4T1-Hsp25shRNA cells (right lane), immunoblotted with anti-Hsp25 (top panel) or β-actin (bottom panel). Data are representative of three independently performed experiments.

FIGS. 2A-B - Effects of gene targeted Hsp25 silencing on 4T1 breast adenocarcinoma cell functions. (FIG. 2A) 4T1-controlshRNA cells (filled circles) or 4T1-Hsp25shRNA cells (open circles) were seeded on day 0 in a 6-well plate at a density of 2.5 x 10^4 cells/well in DMEM supplemented with 10% FBS. At various times from days 1-4, cells were trypsinized and counted with a hemocytometer under a phase-contrast light microscope. Data represent the mean number of cells ± S.D. and is the sum of four independently performed experiments. *, p<0.01 vs 4T1-controlshRNA (Student's t-test). (FIG. 2B) 4T1-controlshRNA or 4T1-Hsp25shRNA cells were trypsinized, counted and added to the upper section of the Boyden chamber according to manufacturer's instruction (BD Biosciences, USA). FBS (1%) was added to the top chamber and 10% FBS added to the lower chamber. Transwell plates were incubated for an additional 20 h at 37°C. Cells on the inside of the transwell inserts were removed with a cotton swab, and cells on the underside of the insert were fixed and stained by using Hema 3 manual staining system (Fisher Scientific).
Photographs of ten random fields were taken, and the cells were counted to calculate the mean number of cells that had transinvaded. Bars represent the mean number of invading cells ± S.D. and is the sum of triplicate wells. *, p<0.01 vs 4T1-controlshRNA (Student's t-test) (bottom panel).

**FIGS. 3A-B - Silencing Hsp25 protein expression enhances prohibitin expression.** (FIG. 3A) 4T1-controlshRNA cells (filled bars) and 4T1-Hsp25shRNA cells (open bars) were used to isolate total RNA and the relative prohibitin mRNA expression was measured using real-time PCR analysis. Data are the mean prohibitin mRNA expression ± SD and is the sum of three independently performed experiments. *, p<0.001 vs 4T1-controlshRNA cells (Student's t-test). (FIG. 3B) 4T1-controlshRNA cells and 4T1-Hsp25shRNA cells were lysed, proteins extracted and subjected to immunoblotting with anti-prohibitin Mab or β-actin. The intensity of the bands were analyzed by densitometry with a video densitometer (Chemilmager™ 5500; Alpha Innotech, San Leandro, CA) using the AAB software (American Applied Biology). Bars represent the mean prohibitin protein expression and is a representative experiment from three independently performed experiments with similar results.

**FIGS. 4A-C - Proteasome activity is increased by silencing Hsp25 protein expression.** (FIG. 4A) 4T1-controlshRNA cells (filled bars) and 4T1-Hsp25shRNA cells (open bars) were used to isolate total RNA and the relative PA28a mRNA expression was measured using real-time PCR analysis. Data are the mean prohibitin mRNA expression ± SD and is the sum of four independently performed experiments. *, p<0.001 vs 4T1-controlshRNA cells (Student's t-test). (FIG. 4B) The intensity of the bands were analyzed by densitometry with a video densitometer (Chemilmager™ 5500; Alpha Innotech) using the AAB software. Bars represent the mean PA28a protein expression and is a representative experiment from three independently performed experiments with similar results. (FIG. 4C) 20S proteasome activity was measured by incubation of cell extracts from 30 µg 4T1-controlshRNA (filled bars) or 4T1-Hsp25shRNA (open bars) for 90 min with a fluorogenic substrate (Suc-LLVY-AMC) in the absence or presence of lactacystin (25 µM). Free AMC fluorescence was measured by using a 380/460 nm filter set in a fluorometer. Data are the mean proteasome activity (% control ± SD) and is the sum of three independently performed experiments. *, p<0.001 vs 4T1-controlshRNA cells (Student's t-test).
FIGS. 5A-B - Silencing hsp25 gene expression in 4T1 cells induces tumor regression. (FIG. 5A) 4Tl-controlshRNA cells or 4T1-Hsp25shRNA cells were injected into the mammary pads of female BALB/c mice and tumor growth was monitored on specific days post tumor cell injection using the MaestroTM in vivo imaging system (CRI). Bars represent the mean GFP signal/exposure (total signal scaled counts/seconds) from 4T1-controlshRNA cells (filled bars) or 4T1-Hsp25shRNA cells (open bars) and is the sum of three mice/group (n=3). *, p0.001 vs 4Tl-controlshRNA cells (Student's t-test) (bottom panel). (FIG. 5B) Colony formation of tumor derived from lungs of mice injected with 4T1-controlshRNA (top panel) or 4T1-Hsp25shRNA cells, was plated at different dilution ratios (1:20-1:320). Plates were stained and the number of cells was counted. Data represent the mean number of colonies ± SD and is a representative experiment from four independently performed experiments. *, p0.001 vs 4Tl-controlshRNA cells (Student's t-test).

FIGS. 6A-B - Silencing hsp25 gene expression augments CD8+- T lymphocyte-dependent tumor recognition and killing. (FIG. 6A) 4T1-Hsp25shRNA cells (10^6) were injected into mammary pads of 6-8 week-old female BALB/c mice. When tumors started regressing at the end of two weeks, and spleen tissues were harvested from the animals and CD8+ and CD8- T lymphocytes were isolated using negative selection technique according to the manufacturer’s instructions (Miltenyi Biotech). 4T1-controlshRNA cells (1.5 x 10^6) were seeded as quintuplicate in 96 well tissue culture plates. CD8+ or CD8- T lymphocytes recovered from mice injected with 4T1-Hsp25shRNA cells were added at different effector/target ratios (10:1 and 30:1), and cytotoxicity was measured by lactate dehydrogenase-cytotoxicity assay kit II, according to the manufacturer’s instructions (BioVision). Data are the sum of three independently performed experiments. *, p<0.001 versus 4Tl-controlshRNA cells (Student's t-test). (FIG. 6B) 4T1-Hsp25shRNA cells (10^6) were injected into the mammary glands of female BALB/c mice and tumor regression was measured using MaestroTM in vivo imaging system. At the end of four weeks splenocytes were collected and CD8+ T lymphocytes were isolated and enriched by negative selection according to manufacturer's instruction (Miltenyi Biotec). The lymphocytes recovered were designated CD8+T cells. The fraction depleted of CD8+T lymphocytes were designated CD8-T cells. Adoptive transfer of 10^6 4T1-Hsp25shRNA reactive CD8+ T cells or CD8- T cells (top panel) was performed via the tail vein on day 5 post TCI into mice injected with 4T1-controlshRNA tumors. Data are fluorescence micropictogram of GFP-tagged tumors (green fluorescence) measured on various days post tumor cell injection (top panel). Bars represent
the mean GFP signal/exposure (total signal scaled counts/seconds) from animals adoptively transferred with CD8+ T cells (filled bars) or CD8+ T cells (open bars) and is the sum of three mice/group (n=3). *pO.001 vs 4T1-controlshRNA cells (Student's t-test) (bottom panel).

FIG. 7 - Mechanism for the free radical initiation on the polysaccharide backbone.

DETAILED DESCRIPTION OF EMBODIMENTS

This study demonstrates that increasing the proteasome activity of 4T1 breast adenocarcinoma cells by lentivirus-mediated hsp25 gene silencing correlates well with increased specific CD8+ T lymphocyte tumor killing. The inventors show that silencing Hsp25 enhances the proteasome regulator PA28α mRNA expression (FIG. 4A) and protein expression (FIG. 4B) and concomitantly increases proteasome chymotrypsin-like activity (FIG. 4C). This is significant since the role for PA28α in antigen presentation has been established. Groettrup and colleagues reported that increased expression of PA28α results in marked enhancement of recognition by virus specific cytotoxic T cells (Groettrup et al, 1996). In addition, an essential role for PA28 was described in the melanoma cell line, Mel-18a. These authors demonstrated that recognition of TRP2-expressing melanoma cells by TRP2360-368-specific CTL directly correlated with the presence of PA28 and impaired epitope presentation on Mel-18a cells could be rescued by transfection of PA28 encoding plasmids (Sun et al, 2002).

4T1 breast adenocarcinoma cells express elevated levels of Hsp25, which is effectively suppressed in Hsp25 silenced cells (FIG. ID). This is important not only because elevated levels of Hsp27 in breast cancer gives rise to aggressive disease and poor prognosis (Budhram-Mahadeo and Heads, 2007), but also because elevated Hsp27 levels have been reported to confer tumor protection against Bortezomib-induced cell death (Chauhan et al, 2003). Bortezomib is characterized as a reversible proteasome inhibitor, with potent anti-cancer effects against multiple myeloma (Mitsiades et al, 2002). Bortezomib was shown to effectively induce apoptotic cell death in DHL6 lymphoma cells (do not express significant Hsp27), but not DHL4 lymphoma cells (expressing high basal levels of Hsp27). Blocking the elevated Hsp27 expression in DHL4 lymphoma cells using antisense against Hsp27 restored sensitivity to Bortezomib, providing a therapeutic advantage to combining agents that
suppress Hsp27 expression to overcome tumors that might be resistant to Bortezomib treatment (Chauhan et al, 2003).

While the inventors do not want to be bound to theory, they hypothesize that high Hsp25 expression in 4Tl breast adenocarcinoma cells represses proteasome activity is substantiated by these findings. However, conclusive proof that silencing Hsp25 improves the quantity and/or quality of peptides presented onto MHC class I for specific CD8+ T lymphocyte recognition, is thus far elusive. This is due to the technical inability to accurately determine the quantity and/or quality of peptides loaded onto MHC class I molecules on tumors. The Srivastava lab previously demonstrated that vaccination of mice with Hsp70 preparations derived from the Meth A sarcoma, but not from normal tissues, renders the mice immune to a further challenge with Meth A sarcoma. An attempt to determine the active peptide chaperoned by the Hsp70 protein by separation of the low-molecular weight material on a C18 reverse-phase column revealed a diverse array of peptides with molecular mass between 1,000 and 5,000 daltons (Udono and Srivastava, 1993).

Data demonstrating that depletion of NK cells in vivo resulted in enhanced anti-tumor killing and complete tumor regression was initially confounding (FIG. 6A). However, further examination of the monoclonal antibody used to deplete NK cells in vivo revealed it to be the 5E6 Mab F(ab')2 fragment, which reacts with Ly-49C, a NK inhibitory receptor expressed on subsets of natural killer cells and NK1.1+ or DX5+ T cells in Balb/c mice (Yu et al, 1996). Studies by Koh and colleagues demonstrated that NK cell-mediated anti-tumor effector functions are increased against syngeneic tumors in vitro and in vivo by blockade of the Ly49C and Ly49I inhibitory receptors using the 5E6 monoclonal antibody (Koh et al, 2001).

A role of CD8+ T lymphocytes was confirmed by the in vivo deletion of mice injected with 4Tl-Hsp25shRNA cells (FIG. 6A). Although tumors grew in these mice in the absence of CD8+ T lymphocytes, there was no metastasis in any of the tissues of mice injected with 4Tl-Hsp25shRNA (data not shown). The possibility exists that the reason for the lack of pulmonary metastasis could be that there is only a concomitant decrease in primary tumor growth. However, the in vitro data presented here on the effect of silencing Hsp25 on migration and invasion (FIG. 2A), as well as, published data demonstrating that Hsp25 is critical for maintaining the integrity of cytoskeleton (Guay et al, 1997), suggests that in the absence of specific CD8+ CTL-mediated killing the tumor is still incapable to leaving the primary tumor foci. The inventors were unable to drastically increase the levels of tumors in the 4Tl-Hsp25shRNA group due to regulation by IACUC. This hypothesis is further
supported by experiments in which 4Tl-Hsp25shRNA tumors grew in BALB/c-nu/nu mice (which do not have functional CD8+ or CD4+ T lymphocytes) however, no metastasis was detected (data not shown).

Proof that silencing Hsp25 improved the quality and/or quantity of peptides loaded onto MHC class I molecules for CD8+ T lymphocyte recognition was therefore obtained indirectly in experiments demonstrating that enhanced proteasome activity correlated with improved specific CD8+, but not CD8- T lymphocyte lysis of 4Tl-controlshRNA target cells (FIG. 6C). In addition, the ability of adoptively transferred 4Tl-Hsp25shRNA reactive CD8+, but not CD8- T lymphocytes to rescue mice injected with 4Tl-controlshRNA tumors (FIG. 5D), which has been demonstrated to succumb from the tumor burden (FIGS. 5A, 6A and 6D) confirms that silencing Hsp25 improved the quality and/or quantity of peptides recognized by CD8+ T lymphocytes via a mechanism dependent on enhanced proteasome activity. Taken together, these results demonstrate that Hsp25 silencing effects on tumor growth are mediated by the immune system and not only by effects on proliferation and invasion capacity of the cells.

The potent efficacious anti-tumor activity observed by silencing Hsp25 strongly suggests that other anti-tumor mechanisms have also been activated. This is supported by reports demonstrating that, 1) Hsp27 plays an essential role in stabilizing actin filaments, a structural protein important for maintaining the integrity of cytoskeleton (Guay et al, 1997); 2) cell cycle progression and proliferation (Farooqui-Kabir et al, 2004), and 3) apoptosis via caspase-3 activation (Rocchi et al, 2005; Rocchi et al, 2006). Taken together, these data have obvious clinical applications in light of recent successful clinical trials with the proteasome inhibitors. The inventors' studies would suggest that screening for patients on the basis of proteasome activity might be an additional benefit and that tumors with high Hsp27 expression might benefit from therapies which increase proteasome activity and concomitantly lower Hsp27 expression. However, it is still necessary to improve delivery of Hsp27 agents to subjects to achieve optimal therapeutic results.

A major challenge in cancer therapy is drug delivery. Drug delivery systems in cancer treatment aim to increase the therapeutic efficacy of the chemotherapeutic agent by modifying its bio-distribution and controlling the rate at which the agent is released from the carrier to the systemic circulation or tissues, thereby minimizing its interaction with non-pathological sites in the body. A variety of means, including protective coats and pH-
dependent polymers, are currently in use with varying success. To date, only available
delivery systems exhibit high toxicity.

As natural biomaterial, polysaccharides are highly stable, safe nontoxic, hydrophilic,
biodegradable, and abundantly available and have low processing costs as compared to
current lipid-based and protein-based delivery systems (Liu et al., 2008). The presence of
hydrophilic groups in natural polysaccharides enable them to form non-covalent bond with
biological tissues (mainly epithelia cells and mucous membranes) forming bioadhesion. This
in turn could prolong the residence time and therefore increase the absorbance of the loaded
drug (Liu et al., 2000). Non-starch, linear polysaccharides are resistant to the digestive action
of the gastrointestinal enzymes and retain their integrity in the upper gastrointestinal tract.
Matrices manufactured from these polysaccharides therefore remain intact in the stomach and
the small intestine, but once they reach the colon they are degraded by the bacterial
polysaccharidases. This property makes these polysaccharides exceptionally suitable for the
formulation of colon-targeted drug delivery systems (Chaurasia et al., 2006; Shirwaikar et al.,
2008).

An exhaustive search of the literature reveals that the use of polysaccharides in drug
delivery system is relatively new and research on its use in cancer chemotherapy is sparse. Some related publications on the use of polysaccharides as a drug delivery system in
chemotherapy includes the use of chitosan as delivery system for siRNA-based anticancer
drugs (Ji et al., 2009) and Angelica polysaccharide-based colon specific drug delivery system
(Zhou et al., 2009). Although chitosan is capable of delivering siRNA to target colorectal
cancer cells, electrophoretic migration assay study showed that higher concentration of the
chitosan at lower pH exhibits significant toxicity (Ji et al., 2009).

I. Heat Shock Proteins

A. General

Heat shock proteins (HSPs) are molecular chaperones that protect cells against a wide
variety of stressors (Lindquist and Craig, 1988). By binding unfolded, mis-folded or mutated
peptides or proteins, and transporting them to the endoplasmic reticulum, HSPs prevent
potential aggregation and/or cell death (Fink, 1999). Recently, additional roles, such as
danger signals that are produced and released when cells are under stress as activators of the
immune system, have been ascribed to HSPs (Asea et al., 2000; Asea, 2003, 2005; Gastpar et
Beginning in the mid-1980s, investigators recognized that many HSPs function as molecular chaperones and thus play a critical role in protein folding, intracellular trafficking of proteins, and coping with proteins denatured by heat and other stresses. Accordingly, the study of stress proteins has undergone explosive growth.

Production of high levels of heat shock proteins can also be triggered by exposure to different kinds of environmental stress conditions, such as infection, inflammation, exercise, exposure of the cell to toxins (ethanol, arsenic, trace metals and ultraviolet light, among many others), starvation, hypoxia (oxygen deprivation), nitrogen deficiency (in plants), or water deprivation. Consequently, the heat shock proteins are also referred to as stress proteins and their upregulation is sometimes described more generally as part of the stress response.

The mechanism by which heat-shock (or other environmental stressors) activates the heat shock factor has not been determined. However, some studies suggest that an increase in damaged or abnormal proteins brings HSPs into action.

Some bacterial heat shock proteins are upregulated via a mechanism involving RNA thermometers such as the FourU thermometer, ROSE element and the Hsp90 cis-regulatory element.

As discussed above, heat shock proteins function as intra-cellular for other proteins. They play an important role in protein-protein interactions such as folding and assisting in the establishment of proper protein (shape) and prevention of unwanted protein aggregation. By helping to stabilize partially unfolded proteins, HSPs aid in transporting proteins across membranes within the cell.

Heat-shock proteins also occur under non-stressful conditions, simply "monitoring" the cell's proteins. Some examples of their role as "monitors" are that they carry old proteins to the cell's proteasome and they help newly synthesised proteins fold properly. These activities are part of a cell's own repair system, called the "cellular stress response" or the "heat-shock response."

Heat shock proteins appear to serve a significant cardiovascular role. Hsp90, hsp84, hsp70, hsp27, hsp20, and alpha-B-crystallin all have been reported as having roles in the cardiovascular. Hsp90 binds both endothelial nitric oxide synthase and soluble guanylate cyclase which in turn are involved in vascular relaxation. A downstream kinase of the nitric oxide cell signalling pathway, protein kinase G, phosphorylates a small heat shock protein, hsp20. Hsp20 phosphorylation correlates well with smooth muscle relaxation and is, one significant phosphorylation involved in the process. Hsp20 appears significant in development...
of the smooth muscle phenotype during development. Hsp20 also serves a significant role in preventing platelet aggregation, cardiac myocyte function and prevention of apoptosis after ischemic injury, and skeletal muscle function and muscle insulin response. Extracellular and membrane bound heat-shock proteins, especially Hsp70, are involved in binding and presenting them to the immune system.

Heat shock proteins (hsp) are overexpressed in different human cancers. Cancer treatments that are in development include inhibitors of different heat shock proteins. Hsp90, Hsp70, and Hsp27 have been implicated in cancer. Inhibitors of these proteins are potential cancer treatments, and siRNAs targeted against each of these is contemplated in methods and compositions described herein. Other heat shock proteins include Hsp60, Hsp72, and Hsp100, which may also be targeted for destruction with an siRNA.

B. Hsp27

In particular embodiments, Hsp27 is the target of one or more siRNA molecules. Hsp27 is a major phosphoprotein during muscle contraction. Hsp27 functions in smooth muscle migration and appears to serve an integral role. Elevated Hsp27 levels have been found in various tumors, including breast, prostate, gastric, uterine, ovarian, head and neck, and tumor arising from the nervous system and urinary system (Budhram-Mahadeo and Heads, 2007). In estrogen receptor (ER)-a positive benign neoplasia, elevated levels of Hsp27 have been shown to promote the progression to more malignant phenotypes (O’Neill et al., 2004). These studies were supported by findings that demonstrate enhanced Hsp27 protein in breast cancer cells correlated well with increased anchorage independent tumor growth (Rust et al., 1999), increased resistance to chemotherapeutic drugs (including cisplatin and doxorubicin) and increased metastatic potential in vitro (Ciocca et al., 1992; Oesterreich et al., 1993; Yamamoto et al., 2001). Together, these studies predict that elevated Hsp27 in breast cancer will give rise to aggressive disease that is refractory to treatment and so have poor prognosis (Budhram-Mahadeo and Heads, 2007). Indeed, elevated Hsp27 expression in tumors correlated with shorter disease free survival and recurrence in node-negative breast cancer (Thor et al., 1991; Storm et al., 1996), whereas its induction following chemotherapy also predicted poor prognosis and shorter disease free survival (Vargas-Roig et al., 1998). With all the background, to date no selective Hsp27 inhibitors have reached clinical trials.

This is important since silencing Hsp27 expression 1) destabilizes actin filaments, a structural protein important for maintaining the integrity of cytoskeleton, making tumors
unable to metastasize or invade tissues (Guay et al., 1997), 2) inhibits cell cycle progression and proliferation (Farooqui-Kabir et al., 2004), and 3) increases apoptosis via caspase-3 activation (Rocchi et al., 2005; Rocchi et al., 2006).

The studies reported below provide a hitherto unknown mechanism by which elevated Hsp27 expression results in aggressive diseases that are not responsive to most chemotherapeutic regimes having a poor prognosis. The data demonstrate that, apart from the functions described above, silencing Hsp27 increases proteasome activity, which in turn results in increased antigen presentation, efficient CD8+ T cell stimulation and resultant inhibition of tumor growth. This is important because there are currently anticancer drugs which are said to function as proteasome inhibitors.

Drugs based on the inhibition of proteasome function seems counter intuitive. This is because in all cells the proteasome system efficiently generates peptides from intracellular antigens, which are loaded onto MHC class I molecules for presentation to T lymphocytes (Kloetzel, 2004) (FIG. 1). Within the proteasome system, the proteasome activator, PA28, is a modulator of the proteasome-catalyzed generation of peptides presented via MHC class I molecules and the selective increase in cellular levels of PA28a results in improved antigen presentation (Dick et al., 1996; Groettrup et al., 1996). In addition, PA28 is essential for the recognition of epitope on melanoma cells by specific CTL (Sun et al, 2002) and may alter the quality of products generated by proteasome cleavage (Stohwasser et al., 2000; Whitby et al, 2000). The overexpression of the PA28α/β subunit enhanced MHC class I-restricted presentation of two viral epitopes and purified PA28α and β subunit accelerated T cell epitope generation by the 20S proteasome in vitro (Groettrup et al, 1996). It is presumed that PA28a facilitates a transfer of antigenic peptides into the endoplasmic reticulum (Groettrup et al., 1995). Together, these data suggest that an efficient, well functioning proteasome system is beneficial for specific CD8+ T lymphocyte recognition of tumors and ultimate cytolysis.
II. Interfering RNAs

The present invention contemplates the use of double-stranded RNAs that target HSPs such as HSP27. RNAi, or RNA interference, is a conserved biological response that is present in many, if not most, eukaryotic organisms. RNAi results in transcript silencing that is both systemic and heritable, permitting the consequences of altering gene expression to be examined throughout the development and life of an animal.

In the RNAi process, long double-stranded RNA molecules (dsRNA) can induce sequence-specific silencing of gene expression in primitive and multicellular organisms. These long dsRNAs are processed by a ribonuclease called Dicer into 21 to 23 nucleotide (nt) guide RNA duplexes termed short interfering RNA (siRNA). The siRNA is subsequently used by an RNA-induced silencing complex (RISC), a protein-RNA effector nuclease complex that uses siRNA as a template to recognize and cleave RNA targets with similar nucleotide sequences. The composition of RISC is not completely defined, but includes argonaute family proteins. The RISC unwinds siRNAs and associates stably with the (antisense) strand that is complementary to the target mRNA. Depending on the degree of homology between a siRNA and its target mRNA, siRNA-RISC complexes inhibit gene function by two distinct pathways. Most siRNAs pair imperfectly with their targets and silence gene expression by translational repression. This RNAi mechanism appears to operate most efficiently when multiple siRNA-binding sites are present in the 3′-untranslated region of the target mRNAs. In some other cases, siRNAs exhibit perfect sequence identity with the target mRNA and inhibit gene function by triggering mRNA degradation. The reduction in transcript level results in lowered levels of the target protein, resulting in phenotypic changes.

While siRNA has been shown to be effective for short-term gene inhibition in certain transformed mammalian cell lines, there may be drawbacks associated with its use in primary cell cultures or for stable transcript knockdown because their suppressive effects are by definition of limited duration. Short hairpin RNAs (shRNA), consisting of short duplex structures, in contrast to siRNAs have been proven as effective triggers of stable gene silencing in plants, *C. elegans*, and in *Drosophila*. These synthetic forms of RNA may be expressed from pol II or pol III promoters and the hairpin structure is recognized and cleaved by Dicer to form siRNA that is subsequently taken up by RISC for silencing of the target gene.

SiRNAs may be designed based on the sequence of the endogenous target using any of a variety of techniques known to those of ordinary skill in the art. Moreover, compositions
and methods for post-transcriptional gene silencing can be found in the inventor's U.S. application 12/673,575, which is hereby incorporated by reference.

B. Analogs

In addition to containing natural bases, the double-stranded RNAs of the present invention may contain non-natural bases and also may contain non-natural backbone linkages. The following discussion describes a variety of non-natural nucleic acid analogs that may be used to construct iRNAs.

A locked nucleic acid (LNA), often referred to as inaccessible RNA, is a modified nucleic acid. The ribose moiety of an LNA nucleotide is modified with an extra bridge connecting the 2' and 4' carbons. The bridge "locks" the ribose in the 3'-endo structural conformation, which is often found in the A-form of DNA or RNA. LNA nucleotides can be mixed with DNA or RNA bases in the oligonucleotide whenever desired. Such oligomers are commercially available. The locked ribose conformation enhances base stacking and backbone pre-organization. This significantly increases the thermal stability (melting temperature) of oligonucleotides (Kaur et al., 2006).

LNA nucleotides are used to increase the sensitivity and specificity of expression in DNA microarrays, FISH probes, real-time PCR probes and other molecular biology techniques based on oligonucleotides. For the in situ detection of miRNA, the use of LNA was as of 2005 the only efficient method. A triplet of LNA nucleotides surrounding a single-base mismatch site maximizes LNA probe specificity unless the probe contains the guanine base of G-T mismatch (You et al., 2006).

Other oligonucleotide modifications can be made to produce the RNAs of the present invention. For example, stability against nuclease degradation has been achieved by introducing a phosphorothioate (P=S) backbone linkage at the 3' end for exonuclease resistance and 2' modifications (2'-OMe, 2'-F and related) for endonuclease resistance (WO 20051 15481; Li et al., 2005; Choung et al., 2006). A motif having entirely of 2'-0-methyl and 2'-fluoro nucleotides has shown enhanced plasma stability and increased in vitro potency (Allerson et al., 2005). The incorporation of 2'-0-MOE and 2'-0-MOE does not have a notable effect on activity (Prakash et al., 2005).

Sequences containing a 4'-thioribose modification have been shown to have a stability 600 times greater than that of natural RNA (Hoshika et al., 2004). Crystal structure studies reveal that 4'-thioriboses adopt conformations very similar to the C3'-endo pucker observed for unmodified sugars in the native duplex (Haeberli et al., 2005). Stretches of 4'-thio-RNA
were well tolerated in both the guide and nonguide strands. However, optimization of both the number and the placement of 4'-thioribonucleosides is necessary for maximal potency.

In the boranophosphate linkage, a non-bridging phosphodiester oxygen is replaced by an isoelectronic borane (BH$_3$) moiety. Boranophosphate siRNAs have been synthesized by enzymatic routes using T7 RNA polymerase and a boranophosphate ribonucleoside triphosphate in the transcription reaction. Boranophosphate siRNAs are more active than native siRNAs if the center of the guide strand is not modified, and they may be at least ten times more nuclease resistant than unmodified siRNAs (Hall et al., 2004; Hall et al., 2006).

Certain terminal conjugates have been reported to improve or direct cellular uptake. For example, nucleic acid analogs conjugated with cholesterol improve in vitro and in vivo cell permeation in liver cells (Rand et al., 2005). Soutschek et al. (2004) have reported on the use of chemically-stabilized and cholesterol-conjugated siRNAs have markedly improved pharmacological properties in vitro and in vivo. Chemically-stabilized siRNAs with partial phosphorothioate backbone and 2'-0-methyl sugar modifications on the sense and antisense strands (discussed above) showed significantly enhanced resistance towards degradation by exo- and endonucleases in serum and in tissue homogenates, and the conjugation of cholesterol to the 3' end of the sense strand of an NAA by means of a pyrrolidine linker does not result in a significant loss of gene-silencing activity in cell culture. These studies demonstrates that cholesterol conjugation significantly improves in vivo pharmacological properties of NAAs.

LNA bases may be included in a RNA backbone, but they can also be in a backbone of 2'-0-methyl RNA, 2'-methoxyethyl RNA, or 2'-fluoro RNA. These molecules may utilize either a phosphodiester or phosphorothioate backbone.

U.S. Patent Publication 2008/0015162, incorporated herein by reference, provide additional examples of nucleic acid analogs useful in the present invention. The following excerpts are derived from that document and are exemplary in nature only:

In certain embodiments, oligomeric compounds comprise one or more modified monomers, including 2'-modified sugars, such as nucleosides and nucleotides, with 2'-substituents such as allyl, amino, azido, thio, O-allyl, O—C$_j$-C$_i$ 0 alkyl, —OCF$_3$, O—(CH$_2$)$_2$—0-CH$_3$, 2'-0(CH$_2$)$_2$SCH$_3$, 0-(CH$_2$)$_2$-0-N(R$_m$)(R$_n$), or 0-CH$_2$-C(=0)-N(R$_m$)(R$_n$), where each R$_m$ and R$_n$ is, independently, H or substituted or unsubstituted C$_j$-C$_i$ 0 alkyl.

Oligomeric compounds provided herein may comprise one or more monomer, including a nucleoside or nucleotide, having a modified sugar moiety. For example, the
furanosyl sugar ring of a nucleoside can be modified in a number of ways including, but not limited to, addition of a substituent group, bridging of two non-geminal ring atoms to form a bicyclic nucleic acid (BNA). Sugars can also be replaced with sugar mimetic groups among others. Methods for the preparations of modified sugars are well known to those skilled in the art. Some representative patents and publications that teach the preparation of such modified sugars include, but are not limited to, U.S. Patents 4,981,957; 5,118,800; 5,319,080; 5,359,044; 5,393,878; 5,446,137; 5,466,786; 5,514,785; 5,519,134; 5,567,811; 5,576,427; 5,591,722; 5,597,909; 5,610,300; 5,627,053; 5,639,873; 5,646,265; 5,658,873; 5,670,633; 5,792,747; 5,700,920; 6,531,584; and 6,600,032; and WO 2005/121371.

In certain embodiments, oligomeric compounds comprise one or more monomers that is a BNA. In certain such embodiments, BNAs include, but are not limited to, (A) oc-L-Methyleneoxy (4'-CH₂-0-2') BNA, (B) β-D-Methyleneoxy (4'-CH₂-0-2') BNA, (C) Ethyleneoxy (4'-CH₂-0-2') BNA, (D) Aminooxy (4'-CH₂-0-N(R)-2') BNA and (E) Oxyamino (4'-CH₂-N(R)-0-2') BNA.

In certain embodiments, BNA compounds include, but are not limited to, compounds having at least one bridge between the 4' and the 2' position of the sugar wherein each of the bridges independently comprises 1 or from 2 to 4 linked groups independently selected from -[C(Rᵢ)(R₂)], -C(Rᵢ)=C(R₂), --C(Rᵢ)=N-- --C=N(Rᵢ)--, --C(=NRᵢ)--, --C(=S)--, -O-, -Si(Rᵢ)₂- =S(=0)ₓ--and --N(Rᵢ)--; wherein: x is 0, 1, or 2; n is 1, 2, 3, or 4; each R₁ and R₂ is, independently, H, a protecting group, hydroxyl, C₃-C₅ alkyl, substituted C₁-C₁₂ alkyl, C₂-C₅ alkanyl, substituted C₂-C₅ alkynyl, C₅-C₂o aryl, substituted C₅-C₂o aryl, heterocycle radical, substituted heterocycle radical, heteroaryl, substituted heteroaryl, C₅-C₇ alicyclic radical, substituted C₅-C₇ alicyclic radical, halogen, OJi, NJJ₂, SJ₁, N₃, COOJ], acyl (C(=0)=H), substituted acyl, CN, sulfonyl (S(=0)-J), or sulfoxyl (S(=0)-J); and each J₁ and J₂ is, independently, H, C₃-C₅ alkyl, substituted C₁-C₁₂ alkyl, C₂-C₅ alkynyl, substituted C₂-C₁₂ alkynyl, C₅-C₂0 aryl; substituted C₅-C₂0 aryl, acyl (C(=0)—H), substituted acyl, a heterocycle radical, a substituted heterocycle radical, C₁-C₅ aminoalkyl, substituted C₁-C₁₂ aminoalkyl or a protecting group.

In one embodiment, each of the bridges of the BNA compounds is, independently, —[C(Rᵢ)(R₂)], -[C(Rᵢ)(R₂)], -C(Rᵢ)=C(R₂), --C(Rᵢ)=N(Rᵢ)--, --C(=NRᵢ)--, --C(=S)--, or -C(Rᵢ)=C(R₂)--O--N(Rᵢ)--. In another embodiment, each of said bridges is, independently, 4'-CH₂-2', 4'-CH₂-2', 4'-
(CH₂)₃-2', 4'-CH ₂-0-2', 4'-(CH₂)₂-0-2', 4'-CH₂-0-N(R)-2' and 4'-CH₂--N(R)-0-2'-
wherein each R is, independently, H, a protecting group or Ci-C₆ alkyl.

Certain BNA's have been prepared and disclosed in the patent literature as well as in scientific literature (Singh et al, 1998; Koshkin et al, 1998; Wahlestedt et al, 2000; Kumar et al, 1998; WO 94/14226; WO 2005/021570; Singh et al, 1998. Examples of issued US patents and published applications that disclose BNA s include, for example, U.S. Patents 7,053,207; 6,268,490; 6,770,748; 6,794,499; 7,034,133; and 6,525,191; and U.S. Patent Publication Nos. 2004/0171570; 2004/0219565; 2004/0014959; 2003/0207841; 2004/01431 14; and 2003/0082807.

Also provided herein are BNAs in which the 2'-hydroxyl group of the ribosyl sugar ring is linked to the 4' carbon atom of the sugar ring thereby forming a methyleneoxy (4'-CH₂-0-2') linkage to form the bicyclic sugar moiety (reviewed in Elayadi et al, 2001; Braasch et al, 2001; and Orum et al, 2001; see also U.S. Patents 6,268,490 and 6,670,461). The linkage can be a methylene (—CH₂—) group bridging the 2' oxygen atom and the 4' carbon atom, for which the term methyleneoxy (4'-CH₂-0-2')

BNA is used for the bicyclic moiety; in the case of an ethylene group in this position, the term ethyleneoxy (4'-CH₂CH₂-0-2') BNA is used (Singh et al, 1998; Morita et al, 2003). Methyleneoxy (4'-CH₂-0-2') BNA and other bicyclic sugar analogs display very high duplex thermal stabilities with complementary DNA and RNA (Tm = +3 to +10°C), stability towards 3'-exonucleolytic degradation and good solubility properties. Potent and nontoxic antisense oligonucleotides comprising BNAs have been described (Wahlestedt et al, 2000).

An isomer of methyleneoxy (4'-CH₂-0-2') BNA that has also been discussed is oc-L-methyleneoxy (4'-CH₂-0-2') BNA which has been shown to have superior stability against a 3'-exonuclease. The oc-L-methyleneoxy (4'-CH₂-0-2') BNA's were incorporated into antisense gapmers and chimeras that showed potent antisense activity (Frieden et al, 2003).

The synthesis and preparation of the methyleneoxy (4'-CH₂-0-2') BNA monomers adenine, cytosine, guanine, 5-methyl-cytosine, thymine and uracil, along with their oligomerization, and nucleic acid recognition properties have been described (Koshkin et al, 1998). BNAs and preparation thereof are also described in WO 98/39352 and WO 99/14226.

Analogs of methyleneoxy (4'-CH₂-0-2') BNA, phosphorothioate-methyleneoxy (4'-CH₂-0-2') BNA and 2'-thio-BNAs, have also been prepared (Kumar et al, 1998). Preparation of locked nucleoside analogs comprising oligodeoxyribonucleotide duplexes as
substrates for nucleic acid polymerases has also been described (Wengel et al., WO 99/14226). Furthermore, synthesis of 2'-amino-BNA, a novel conformationally restricted high-affinity oligonucleotide analog has been described in the art (Singh et al., 1998). In addition, 2'-amino- and 2'-methylamino-BNA's have been prepared and the thermal stability of their duplexes with complementary RNA and DNA strands has been previously reported.

In certain embodiments, the oligomeric compounds comprise one or more high affinity monomers provided that the oligomeric compound does not comprise a nucleotide comprising a 2'-0(CH$_2$n)$_n$H, wherein n is one to six. In certain embodiments, the oligomeric compounds including, but not limited to short antisense compounds of the present invention, comprise one or more high affinity monomer provided that the oligomeric compound does not comprise a nucleotide comprising a 2'-OCH$_3$ or a 2'-0(CH$_2$)$_2$OCH$_3$. In certain embodiments, the oligomeric compounds including, but not limited to short antisense compounds of the present invention, comprise one or more high affinity monomer provided that the oligomeric compound does not comprise a a-L-Methyleneoxy (4'-CH$_2$-0-2') BNA. In certain embodiments, the oligomeric compounds including, but not limited to short antisense compounds of the present invention, comprise one or more high affinity monomer provided that the oligomeric compound does not comprise a a-L-Methyleneoxy (4'-CH$_2$-0-2') BNA or a β-D-Methyleneoxy (4'-CH$_2$-0-2') BNA.

The naturally occurring base portion of a nucleoside is typically a heterocyclic base. The two most common classes of such heterocyclic bases are the purines and the pyrimidines. For those nucleosides that include a pentofuranosyl sugar, a phosphate group can be linked to the 2', 3' or 5' hydroxyl moiety of the sugar. In forming oligonucleotides, those phosphate groups covalently link adjacent nucleosides to one another to form a linear polymeric compound. Within oligonucleotides, the phosphate groups are commonly referred to as forming the internucleotide backbone of the oligonucleotide. The naturally-occurring linkage or backbone of RNA is a 3' to 5' phosphodiester linkage.

In addition to "unmodified" or "natural" nucleobases such as the purine nucleobases adenine (A) and guanine (G), and the pyrimidine nucleobases thymine (T), cytosine (C) and uracil (U), many modified nucleobases or nucleobase mimetics known to those skilled in the art are amenable with the compounds described herein. In certain embodiments, a modified
nucleobase is a nucleobase that is fairly similar in structure to the parent nucleobase, such as for example a 7-deaza purine, a 5-methyl cytosine, or a G-clamp. In certain embodiments, nucleobase mimetic include more complicated structures, such as for example a tricyclic phenoxazine nucleobase mimetic. Methods for preparation of the above noted modified nucleobases are well known to those skilled in the art.

In one embodiment, each of the substituted groups, is, independently, mono- or poly-substituted with optionally protected substituent groups independently selected from halogen, oxo, hydroxyl, OJi, NJiJ2, SJi, N3, OC(=X)Ji, OC(=X)NJiJ2, NJ3C(=X)NJiJ2 and CN, wherein each J1, J2 and J3 is, independently, H or Ci-C6 alkyl, and X is O, S or NJ1.

In certain such embodiments, each of the substituted groups, is, independently, mono- or poly-substituted with substituent groups independently selected from halogen, oxo, hydroxyl, OJ1, NJiJ2, SJi, N3, OC(=X)Ji, and NJ3C(=X)NJiJ2, wherein each J1, J2 and J3 is, independently, H, Ci-C6 alkyl, or substituted Ci-C6 alkyl and X is O or NJi.

In one embodiment, each of the substituted groups, is, independently, mono or poly substituted with optionally protected substituent groups independently selected from halogen, oxo, hydroxyl, OJi, NJiJ2, SJi, N3, OC(=X)Ji, OC^NJ^, NJ3C(=X)NJiJ2 and CN, wherein each Ji, J2 and J3 is, independently, H or Ci-C6 alkyl, and X is O, S or NJ1.

In one embodiment, each of the substituted groups, is, independently, mono- or poly-substituted with substituent groups independently selected from halogen, oxo, hydroxyl, OJi, NJJ2, SJi, N3, OC(=X)Ji, and NJ3C(=X)NJiJ2, wherein each J1, J2 and J3 is, independently, H or Ci-C6 alkyl, and X is O or NJ1.

Described herein are linking groups that link monomers (including, but not limited to, modified and unmodified nucleosides and nucleotides) together, thereby forming an oligomeric compound. The two main classes of linking groups are defined by the presence or absence of a phosphorus atom. Representative phosphorus containing linkages include, but are not limited to, phosphodiesters (P=0), phosphotriesters, methylphosphonates, phosphoramidate, and phosphorothioates (P=S). Representative non-phosphorus containing linking groups include, but are not limited to, methylenemethylimino (—CH2—N(CH3)—O—CH2—), thiodiester (—O-C(O)-S—), thionocarbamate (—O-C(0)(NH)-S—); siloxane (—O-Si(H)2-O—); and N,N'-dimethylhydrazine (—CH2-N(CH3)-N(CH3)-). Oligomeric compounds having non-phosphorus linking groups are referred to as oligonucleosides. Modified linkages, compared to natural phosphodiester linkages, can be used to alter, typically increase, nuclease resistance of the oligomeric compound. In certain embodiments,
linkages having a chiral atom can be prepared a racemic mixtures, as separate enantiomers. Representative chiral linkages include, but are not limited to, alkylphosphonates and phosphorothioates. Methods of preparation of phosphorous-containing and non-phosphorous-containing linkages are well known to those skilled in the art.

The oligomeric compounds described herein contain one or more asymmetric centers and thus give rise to enantiomers, diastereomers, and other stereoisomeric configurations that may be defined, in terms of absolute stereochemistry, as (R) or (S), a or β such as for sugar anomers, or as (D) or (L) such as for amino acids et al. Included in the antisense compounds provided herein are all such possible isomers, as well as their racemic and optically pure forms.

In certain embodiments, provided herein are oligomeric compounds having reactive phosphorus groups useful for forming linkages including for example phosphodiester and phosphorothioate internucleoside linkages. Methods of preparation and/or purification of precursors or oligomeric compounds are not a limitation of the compositions or methods provided herein. Methods for synthesis and purification of oligomeric compounds including DNA, RNA, oligonucleotides, oligonucleosides, and antisense compounds are well known to those skilled in the art.

Generally, oligomeric compounds comprise a plurality of monomeric subunits linked together by linking groups. Nonlimiting examples of oligomeric compounds include primers, probes, antisense compounds, antisense oligonucleotides, external guide sequence (EGS) oligonucleotides, alternate splicers, and siRNAs. As such, these compounds can be introduced in the form of single-stranded, double-stranded, circular, branched or hairpins and can contain structural elements such as internal or terminal bulges or loops. Oligomeric double-stranded compounds can be two strands hybridized to form double-stranded compounds or a single strand with sufficient self complementarity to allow for hybridization and formation of a fully or partially double-stranded compound.

In certain embodiments, the present invention provides chimeric oligomeric compounds. In certain such embodiments, chimeric oligomeric compounds are chimeric oligonucleotides. In certain such embodiments, the chimeric oligonucleotides comprise differently modified nucleotides. In certain embodiments, chimeric oligonucleotides are mixed-backbone antisense oligonucleotides. In general, a chimeric oligomeric compound will have modified nucleosides that can be in isolated positions or grouped together in regions that will define a particular motif. Any combination of modifications and/or mimetic groups can
comprise a chimeric oligomeric compound as described herein. In certain embodiments, chimeric oligomeric compounds typically comprise at least one region modified so as to confer increased resistance to nuclease degradation, increased cellular uptake, and/or increased binding affinity for the target nucleic acid. In certain embodiments, an additional region of the oligomeric compound may serve as a substrate for enzymes capable of cleaving RNA:DNA or RNA:RNA hybrids. By way of example, RNase H is a cellular endonuclease that cleaves the RNA strand of an RNA:DNA duplex. Activation of RNase H, therefore, results in cleavage of the RNA target, thereby greatly enhancing the efficiency of inhibition of gene expression. Consequently, comparable results can often be obtained with shorter oligomeric compounds when chimeras are used, compared to for example phosphorothioate deoxyoligonucleotides hybridizing to the same target region. Cleavage of the RNA target can be routinely detected by gel electrophoresis and, if necessary, associated nucleic acid hybridization techniques known in the art.

III. Polysaccharide Compositions

Embodiments involve a plant-based food grade polysaccharide-based drug delivery system (LPDS) derived from *Trigonella foenum graecum*, commonly called fenugreek. The structure and properties of the fenugreek polysaccharides (FP) are described in Rajani and Mishra, 2008 and Mathur and Mathur, 2005, which are hereby incorporated by reference. FP is a neutral polysaccharide and inert to pH changes. It has been used as flocculent for water purification (Rajani and Mishra, 2008). This is first time FP is being used as a drug delivery system for siRNA based anti-cancer drugs. This delivery system can be used as a platform technology for other cancer drugs which are in need of suitable, effective nontoxic carriers.

The endosperm galactomannans are reserve seed polysaccharides in fenugreek. Galactomannans generally consist of a β-(1→4)-linked D-mannose units (mannans) to which single galactose moieties are linked by a, 1→6 glycoside bond. Galactomannans are a hemicellulose, which is described in Hoch 2007, which is hereby incorporated by reference for its discussion of hemicelluloses, which are embodiments of the invention, and of galactomannans and galactoglucomannans in particular. FP contains mannose, galactose and a small amount of other sugars. There are typically an average of 180-190 monosaccharide (mannose + galactose) units in a molecule. The linear mannan backbone of FG averages 90-95 β-(1→4)-linked manno pyranosyl units and each backbone monomer has an a, 1→6 linked
galactopyranosyl group. Structures of these different groups (as unattached moieties) are provided. Any of these structures is contemplated for use in embodiments described herein.

Mannose backbone (below) with branching galactose

LPDS is a highly efficient drug delivery system which induces significantly less cellular toxicity than currently available delivery systems like lipofectamin and chitosan for siRNA based anticancer drug because plant derived polysaccharide FP is neutral and possess
properties like capability of passing through smallest capillary vessels avoiding rapid clearance by phagocytes prolonging their duration in the blood stream; ability to penetrate cells and tissue gap to arrive at target organs such as liver, spleen, lung, spinal cord and lymph; capability of showing controlled release property due to biodegradability, pH, ion and/or temperature sensibility of materials; and reduction of toxicity of the drugs. This delivery system addresses the 3 fundamental problems with siRNA therapeutics: 1) relatively large size, 2) negative charge, and 3) susceptibility to enzymatic degradation.

IV. Treatment of Inflammatory Disease

In accordance with the present invention, one may treat an inflammatory disorder with an HSP siRNA that is formulated with the polysaccharide compositions, discussed above. Various aspects of therapeutic intervention are discussed below.

A. Inflammatory Disease States

Inflammation is part of the complex biological response of vascular tissues to harmful stimuli, such as pathogens, damaged cells, or irritants. Inflammation is a protective attempt by the organism to remove the injurious stimuli and to initiate the healing process. Without inflammation, wounds and infections would never heal. Similarly, progressive destruction of the tissue compromises the survival of the organism, and chronic inflammation can also lead to a host of diseases, such as hay fever, atherosclerosis, rheumatoid arthritis and cancer. It is for that reason that inflammation is normally closely regulated by the body, and when it is not, it can lead to a variety of devastating conditions. The following is a non-inclusive discussion of some important inflammatory disorders.

Sepsis. Sepsis is a serious medical condition caused by infection and characterized by a whole-body inflammatory state caused. The term sepsis has traditionally been used interchangeably with septicemia and septicemia ("blood poisoning"). However, these terms are no longer considered synonymous; septicemia is considered a subset of sepsis.

Symptoms of sepsis are often related to the underlying infectious process. When the infection crosses into sepsis, the resulting symptoms are that of systemic inflammatory response syndrome (SIRS): general inflammation, fever, elevated white blood cell count (leukocytosis), and raised heart rate (tachycardia) and breathing rate (tachypnea). Secondary to the above, symptoms also include flu like chills.
The immunological response that causes sepsis is a systemic inflammatory response causing widespread activation of inflammation and coagulation pathways. This may progress to dysfunction of the circulatory system and, even under optimal treatment, may result in the multiple organ dysfunction syndrome and eventually death.

Sepsis is currently considered present if infection is highly suspected or proven and two or more of the following (SIRS) criteria are met:

- Heart rate > 90 beats per minute
- Body temperature < 36 (96.8°F) or > 38°C (100.4°F)
- Hyperventilation (high respiratory rate) > 20 breaths per minute or, on blood gas, a P_aCO_2 less than 32 mmHg
- A white blood cell count < 4000 cells/µL or > 12000 cells/µL (> 4 x 10^9 or > 12 x 10^9 cells/µL), or greater than 10% band forms (immature white blood cells).

The more critical subsets of sepsis are severe sepsis (sepsis with acute organ dysfunction) and septic shock (sepsis with refractory arterial hypotension). Alternatively, when two or more of the systemic inflammatory response syndrome criteria are met without evidence of infection, patients may be diagnosed simply with "SIRS." Patients with SIRS and acute organ dysfunction may be termed "severe SIRS."

Patients are defined as having "severe sepsis" if they have sepsis plus signs of systemic hypoperfusion; either end organ dysfunction or a serum lactate greater than 4 mmol/dL. Patients are defined as having septic shock if they have sepsis plus hypotension after an appropriate fluid bolus (typically 20 ml/kg of crystalloid). The criteria for diagnosing an adult with sepsis do not apply to infants under one month of age. In infants, only the presence of infection plus a "constellation" of signs and symptoms consistent with the systemic response to infection are required for diagnosis.

Sepsis treatment relies on antibiotics, surgical drainage of infected fluid collections, fluid replacement and appropriate support for organ dysfunction. This may include hemodialysis in kidney failure, mechanical ventilation in pulmonary dysfunction, transfusion of blood products, and drug and fluid therapy for circulatory failure. Ensuring adequate nutrition, if necessary by parenteral nutrition, is important during prolonged illness.

A problem in the adequate management of septic patients has been the delay in administering therapy after sepsis has been recognized. Published studies have demonstrated that for every hour delay in the administration of appropriate antibiotic therapy there is an associated 7% rise in mortality. A large international collaboration was established to educate
people about sepsis and to improve patient outcomes with sepsis, entitled the "Surviving Sepsis Campaign." The Campaign has published an evidence-based review of management strategies for severe sepsis, with the aim to publish a complete set of guidelines in subsequent years.

Most therapies aimed at the inflammatory process itself have failed to improve outcome, however drotrecogin alfa (activated protein C, one of the coagulation factors) has been shown to decrease mortality from about 31% to about 25% in severe sepsis. To qualify for drotrecogin alfa, a patient must have severe sepsis or septic shock with an APACHE II score of 25 or greater and a low risk of bleeding. Low dose hydrocortisone treatment has shown promise for septic shock patients with relative adrenal insufficiency as defined by ACTH stimulation testing.

Standard treatment of infants with suspected sepsis consists of supportive care, maintaining fluid status with intravenous fluids, and the combination of a β-lactam antibiotic (such as ampicillin) with an aminoglycoside such as gentamicin.

**Trauma** is a serious and body-altering physical injury, such as the removal of a limb. Blunt force trauma, a type of physical trauma caused by impact or other force applied from or with a blunt object, whereas penetrating trauma is a type of physical trauma in which the skin or tissues are pierced by an object. Trauma can also be described as both unplanned, such as an accident, or planned, in the case of surgery. Both can be characterized by mild to severe tissue damage, blood loss and/or shock, and both may lead to subsequent infection, including sepsis. The present invention provides to treatment of trauma, including both pre-treatment (in the case of a medical procedure) and treatment after trauma injury as occurred.

**Surgery.** Surgery uses operative manual and instrumental techniques on a patient to investigate and/or treat a pathological condition such as disease or injury, to help improve bodily function or appearance, or sometimes for some other reason. The present embodiments can address trauma resulting from surgeries, as defined further below.

As a general rule, a procedure is considered surgical when it involves cutting of a patient's tissues or closure of a previously sustained wound. Other procedures that do not necessarily fall under this rubric, such as angioplasty or endoscopy, may be considered surgery if they involve common surgical procedure or settings, such as use of a sterile environment, anesthesia, antiseptic conditions, typical surgical instruments, and suturing or stapling. All forms of surgery are considered invasive procedures; so-called noninvasive surgery usually refers to an excision that does not penetrate the structure being addressed.
(e.g., laser ablation of the cornea) or to a radiosurgical procedure (e.g., irradiation of a tumor). Surgery can last from minutes to hours.

Surgical procedures are commonly categorized by urgency, type of procedure, body system involved, degree of invasiveness, and special instrumentation. Elective surgery is done to correct a non-life-threatening condition, and is carried out at the patient's request, subject to the surgeon's and the surgical facility's availability. Emergency surgery is surgery which must be done quickly to save life, limb, or functional capacity. Exploratory surgery is performed to aid or confirm a diagnosis. Therapeutic surgery treats a previously diagnosed condition.

Amputation involves cutting off a body part, usually a limb or digit. Replantation involves reattaching a severed body part. Reconstructive surgery involves reconstruction of an injured, mutilated, or deformed part of the body. Cosmetic surgery is done to improve the appearance of an otherwise normal structure. Excision is the cutting out of an organ, tissue, or other body part from the patient. Transplant surgery is the replacement of an organ or body part by insertion of another from different human (or animal) into the patient. Removing an organ or body part from a live human or animal for use in transplant is also a type of surgery.

When surgery is performed on one organ system or structure, it may be classed by the organ, organ system or tissue involved. Examples include cardiac surgery (performed on the heart), gastrointestinal surgery (performed within the digestive tract and its accessory organs), and orthopedic surgery (performed on bones and/or muscles).

Minimally invasive surgery involves smaller outer incision(s) to insert miniaturized instruments within a body cavity or structure, as in laparoscopic surgery or angioplasty. By contrast, an open surgical procedure requires a large incision to access the area of interest. Laser surgery involves use of a laser for cutting tissue instead of a scalpel or similar surgical instruments. Microsurgery involves the use of an operating microscope for the surgeon to see small structures. Robotic surgery makes use of a surgical robot, such as Da Vinci or Zeus surgical systems, to control the instrumentation under the direction of the surgeon.

**Traumatic Hemorrhage.** Traumatic hemorrhage accounts for much of the wide ranging international impact of injury, causing a large proportion of deaths and creating great morbidity in the injured. Despite differences in pre-hospital care, the acute management of traumatic hemorrhage is similar around the world and follows well accepted published guidelines. A critically injured patient's care occurs as four, often overlapping segments: the resuscitative, operative, and critical care phases. The diagnosis and control of bleeding
should be a high priority during all of the phases of trauma care and is especially important in
the patient who is in hemorrhagic shock. Early attempts at hemorrhage control include direct
control of visible sources of severe bleeding with direct pressure, pressure dressings, or
tourniquets; stabilization of long bone and pelvic fractures; and keeping the patient warm.

During the resuscitative phase, warmed intravenous fluids, hypotensive resuscitation prior to
surgical control of hemorrhage, and appropriate transfusion of blood and blood products are
provided. In the operative phase, surgical control of the hemorrhage and any other injury,
and additional transfusion is provide. Finally, the critical care phase provides for post-
 operative support and tissue perfusion.

**Acute Pancreatitis.** Acute pancreatitis is rapidly-onset inflammation of the pancreas.
Depending on its severity, it can have severe complications and high mortality despite
treatment. While mild cases are often successfully treated with conservative measures or
laparoscopy, severe cases require invasive surgery (often more than one intervention) to
contain the disease process.

**Acute Respiratory Distress Syndrome.** Acute respiratory distress syndrome
(ARDS), also known as respiratory distress syndrome (RDS) or adult respiratory distress
syndrome (in contrast with IRDS) is a serious reaction to various forms of injuries to the
lung. This is the most important disorder resulting in increased permeability pulmonary
edema.

ARDS is a severe lung disease caused by a variety of direct and indirect insults. It is
characterized by inflammation of the lung parenchyma leading to impaired gas exchange with
concomitant systemic release of inflammatory mediators causing inflammation, hypoxemia
and frequently resulting in multiple organ failure. This condition is life threatening and often
lethal, usually requiring mechanical ventilation and admission to an intensive care unit. A
less severe form is called acute lung injury (ALI).

ARDS can occur within 24 to 48 hours of an injury or attack of acute illness. In such a
case the patient usually presents with shortness of breath, tachypnea, and symptoms related to
the underlying cause, *i.e.*, shock. Long term illnesses can also trigger it, such as malaria. The
ARDS may then occur sometime after the onset of a particularly acute case of the infection.

An arterial blood gas analysis and chest X-ray allow formal diagnosis by inference
using the aforementioned criteria. Although severe hypoxemia is generally included, the
appropriate threshold defining abnormal PaO$_2$ has never been systematically studied. Any
cardiogenic cause of pulmonary edema should be excluded. This can be done by placing a
pulmonary artery catheter for measuring the pulmonary artery wedge pressure. However, this
is not necessary and is now rarely done as abundant evidence has emerged demonstrating that
the use of pulmonary artery catheters does not lead to improved patient outcomes in critical
illness including ARDS. Plain chest X-rays are sufficient to document bilateral alveolar
infiltrates in the majority of cases. While CT scanning leads to more accurate images of the
pulmonary parenchyma in ARDS, its has little utility in the clinical management of patients
with ARDS, and remains largely a research tool.

Acute respiratory distress syndrome is usually treated with mechanical ventilation in
the Intensive Care Unit. Ventilation is usually delivered through oro-tracheal intubation, or
tracheostomy whenever prolonged ventilation (≥ 2 weeks) is deemed inevitable. The
possibilities of non-invasive ventilation are limited to the very early period of the disease or,
better, to prevention in individuals at risk for the development of the disease (atypical
pneumonias, pulmonary contusion, major surgery patients). Treatment of the underlying
cause is imperative, as it tends to maintain the ARDS picture. Appropriate antibiotic therapy
must be administered as soon as microbiological culture results are available. Empirical
therapy may be appropriate if local microbiological surveillance is efficient. More than 60%
ARDS patients experience a (nosocomial) pulmonary infection either before or after the onset
of lung injury. The origin of infection, when surgically treatable, must be operated on. When
sepsis is diagnosed, appropriate local protocols should be enacted.

**Ischemia-Reperfusion Injury.** Reperfusion injury refers to damage to tissue caused
when blood supply returns to the tissue after a period of ischemia. The absence of oxygen and
nutrients from blood creates a condition in which the restoration of circulation results in
inflammation and oxidative damage through the induction of oxidative stress rather than
restoration of normal function.

The damage of reperfusion injury is due in part to the inflammatory response of
damaged tissues. White blood cells carried to the area by the newly returning blood release a
host of inflammatory factors such as interleukins as well as free radicals in response to tissue
damage. The restored blood flow reintroduces oxygen within cells that damages cellular
proteins, DNA, and the plasma membrane. Damage to the cell's membrane may in turn cause
the release of more free radicals. Such reactive species may also act indirectly in redox
signaling to turn on apoptosis. Leukocytes may also build up in small capillaries, obstructing
them and leading to more ischemia.
Reperfusion injury plays a part in the brain's ischemic cascade, which is involved in stroke and brain trauma. Repeated bouts of ischemia and reperfusion injury also are thought to be a factor leading to the formation and failure to heal of chronic wounds such as pressure sores and diabetic foot ulcers. Continuous pressure limits blood supply and causes ischemia, and the inflammation occurs during reperfusion. As this process is repeated, it eventually damages tissue enough to cause a wound.

In prolonged ischemia (60 min or more), hypoxanthine is formed as breakdown product of ATP metabolism. The enzyme xanthine dehydrogenase is converted to xanthine oxidase as a result of the higher availability of oxygen. This oxidation results in molecular oxygen being converted into highly reactive superoxide and hydroxyl radicals. Xanthine oxidase also produces uric acid, which may act as both a prooxidant and as a scavenger of reactive species such as peroxynitrite. Excessive nitric oxide produced during reperfusion reacts with superoxide to produce the potent reactive species peroxynitrite. Such radicals and reactive oxygen species attack cell membrane lipids, proteins, and glycosaminoglycans, causing further damage. They may also initiate specific biological processes by redox signaling.

**Cardiovascular Disease.** Cardiovascular disease refers to the class of diseases that involve the heart or blood vessels (arteries and veins). While the term technically refers to any disease that affects the cardiovascular system, it is usually used to refer to those related to atherosclerosis (arterial disease). These conditions have similar causes, mechanisms, and treatments. Treatment of cardiovascular disease depends on the specific form of the disease in each patient, but effective treatment always includes preventive lifestyle changes discussed above. Medications, such as blood pressure reducing medications, aspirin and the statin cholesterol-lowering drugs may be helpful. In some circumstances, surgery or angioplasty may be warranted to reopen, repair, or replace damaged blood vessels.

Most Western countries face high and increasing rates of cardiovascular disease. Each year, heart disease kills more Americans than cancer. Diseases of the heart alone caused 30% of all deaths, with other diseases of the cardiovascular system causing substantial further death and disability. Up until the year 2005, it was the number 1 cause of death and disability in the United States and most European countries. A large histological study (P-Day) showed vascular injury accumulates from adolescence, making primary prevention efforts necessary from childhood.
Some biomarkers are thought to offer a more detailed risk of cardiovascular disease. However, the clinical value of these biomarkers is questionable. Currently, biomarkers which may reflect a higher risk of cardiovascular disease include:

- higher fibrinogen and PAI-1 blood concentrations
- elevated homocysteine, or even upper half of normal
- elevated blood levels of asymmetric dimethylarginine
- high inflammation as measured by C-reactive protein
- elevated blood levels of B-type natriuretic peptide (BNP)

Various forms of cardiovascular disease include aneurysms, angina, arrhythmia, atherosclerosis, cardiomyopathy, cerebrovascular disease, congenital heart disease, congestive heart failure, myocarditis, valve disease, coronary artery disease, dilated cardiomyopathy, diastolic dysfunction, endocarditis, high blood pressure (hypertension), hypertrophic cardiomyopathy, nitral valve prolapse, myocardial infarction, and venous thromboembolism.

**Autoimmune/Inflammatory Disease.** The present invention contemplates the treatment of a variety of autoimmune and/or inflammatory disease states such as spondyloarthritis, ankylosing spondylitis, psoriatic arthritis, reactive arthritis, enteropathic arthritis, ulcerative colitis, Crohn's disease, irritable bowel disease, inflammatory bowel disease, rheumatoid arthritis, juvenile rheumatoid arthritis, familial Mediterranean fever, amyotrophic lateral sclerosis, Sjogren's syndrome, early arthritis, viral arthritis, multiple sclerosis, or psoriasis. The diagnosis and treatment of these diseases are well documented in the literature.

**Chemotherapy, Radiotherapy and Cytokine Therapy Toxicity.** Various forms of cancer therapy, including chemotherapy, radiation, and cytokines, are associated with toxicity, sometimes severe, in the cancer patient. To the extent that the toxicity is caused at least in part by the extracellular actions of histones, the present invention seeks to reduce this toxicity using the pharmaceutical compositions of the present invention, thereby reducing or alleviating discomfort on the part of the patient, as well as permitting higher doses of the therapy.

**BURNS.** In medicine, a burn may be an injury caused by heat, cold, electricity, chemicals, friction or radiation. First-degree burns are usually limited to redness (erythema), a white plaque, and minor pain at the site of injury. These burns usually extend only into the epidermis. Second-degree burns additionally fill with clear fluid, have superficial blistering
of the skin, and can involve more or less pain depending on the level of nerve involvement. Second-degree burns involve the superficial (papillary) dermis and may also involve the deep (reticular) dermis layer. Third-degree burns additionally have charring of the skin, and produce hard, leather-like eschars. An eschar is a scab that has separated from the unaffected part of the body. Frequently, there is also purple fluid. These types of burns are often painless, because nerve endings have been destroyed in the burned areas. Serious burns, especially if they cover large areas of the body, can cause death; any hint of burn injury to the lungs (e.g., through smoke inhalation) is a medical emergency.

Burns that injure the tissues underlying the skin, such as the muscles or bones, are sometimes categorized as fourth-degree burns. These burns are broken down into three additional degrees: fourth-degree burns result in the skin being irretrievably lost, fifth-degree burns result in muscle being irretrievably lost, and sixth-degree burns result in bone being charred.

A newer classification of "Superficial Thickness," "Partial Thickness" (which is divided into superficial and deep categories) and "Full Thickness" relates more precisely to the epidermis, dermis and subcutaneous layers of skin and is used to guide treatment and predict outcome.

Chemical burns are usually caused by chemical compounds, such as sodium hydroxide (lye), silver nitrate, and more serious compounds (such as sulfuric acid). Most chemicals (but not all) that can cause moderate to severe chemical burns are strong acids or bases. Nitric acid, as an oxidizer, is possibly one of the worst burn-causing chemicals. Hydrofluoric acid can eat down to the bone and its burns are often not immediately evident. Most chemicals that can cause moderate to severe chemical burns are called caustic.

Electrical burns are generally symptoms of electric shock, being struck by lightning, being defibrillated or cardioverted without conductive gel, etc. The internal injuries sustained may be disproportionate to the size of the "burns" seen - as these are only the entry and exit wounds of the electrical current.

Burns are assessed in terms of total body surface area (TBSA), which is the percentage affected by partial thickness or full thickness burns (superficial thickness burns are not counted). The rule of nines is used as a quick and useful way to estimate the affected TBSA. The first step in managing a person with a burn is to stop the burning process. With dry powder burns, the powder should be brushed off first. With other burns, the affected area should be rinsed with a large amount of clean water to remove foreign bodies and help stop
the burning process. Cold water should never be applied to any person with extensive burns, as it may severely compromise the burn victim's temperature status. At this stage of management, it is also critical to assess the airway status. If the patient was involved in a fire, then it must be assumed that he or she has sustained inhalation injury until proven otherwise, and treatment should be managed accordingly.

Once the burning process has been stopped, and airway status is ensured, the patient should be volume resuscitated according to the Parkland formula. This formula dictates that the amount of Lactated Ringer's solution to deliver in the first twenty four hours after time of injury is:

\[
\text{fluid} = 4 \text{cc} \times \% \text{TBSA} \times \text{weight in kg}
\]

\% TBSA excludes any first degree burn

Half of this fluid should be given in the first eight hours post injury and the rest in the subsequent sixteen hours. The formula is a guide only and infusions must be tailored to urine output and central venous pressure. Inadequate fluid resuscitation causes renal failure and death. Severe edema in full thickness burns may be treated by escharotomy.

Cancer. Cancer results from the outgrowth of a clonal population of cells from tissue. The development of cancer, referred to as carcinogenesis, can be modeled and characterized in a number of ways. An association between the development of cancer and inflammation has long been appreciated. The inflammatory response is involved in the host defense against microbial infection, and also drives tissue repair and regeneration. Considerable evidence points to a connection between inflammation and a risk of developing cancer, i.e., chronic inflammation can lead to dysplasia.

Studies have estimated that nearly 15% of worldwide cancer is associated with microbial infection. Organisms such as human papilloma virus (HPV), hepatitis B and C virus, HIV, and *Helicobacter pylori* all have been linked to cancer. In other cases, environmental conditions causing chronic irritation and subsequent inflammation can also predispose to cancer, including cigarette smoke, asbestos and silica.

In the case of some types of viral infection, virally-encoded genes can contribute to cellular transformation. An example is the HPV oncoproteins E6 and E7. However, other microbes associated with cancer do not operate in this fashion as they are not transforming. For example, certain strains of *H. pylori* contain factors that affect host cell signaling but do not contain oncogenes.Interestingly, it has been observed that *H. pylori* induces MUC 1.
Other ways in which chronic inflammatory states can lead to genomic lesions and tumor initiation are chemical. For example, host cells fight microbial infection by the production of free radicals. In addition to their anti-microbial effects, these molecules lead to oxidative damage and nitration of DNA bases which increases the risk of DNA mutations even in host cells.

Yet another path to cellular dysregulation may result from the cell death that occurs in infection or other inflammatory insult. Lost cells must be repopulated by the expansion of other cells, sometimes undifferentiated precursor cells such as tissue stem cells. Not surprisingly, many inflammatory pathways function to mediate survival and proliferation. Thus, in attempting to mediating tissue repair, the inflammatory response may unwittingly provide excessive survival and proliferative signals to cells, thus leading to tumorigenesis.

Because of the link between cancer and inflammation, the ability of the peptides and peptide analogs of the present invention to reduce inflammatory signalling pathways can be exploited in a pre-cancer or cancer risk situation to prevent or delay the onset of dysplastic growth.

B. Formulations and Routes of Administration

The present invention also involves the treatment of inflammatory conditions. By treatment, it is not necessary that all symptoms of the disease be addressed, or that any degree of "cure" be achieved. Rather, to accomplish a meaningful treatment, all that is required is that one or more symptoms of the disease or condition be ameliorated to some degree, an advantageous effect be provided in combination with another therapy, or that the disease progression be slowed.

Where clinical applications are contemplated, it will be necessary to prepare pharmaceutical compositions in a form appropriate for the intended application. Generally, this will entail preparing compositions that are essentially free of pyrogens, as well as other impurities that could be harmful to humans or animals. One will generally desire to employ appropriate salts, buffers, and lipids to render delivery of the oligonucleotides to allow for uptake by target cells. Such methods an compositions are well known in the art, for example, as disclosed in U.S. Patents 6,747,014 and 6,753,423. Compositions of the present invention comprise an effective amount of the oligonucleotide to cells, dissolved or dispersed in a pharmaceutically acceptable carrier or medium.

The phrase "pharmaceutically or pharmacologically acceptable" refer to molecular entities and compositions that do not produce adverse, allergic, or other untoward reactions.
when administered to an animal or a human. As used herein, "pharmaceutically acceptable carrier" includes any and all solvents, dispersion media, coatings, antibacterial and antifungal agents, isotonic and absorption delaying agents, liposomes, cationic lipid formulations, microbubble nanoparticles, and the like. The use of such media and agents for pharmaceutically active substances is well-known in the art. Except insofar as any conventional media or agent is incompatible with the vectors or cells of the present invention, its use in therapeutic compositions is contemplated. Supplementary active ingredients also can be incorporated into the compositions.

The active compositions of the present invention may include classic pharmaceutical preparations. Administration of these compositions according to the present invention will be via any common route so long as the target tissue is available via that route. This includes oral or parenteral routes, including intracranial (including intraparenchymal and intraventricular), intrathecal, epidural, intravenous, intraarterial, intramuscular, intraperitoneal, subcutaneous, intratumoral, transdermal, airway (aerosol), nasal, rectal, vaginal and topical (including buccal and sublingual) administration. Compositions would normally be administered as pharmaceutically acceptable compositions, described supra.

The pharmaceutical forms suitable for injectable use include sterile aqueous solutions or dispersions and sterile powders for the extemporaneous preparation of sterile injectable solutions or dispersions. In all cases the form must be sterile and must be fluid to the extent that easy syringability exists. It must be stable under the conditions of manufacture and storage and must be preserved against the contaminating action of microorganisms, such as bacteria and fungi. The carrier can be a solvent or dispersion medium containing, for example, water, ethanol, polyol (for example, glycerol, propylene glycol, and liquid polyethylene glycol, and the like), suitable mixtures thereof, and vegetable oils. The proper fluidity can be maintained, for example, by the use of a coating, such as lecithin, by the maintenance of the required particle size in the case of dispersion and by the use of surfactants. The prevention of the action of microorganisms can be brought about by various antibacterial and antifungal agents, for example, parabens, chlorobutanol, phenol, sorbic acid, thimerosal, and the like. In many cases, it will be preferable to include isotonic agents, for example, sugars or sodium chloride. Prolonged absorption of the injectable compositions can be brought about by the use in the compositions of agents delaying absorption, for example, aluminum monostearate and gelatin.
Sterile injectable solutions are prepared by incorporating the active compounds in the required amount in the appropriate solvent with various of the other ingredients enumerated above, as required, followed by filtered sterilization. Generally, dispersions are prepared by incorporating the various sterilized active ingredients into a sterile vehicle which contains the basic dispersion medium and the required other ingredients from those enumerated above. In the case of sterile powders for the preparation of sterile injectable solutions, the preferred methods of preparation are vacuum-drying and freeze-drying techniques which yield a powder of the active ingredient plus any additional desired ingredient from a previously sterile-filtered solution thereof.

As used herein, "pharmaceutically acceptable carrier" includes any and all solvents, lipids, nanoparticles, dispersion media, coatings, antibacterial and antifungal agents, isotonic and absorption delaying agents and the like. The use of such media and agents for pharmaceutical active substances is well known in the art. Except insofar as any conventional media or agent is incompatible with the active ingredient, its use in the therapeutic compositions is contemplated. Supplementary active ingredients can also be incorporated into the compositions.

For oral administration the NAAs of the present invention may be incorporated with excipients. The compositions of the present invention may be formulated in a neutral or salt form. Pharmaceutically-acceptable salts include the acid addition salts (formed with the free amino groups of the protein) and which are formed with inorganic acids such as, for example, hydrochloric or phosphoric acids, or such organic acids as acetic, oxalic, tartaric, mandelic, and the like. Salts formed with the free carboxyl groups can also be derived from inorganic bases such as, for example, sodium, potassium, ammonium, calcium, or ferric hydroxides, and such organic bases as isopropylamine, trimethylamine, histidine, procaine and the like.

Upon formulation, solutions will be administered in a manner compatible with the dosage formulation and in such amount as is therapeutically effective. The formulations are easily administered in a variety of dosage forms such as injectable solutions, drug release capsules and the like. For parenteral administration in an aqueous solution, for example, the solution should be suitably buffered if necessary and the liquid diluent first rendered isotonic with sufficient saline or glucose. These particular aqueous solutions are especially suitable for intravenous, intraarterial, intramuscular, subcutaneous, intraperitoneal, intrathecal, epidural and intracranial (including intraparenchymal and intraventricular) administration. In this connection, sterile aqueous media which can be employed will be known to those of skill
in the art in light of the present disclosure. For example, one dosage could be dissolved in 1 ml of isotonic NaCl solution and either added to 1000 ml of hypodermoclysis fluid or injected at the proposed site of infusion, (see for example, "Remington's Pharmaceutical Sciences" 15th Edition, pages 1035-1038 and 1570-1580). Some variation in dosage will necessarily occur depending on the condition of the subject being treated. The person responsible for administration will, in any event, determine the appropriate dose for the individual subject. Moreover, for human administration, preparations should meet sterility, pyrogenicity, general safety and purity standards as required by FDA Office of Biologies standards.

C. Combination Therapies

It is common in many fields of medicine to treat a disease with multiple therapeutic modalities, often called "combination therapies." Therapies would be provided in a combined amount effective to achieve a reduction in one or more disease parameter. This process may involve contacting subjects with the both agents/therapies at the same time, e.g., using a single composition or pharmacological formulation that includes both agents, or by contacting the subject with two distinct compositions or formulations, at the same time, wherein one composition includes the double-stranded RNAs of the present invention and the other includes the other agent.

Alternatively, the double-stranded RNA therapy may precede or follow the other treatment by intervals ranging from minutes to weeks. One would generally ensure that a significant period of time did not expire between the time of each delivery, such that the therapies would still be able to exert an advantageously combined effect on the subject. In such instances, it is contemplated that one would contact the subject with both modalities within about 12-24 hours of each other, within about 6-12 hours of each other, or with a delay time of only about 12 hours. In some situations, it may be desirable to extend the time period for treatment significantly; however, where several days (2, 3, 4, 5, 6 or 7) to several weeks (1, 2, 3, 4, 5, 6, 7 or 8) lapse between the respective administrations.

It also is conceivable that more than one administration of either the double-stranded RNA therapy or the other therapy will be desired. Various combinations may be employed, where the double-stranded RNA is "A," and the other therapy is "B," as exemplified below:

A/B/A B/A/B B/B/A A/A/B B/A/A A/B/B B/B/B/A B/B/A/B
A/A/B/B A/B/A/B A/B/B/A B/B/A/A B/A/B/A B/A/A/B B/B/B/A
Other combinations are contemplated. Agents or factors suitable for use in a combined therapy include those described above for the various polyglutamine repeat diseases.

The skilled artisan is directed to "Remington's Pharmaceutical Sciences" 15th Edition, chapter 33, in particular pages 624-652. Some variation in dosage will necessarily occur depending on the condition of the subject being treated. The person responsible for administration will, in any event, determine the appropriate dose for the individual subject. Moreover, for human administration, preparations should meet sterility, pyrogenicity, general safety and purity standards as required by FDA Office of Biologies standards.

V. Examples

The following examples are included to demonstrate preferred embodiments of the invention. It should be appreciated by those of skill in the art that the techniques disclosed in the examples which follow represent techniques discovered by the inventor to function well in the practice of the invention, and thus can be considered to constitute preferred modes for its practice. However, those of skill in the art should, in light of the present disclosure, appreciate that many changes can be made in the specific embodiments which are disclosed and still obtain a like or similar result without departing from the spirit and scope of the invention.

Example 1 - Materials & Methods

Cells and culture conditions. 4T1 is a highly metastatic breast cancer cell line derived from a spontaneously arising BALB/c mammary tumor purchased from ATCC (Manassas, VA). 4T1 and 293FT cells were maintained in monolayer cultures in DMEM supplemented with 10% fetal bovine serum. Cells were maintained at 37°C humidified atmosphere with 5% CO₂.

Animals and tumor challenge. Female BALB/c mice (6-8 weeks old) were purchased from Charles River Laboratories (Wilmington, MA) and housed under pathogen-free conditions in laminar flow isolation units in the Scott & White Hospital's vivarium under alternate dark and light cycles. Animals were maintained on food and water ad libitum. Mice were challenged s.c. in the abdominal mammary gland with 104 4T1 cells and tumor volume was measured at regular intervals using an electronic caliper. The tumor volume was...
estimated using the formula of an ellipsoid (length x width x height x 0.5236). All animals
were treated humanely and in accordance with the guidelines of the Committee on the Care
and Use of Laboratory Animals of the Institute of Animal Resources, National Research
Council and Institutional Animal Care and Use Committee (IACUC) of Scott & White
Hospital.

**Preparation of small hairpin RNA of mouse Hsp25 lentivirus gene transfer vector.** A HIV derived three plasmid system was kindly provided by Dr. Trono (Department
of Microbiology and Molecular Medicine, University of Geneva, Switzerland). Briefly, the
plasmid pLVTHM was digested with Mlu I and Cla I and ligated to an oligonucleotide pair
containing Hsp25shRNA or controlshRNA carrying Mlu I and Cla I restriction overhangs and
transformed into Max Stbl2 competent cells. The positive clones were identified by digesting
the control pLVTHM vector and the vector containing Hsp25shRNA inserts using MluI and
Xba I enzymes. Alternatively, positive clones were also identified by DNA sequencing.

**Lentivirus production and transduction.** Lentivirus transfection was carried out
according to the standard protocol (Naldini *et al.*, 1996). Briefly, 4T1 cells were plated into
six-well plates (3 x 10^4 cells/well), 1-ml concentrated high titer virus (5 x 10^8) was directly
overlayed on cells and polybrene was added at a final concentration of 8 µg/ml. Five days later
the cells were harvested and analyzed by fluorescence-activated cell sorting (FACS).

**Western blot analysis.** Total cell extracts (50 µg) from 4T1-controlshRNA and 4T1-
Hsp25shRNA cells were isolated according to standard protocol (Cell Signaling) and
fractionated by electrophoresis on 10% SDS-PAGE and electroblotted to PVDF membrane
(Amersham Biosciences) and probed with anti-Hsp25 (Santa Cruz), anti PA28a (Cell
Signaling) and anti-prohibitin (Cell Signaling). Protein loading control was used as β-actin
(Abeam). Appropriate secondary antibodies (Santa Cruz and Sigma) were used in the study.

**RNA isolation and real-time PCR analysis.** Total RNA was isolated from 4T1-
controlshRNA and 4T1-Hsp25shRNA cells using Qiagen RNeasy kit. Oligo-dT primed 5 µg
of total RNA was converted into cDNA according to manufacturer's protocol (SA Biosciences). Real-time PCR was performed using gene specific primers (SA Biosciences).

**Two-dimensional SDS-PAGE.** Proteins from 4T1-controlshRNA and 4T1-
Hsp25shRNA cells were analyzed by two-dimensional PAGE. Briefly, proteins were
solubilized with lysis buffer, containing 8M urea, 4% CHAPS, 50 mM DTT and 0.5% IPG
buffer (GE Healthcare) and protease inhibitors (Roche) and halt-phosphatase inhibitors
(Pierce Protein Research Products). Isoelectric focusing was carried out using pH 3-10 NL,
pH 4-7 NL, 11 cm IPG strips (GE Healthcare) for 30,000 Vhrs at room temperature using IPG 3 Ettan unit (GE Healthcare). The focused IPG strips were equilibrated in a second dimension sample buffer (25 mM Tris (pH 6.8) containing 20% glycerol, 2% SDS, 2% DTT for 15 minutes, and equilibrated with the same buffer containing 2.5% of iodoacetamide (IAA) for 15 minutes. The second dimension gel electrophoresis was performed on 8-16% polyacrylamide gradient SDS gel (Bio-Rad Laboratories) and the samples were electrophoresed until the dye front reached the opposite end of the gel. Gel was fixed for 20 h with fixing solution containing 50% ethanol and 1% phosphoric acid. Thereafter, gels were stained with Bio-Safe Coomassie Blue Stain (Bio-Rad Laboratories) for three days and destained with MilliQ water to remove the background staining.

Mass spectrometric analysis of tryptic peptides. The gel spots were cut using Bio-Rad's EXQuest Spot Cutter and proteins were digested in-gel, and peptides were extracted and analyzed as described earlier (Bhat et al, 2005).

Proteasome activity assay. A whole cell extract was prepared as follows briefly, cells were harvested, washed with phosphate buffered saline twice, and 10^7 cells were homogenized in 0.5 ml cell lysis buffer (50 mM HEPES, pH7.5, 5 mM EDTA, 150 mM NaCl, 1% Triton X-100 and 2 mM ATP) for 30 min at 4°C. Cell lysates were centrifuged at 14,000 x g for 30 min and the clear supernatant were collected as whole extracts. The proteasome activity assay was performed by using 20S proteasome activity assay kit (Millipore). Briefly, a whole cell extract (30 µg) of 4T1-controlshRNA and 4T1-Hsp25shRNA cells was incubated for 90 min at 37°C with fluorogenic proteasome substrate, Suc-LLVY-AMC in 100 µl of the assay buffer with or without lactacystin proteasome inhibitor (25 µM). The hydrolyzed AMC were quantified using 380/460nm filter set in a Fluoroskan Ascent fluorometer (Thermo Scientific).

Live animal imaging. 4T1-controlshRNA and 4T1-Hsp25shRNA cells (10^4) were injected into the mammary pad of female BALB/c mice. The mice were anesthetized using isoflurane gas before imaging. On the indicated days spectral fluorescence images were captured using the Maestro™ in vivo imaging system (CRI, Woburn, MA). An excitation band pass filter from 445 to 490 nm and an emission filter over 515 nm were used in the study. The tunable filter was automatically spaced in 10nm increments from 500-720 nm while the camera captured fluorescence images at each wavelength with constant exposure. RGB (red-green-blue) color fluorescence images were synthesized from the spectral cube by mapping the spectral data into those color channels. All the fluorescence images obtained as RGB
images in the present study were derived from the spectral datasets. Spectral unmixing was performed to segregate skin and hair auto fluorescence and to measure the true GFP signal.

**Flow cytometry.** FACS experiments were conducted on a BD FACSARia flow cytometer (BD Biosciences) equipped with a 488 nm argon laser. Emission filter was 515-545 for GFP. For GFP sorting, 4Tl-controlshRNA and 4Tl-Hsp25shRNA cells were harvested and suspended in PBS buffer containing 2% FBS to a concentration of $10^7$ cells/ml. Cells were appropriately gated by forward/size scatter and 2-3% cells gated events were collected per sample. Post-sorted cells were collected in cell culture medium containing 20% FBS and plated in 4T1 complete medium.

**Fluorescence microscopy.** Standard fluorescence microscopy was performed using an Olympus CKX41 microscope. DP71 CCD camera was used to capture phase contrast and GFP fluorescence images with DP71 image acquisition interface software (Olympus).

**Haematoxylin & eosin and immunohistochemical staining.** At the end of the experiment, animals were sacrificed using euthasol injection. The lungs, heart, liver, kidneys, brain, spleen and hind limbs were incised and fixed in 10% formalin. All tissues were embedded in paraffin. Histological sections were prepared by standard conventional processing and stained with H&E and digital pictographs were taken using the Olympus microscope equipped with DP71 CCD camera.

**Clonogenic assay.** The lung metastasis study was performed by clonogenic assay as previously described (Bausero et al, 2004). Briefly, lung tissue (n=5) was aseptically removed, minced with trypsin and seeded in triplicate after dilution series (1:20 to 1:320) in 60-mm² Petri dish and incubated at 37°C in a 5% CO2 air atmosphere. Ten to twelve days later, the plates were washed twice with PBS and colonies were stained with crystal violet and counted.

**In vivo antibody depletion.** Anti-CD4 (L3T4), anti-CD8 (Ly-2) and anti-NK (5E6) antibodies were purchased from BD Biosciences Pharmingen. Antibody depletion was started four days before injection of 4Tl-controlshRNA and 4Tl-Hsp25shRNA cells into the abdominal mammary pads of mice. Depletion of CD4, CD8 T-cell subsets and NK cells was accompanied by i.p. injection of 100 µg antibody/mice for every week. Effective depletion of cell subsets was confirmed by flow cytometric analysis of splenocytes one day before tumor challenge and maintained by the antibody injections once a week for the duration of the tumor challenge experiment. PBS was used as control.
**In vitro cytotoxicity assays.** 4T1-Hsp25shRNA cells (10⁴) were injected s.c. into mammary pads of 6-8 week old female BALB/c mice and tumor growth was monitored by using the MaestroTM in vivo animal imaging system (CRI). Tumors started regressing at the end of two weeks, and spleen tissues were harvested from the animals and CD8+ and CD8- T lymphocytes were isolated by using protocol of Miltenyi Biotec. Cytotoxicity was measured by lactate dehydrogenase cytotoxicity assay kit II (BioVision). 4T1-control shRNA cells were used as target. 4T1-control shRNA cells (1.5 x 10⁴) were seeded as quintuplicate in 96 well tissue culture plates. CD8+ or CD8- T lymphocytes were added at different effector/target ratios (10:1 and 30:1), and absorbance was measured for all controls and samples using a plate reader.

**In vivo adoptive transfer assay.** 4T1-control shRNA cells (10⁴) were subcutaneously (s.c.) injected into mammary glands of BALB/c mice. Briefly, 10⁴ 4T1-Hsp25shRNA cells were injected s.c. into mammary glands of BALB/c mice, and animals were monitored for tumor regression by using the Maestro™ in vivo animal imaging system (CRI). At the end of two weeks splenocytes were collected and CD8+ or CD8- T lymphocytes were isolated according to manufacturer’s instructions (Milteny Biotec). Adoptive transfer of 4T1-Hsp25shRNA reactive CD8+ or CD8- T lymphocytes (10⁶) via the tail vein was done on day 5 following 4T1-control shRNA tumor cell injections. Tumor regression and development was monitored by using Maestro™ in vivo imaging system (CRI).

**Statistical analysis.** For comparisons between groups, Dunn multiple comparison tests and student t-test and one-way analysis of variance (ANOVA) were used in this study (p values <0.05 were considered significant).

**Example 2 - Results**

**Hsp25shRNA permanently silences hsp25 gene expression.** The inventors used a lentivirus-based vector (pLVTHM) that expresses RNAi inducing the twenty-five kilo Dalton heat shock protein (Hsp25)shRNA (Hsp25shRNA) under the control of the H1 promoter (FIG. 1). This bicistronic vector was engineered to coexpress enhanced green fluorescent protein (GFP) as a reporter gene under the tight control of the elongation factor-1 alpha (EF-la) promoter, permitting transduced/infected target cells to be tracked using in vivo imaging. Stable silencing of hsp25 gene expression in 4T1 tumor cells was achieved by subcloning the Hsp25shRNA cassette into pLVTHM, a self-inactivating (SIN) lentiviral vector using Mlu I and Cla I restriction sites (4T1-Hsp25shRNA hairpin loop sequence) (FIG. 1). The inventors
also constructed control/scrambled shRNA containing lentiviral vector which does not have sequence homology to the mouse genome (4T1-controlshRNA hairpin loop sequence) (FIG. 1). These constructs were introduced into 293FT viral packaging cells to make lentivirus. The concentrated lentivirus preparation was used to infect target 4T1 breast adenocarcinoma cells. The resulting GFP expression was assessed 4 days post-infection by flow cytometry and further enriched for only highly expressing GFP-positive cells. The resulting sorted 4T1-Hsp25shRNA cells were 96.7% positive for GFP. The high GFP expression exhibited by both 4T1-controlshRNA and Hsp25shRNA stable transfected cells remained high even after 6 weeks of culture. The inventors confirmed that high GFP expression in 4T1-Hsp25shRNA cells corresponded to efficient silencing of Hsp25 protein expression consistently by >98% after 6-8 weeks in vitro cell culture.

**Silencing Hsp25 protein suppresses tumor proliferation and metastasis.** The uncontrollable growth of tumors and their ability to metastasize and invade distant organs is a serious problem. The inventors demonstrated that silencing Hsp25 protein expression in 4T1 cells (4T1-Hsp25shRNA) significantly suppressed cell proliferation by 40% and 53% as compared to control cells (4T1-controlshRNA) by day 3 and 4, respectively (FIG. 2A). They further demonstrated that Hsp25shRNA treatment adversely affects the directional cell migration of 4T1 cells in vitro, almost to the same extent as serum starvation, as judged by the wound healing experiment. These results correlated well with the inability of 4T1-Hsp25shRNA cells to invade extracellular matrix in vitro as compared to 4T1-controlshRNA cells (FIG. 2B). The inventors demonstrated that silencing the hsp25 gene significantly downregulated the expression of MMP-9 as compared to 4T1-controlshRNA cells. The expression of additional genes involved in cell survival, migration and metastasis, including COX2, PARI, TWIST ID1 and SPARC were amplified by RT-PCR; however, no significant differences in gene expression levels were observed between 4T1-controlshRNA and 4T1-Hsp25shRNA cells. Together, these results indicate that silencing the expression of Hsp25 in 4T1 breast adenocarcinoma tumors interferes with its ability to proliferate and metastasize in vitro.

**High expression of Hsp25 represses proteasome activity and tumor suppressor genes.** To obtain an integrative understanding on the effect of Hsp25 silencing on protein expression in 4T1 breast adenocarcinoma cells, the inventors used 2D SDS-PAGE combined with LC-MS/MS techniques to compare the protein profiles between controlshRNA and Hsp25shRNA stably transfected 4T1 cells. Three unique spots were selected from 4T1-
Hsp25shRNA cells which were absent in 4T1-controlshRNA cells. Further characterization using LC-MS/MS and bioinformatics revealed that the unique proteins were NG,Ng-dimethylarginine dimethylaminohydrolase 2 and prohibitin (Table 1; square), PA28α, PA28γ and mitochondrial ribosomal protein L46 (Table 1; circle). Proteins expressed within the triangle could not be identified, possibly due to the highly glycosidic nature of the proteins (Table 1; triangle).

Due to the obvious relevance to tumor growth and metastasis, the inventors chose to validate prohibitin and PA28a by real-time PCR and Western blot analysis. They demonstrated that silencing the hsp25 gene increased prohibitin mRNA expression by 3-fold (FIG. 3A). mRNA expression levels correlated well with a 2.5-fold increase in prohibitin protein expression as judged by Western blot analysis (FIG. 3B). Similar increases were observed for PA28a mRNA expression which was upregulated by 1.5-fold, as judged by real-time PCR (FIG. 4A) and by 2-fold as judged by Western blot analysis, as compared to controls (FIG. 4B). There was no significant alteration in PA28γ protein and RNA levels. To further validate the findings that silencing Hsp25 protein expression increases the proteasome activity, the inventors measured the chymotrypsin-like activity of 20S proteasome in 4T1-controlshRNA and 4T1-Hsp25shRNA cell extracts. The inventors demonstrated that 4T1-Hsp25shRNA cells showed 50% more proteasome activity than 4T1-controlshRNA tumor cells (FIG. 4C). Together, these results indicate that silencing of Hsp25 enhances the tumor suppressor gene prohibitin and proteasome function via PA28a.

**Silencing Hsp25 expression induces tumor regression via enhanced specific CD8+ cytotoxic T lymphocyte (CTL) function.** To determine the consequence of lentivirus-mediated hsp25 gene silencing in vivo, 4T1-controlshRNA and 4T1-Hsp25shRNA tumor cells were injected subcutaneously (s.c.) into the mammary pad of female BALB/c mice. As early as 7 days post-tumor cells injection (TCI), tumors could be visualized growing in the mammary pad of all mice. Mice injected with 4T1-controlshRNA tumors grew progressively and were sacrificed by day 34 post-TCI, due to the tumor burden (FIG. 5A). In contrast, mice injected with 4T1-Hsp25shRNA tumor cells demonstrated a steady regression of tumors after day 7 post-tumor cell inoculation with no detectable GFP signal after day 25 (FIG. 5A). Efficient Hsp25 silencing (>95%) could still be demonstrated in 4T1-Hsp25shRNA tumor before they completely disappeared (day 13 post-tumor cell injection). At the end of the experiment (day 34 post-TCI), gross pathology of multiple organs, including lungs, brain, bone and liver demonstrated an absence of tumor metastasis in mice injected with 4T1-
Hsp25shRNA, but not 4T1-controlshRNA mice. H&E staining of lungs from mice injected with 4T1-controlshRNA revealed micrometastasis in lung tissues.

In contrast, lungs of mice injected with 4T1-Hsp25shRNA had no visible micrometastasis. To confirm that micrometastasis undetectable by light microscopy did not exist in 4T1-Hsp25shRNA injected mice, the inventors performed colonogenisity assays on lung tissues in the presence of complete media containing 6-thioguanine. 4T1 breast adenocarcinoma cells are resistant to 6-thioguanine, however, all other contaminating cells will be destroyed. Mice injected with the 4T1-controlshRNA cells exhibited large numbers of colonies at all dilution, reflecting robust metastasis of tumors to the lungs (FIG. 5B). In contrast, no colonies were observed in dishes plated with lung tissue harvested from mice injected with 4T1-Hsp25shRNA cells (FIG. 5B). Together, these data suggest that permanent silencing of Hsp25 results in tumor regression and inhibition of metastasis in vivo.

To determine the cells responsible for tumor regression following silencing of Hsp25 expression in 4T1 breast adenocarcinoma cells, prior to TCI, the inventors performed in vivo depletion of cells known to play an important role in tumor regression. Here, the inventors demonstrated that in vivo depletion of CD8+ T lymphocytes prior to injection with 4T1-controlshRNA cells, drastically increased tumor growth rate and by day 34 post-TCI the size of the tumors were approximately 10 times larger than mice injected with PBS only. The in vivo depletion of CD4+ T lymphocytes did not significantly alter tumor growth rate or tumor volume in mice injected with 4T1-controlshRNA cells. Unexpectedly, using similar mice, the in vivo depletion of NK cells using the 5E6 monoclonal antibody induced complete tumor regression.

In mice injected with 4T1-Hsp25shRNA cells, no tumor growth was seen in any of the mice by the end of the experiment. As expected, the in vivo depletion of CD8+ T lymphocytes and NK cells, prior to injection with 4T1-Hsp25shRNA cells resulted in tumor growth. Similar depletion of CD4+ T lymphocytes initially resulted in increased tumor growth, followed by tumor regression. Interestingly, although the in vivo depletion of CD8+ T lymphocytes prior to injection with 4T1-Hsp25shRNA cells resulted in increased tumor growth, gross pathology of lung, brain and bone did not reveal any signs of metastasis to the lungs. Similarly, injection of 4T1-Hsp25shRNA cells into the breast pad of BALB/c-nu/nu mice resulted in tumor growth without metastasis.

To confirm that CD8+ T lymphocytes mediated the enhanced cytolytic effects after silencing Hsp25, reactive CD8+ T lymphocytes were harvested from the spleen of mice
which had been injected with 4Tl-Hsp25shRNA cells and were tumor-free (days 21-28 post-TCI) and the specific T-cell cytotoxicity measured against 4Tl-controlRNA target cells ex vivo. Extracted splenic CD8+ T lymphocytes were enriched using negative selection by magnetic beads and consistently exhibited >95% purity, as judged by flow cytometry.

The inventors demonstrated that reactive CD8+ T lymphocytes, but not CD8- T lymphocytes effector cells harvested from the spleen of mice injected with 4Tl-Hsp25shRNA cells exhibited potent-specific lysis against 4Tl-controlshRNA tumor target cells at various effector: target ratios in vitro (FIG. 6A). As expected, both CD8+ and CD8- T lymphocytes from mice injected with 4Tl-controlshRNA cells did not mediate significant lysis above base-line levels against 4Tl-controlshRNA targets. To determine whether 4Tl-Hsp25shRNA reactive CD8+ T lymphocytes could rescue mice injected with 4Tl-controlshRNA cells, 4Tl-Hsp25shRNA reactive CD8+ T lymphocytes were adoptively transferred into 4Tl-controlshRNA tumor-bearing mice. As predicted, the adoptive transfer of 4Tl-Hsp25shRNA reactive CD8+ T lymphocytes into 4Tl-controlshRNA tumor-bearing mice induced significant tumor regression starting by day 17 post-TCI and by day 28 there was no detectable tumor growth (FIG. 6A). In contrast, 4Tl-controlshRNA tumor-bearing mice adoptively transferred with CD8- T cell fraction were not protected and mice rapidly developed tumors (FIG. 6B) and metastasis.

**Delivery of siRNA using LPDS.** Extracted polysaccharides are used to prepare polysaccharide drug (siRNA) composites using free radical grafting method. The polysaccharide drug (siRNA) composites are synthesized by grafting drugs onto purified polysaccharide by free radical polymerization method in aqueous system using eerie ion, nitric acid redox or potassium persulfate, water initiator (FIG. 7). Table 1 below represents effects of the variation in drug-to-polysaccharide concentration on transfection efficiency. From the results it can be seen that increase in the concentration of the polysaccharide increases the drug payload, indicated by increase in the transfection efficiency.

<table>
<thead>
<tr>
<th>Polysaccharide concentration (g/mL)</th>
<th>Amount of kps (initiator) added</th>
<th>Vol. of the siRNA (drug) (nm)</th>
<th>Total cell count</th>
<th>Percent transfection</th>
<th>Cells lines used</th>
<th>Total volume (mL)</th>
</tr>
</thead>
<tbody>
<tr>
<td>6</td>
<td>0.008 g</td>
<td>50</td>
<td>300,000</td>
<td>-35%</td>
<td>4T1</td>
<td>1</td>
</tr>
<tr>
<td>9</td>
<td>0.008 g</td>
<td>50</td>
<td>300,000</td>
<td>-47%</td>
<td>4T1</td>
<td>1</td>
</tr>
<tr>
<td>15</td>
<td>0.008 g</td>
<td>50</td>
<td>300,000</td>
<td>-56%</td>
<td>4T1</td>
<td>1</td>
</tr>
</tbody>
</table>
Increase in the bond strength of the polysaccharide siRNA composites will be achieved by varying the ratios of polysaccharide, drug and initiator concentrations till the maximum % grafting is achieved. The % grafting will be calculated by the equation where \( W_0 \) is weight of the resulting polysaccharide drug composite; \( W_1 \) is weight of the added polysaccharide and \( W_2 \) is weight of the added drug:

\[
\% \text{ Grafting} = \left( \frac{(W_0 - W_1)}{(W_2)} \right) \times 100
\]

Based on the preliminary experiments (Table 2), the concentration ratios will be increased from 1 \( \mu \text{g/mL} \) to 30 \( \mu \text{g/mL} \) of polysaccharide: 0.002g to O.lg of initiator: and 25 nm to 100 nm of siRNA based drug till the maximum grafting efficiency is achieved. This is because change in the ratio of polysaccharide and siRNA also effects the % grafting efficiency thus the bond strength (Rajani et al, 2002)

<table>
<thead>
<tr>
<th>Polysaccharide concentration (( \mu \text{g/mL} ))</th>
<th>Amount of kps (initiator) added</th>
<th>Vol. of the siRNA(drug) (nm)</th>
<th>Total cell count</th>
<th>Percent transfection</th>
<th>Cell lines used</th>
<th>Total volume (mL)</th>
</tr>
</thead>
<tbody>
<tr>
<td>15</td>
<td>0.002 g</td>
<td>50</td>
<td>300,000</td>
<td>~15%</td>
<td>4T1</td>
<td>1</td>
</tr>
<tr>
<td>15</td>
<td>0.008g</td>
<td>50</td>
<td>300,000</td>
<td>-56%</td>
<td>4T1</td>
<td>1</td>
</tr>
<tr>
<td>10</td>
<td>0.002g</td>
<td>25</td>
<td>300,000</td>
<td>-20%</td>
<td>4T1</td>
<td>1</td>
</tr>
</tbody>
</table>

These data demonstrate that grafting GFP-tagged siRNA drug with the fenugreek polysaccharide at different concentration ratios of drug: polysaccharide: initiator concentration increased the bond strength increased from 25% to 50%. Our data shows that the concentration ratios will be increased from 1 \( \mu \text{g/mL} \) to 30 \( \mu \text{g/mL} \) of polysaccharide: 0.002g to O.lg of initiator: and 25nm to 100 nm of siRNA-based drug till the maximum grafting efficiency is achieved.

The inventors next demonstrated that LPDS efficiently binds AA1907 (siRNA-based drug for triple negative breast cancer). In this experiment, grafting was achieved by using potassium persulfate redox initiation in the inert gas atmosphere. Characterization of the polysaccharide drug conjugate using FTIR demonstrates that grafting successfully combined the drug to the polysaccharide LPDS backbone.

One of the major concerns as pointed out in the beginning is the large size of siRNA-based drug. In vitro experiments were performed with GFP-tagged siRNA and LPDS to study
the intracellular internalization of AA1907N/LPDS. Tumor cells were grown on microscopic slides and incubated with GFP-tagged siRNA and LPDS (FP based polysaccharide). After transfection the intracellular distribution of FAM labeled siRNA in the tumor cells was visualized by fluorescence. Both the cytoplasm and nucleus showed the distribution of the labeled siRNA. This provides us with the evidence that LPDS is capable of delivering the drug inside the cytoplasm and nucleus thus overcoming the large size issue of the AA1907.

Finally, the inventors demonstrated that the LPDS efficiently binds siRNA and delivers it into tumors without significant toxicity. In this experiment although the "gold standard" lipofectamine delivery system showed superior transfection efficiency (98%) as compared to the LPDS (56%), this was accompanied by significant cell death (87%), as compared to LPDS (0%). Studies are designed to increase the efficiency of LPDS and maintain its relatively nontoxic abilities.

All of the compositions and/or methods disclosed and claimed herein can be made and executed without undue experimentation in light of the present disclosure. While the compositions and methods of this invention have been described in terms of preferred embodiments, it will be apparent to those of skill in the art that variations may be applied to the compositions and/or methods and in the steps or in the sequence of steps of the method described herein without departing from the concept, spirit and scope of the invention. More specifically, it will be apparent that certain agents which are both chemically and physiologically related may be substituted for the agents described herein while the same or similar results would be achieved. All such similar substitutes and modifications apparent to those skilled in the art are deemed to be within the spirit, scope and concept of the invention as defined by the appended claims.
VI. References

The following references, to the extent that they provide exemplary procedural or other details supplementary to those set forth herein, are specifically incorporated herein by reference.

U.S. Patent 4,981,957
U.S. Patent 5,118,800
U.S. Patent 5,319,080
U.S. Patent 5,359,044
U.S. Patent 5,393,878
U.S. Patent 5,446,137
U.S. Patent 5,466,786
U.S. Patent 5,514,785
U.S. Patent 5,519,134
U.S. Patent 5,567,811
U.S. Patent 5,576,427
U.S. Patent 5,591,722
U.S. Patent 5,597,909
U.S. Patent 5,610,300
U.S. Patent 5,627,053
U.S. Patent 5,639,873
U.S. Patent 5,646,265
U.S. Patent 5,658,873
U.S. Patent 5,670,633
U.S. Patent 5,700,920
U.S. Patent 5,792,747
U.S. Patent 6,268,490
U.S. Patent 6,525,191
U.S. Patent 6,531,584
U.S. Patent 6,600,032
U.S. Patent 6,670,461
U.S. Patent 6,747,014
U.S. Patent 6,753,423
PCT Appln. WO 2005/021570
PCT Appln. WO 2005/1 15481
PCT Appln. WO 2005/121371
PCT Appln. WO 94/14226
PCT Appln. WO 98/39352
PCT Appln. WO 99/14226


What is Claimed:

1. A pharmaceutical composition comprising:
   a) a nucleic acid;
   b) a purified water soluble polysaccharide comprising galactomannan or galacturonic acid; and
   c) a pharmaceutically acceptable formulation.

2. The pharmaceutical composition of claim 1, wherein the nucleic acid is a double-stranded RNA 10-130 nucleotides in length comprising one strand with a sequence that is at least 85% complementary to a mammalian heat shock protein coding sequence.

3. The pharmaceutical composition of claim 2, wherein the RNA is 17-30 nucleotides in length.

4. The pharmaceutical composition of claim 3, wherein the RNA comprises two separate but hybridized strands.

5. The pharmaceutical composition of claim 2, wherein the RNA is blunt-ended.

6. The pharmaceutical composition of claim 2, wherein the RNA is a hairpin.

7. The pharmaceutical composition of claim 2, wherein the RNA has a 5' and/or 3' overhang.

8. The pharmaceutical composition of claim 7, wherein the RNA comprises one or more modified nucleotides.

9. The pharmaceutical composition of claim 1, wherein the polysaccharide comprises a galactomannan moiety.

10. The pharmaceutical composition of claim 9, wherein the galactomannan moiety has the following structure:
wherein R1 or R2 is an OH, a sugar (such as glucose, mannose, etc), a substituted sugar, -OR (R is alkyl, substituted alkyl, alkene, substituted alkene, alkyne, or substituted alkyne and x + y is a number from 50 to 150 and the ratio of x:y is about 1:1 to about 1:5.

12. The pharmaceutical composition of claim 10, wherein the ratio of x:y is about 1:1.

13. The pharmaceutical composition of claim 12, wherein the polysaccharide has the structure of a polysaccharide obtained from Trigonellafoenum-graecum.

14. The pharmaceutical composition of claim 12, wherein the polysaccharide is obtained from the seeds of fenugreek.

15. The pharmaceutical composition of claim 1, wherein the polysaccharide comprises a galacturonic acid moiety.

16. The pharmaceutical composition of claim 15, wherein the polysaccharide further comprises a glucose moiety.

17. The pharmaceutical composition of claim 15, wherein the polysaccharide further comprises a glucopyranose moiety or a galactopyranose moiety.
18. The pharmaceutical composition of claim 15, wherein the polysaccharide has the structure of a polysaccharide obtained from psyllium (Plantago psyllium), okra (Hibiscus esculentus), prickly pear cactus (Opuntia Ficus -Indica) and Aloe vera (Aloe barbadensis).

19. The pharmaceutical composition of claim 2, wherein the heat shock protein is human heat shock factor 27.

20. The pharmaceutical composition of claim 19, wherein the RNA has at least 90% sequence identity to SEQ ID NO:2.

21. The pharmaceutical composition of claim 19, wherein the RNA has at least 95% sequence identity to SEQ ID NO:2.

22. The pharmaceutical composition of claim 19, wherein the RNA is identical to SEQ ID NO:2.

23. The pharmaceutical composition of claim 1, wherein the RNA comprises the sequence of SEQ ID NO:2.

24. The pharmaceutical composition of claim 1 comprising multiple different polysaccharides.

25. The pharmaceutical composition of claim 24, wherein at least two of the different polysaccharides comprise a galactomannan or a galacturonic acid moiety.

26. The pharmaceutical composition of claim 25, wherein the at least two polysaccharides can be obtained from psyllium (Plantago psyllium), okra (Hibiscus esculentus), fenugreek (Trigonella foenum-graecum), prickly pear cactus (Opuntia Ficus -Indica), or Aloe vera (Aloe barbadensis).

27. The pharmaceutical composition of claim 1, wherein RNA and polysaccharides are directly attached.

28. The pharmaceutical composition of claim 27, wherein RNA and polysaccharides are chemically conjugated.

29. The pharmaceutical composition of claim 28, wherein more than one RNA molecule is conjugated to a polysaccharide.
30. The pharmaceutical composition of claim 1, wherein the composition comprises nanoparticles comprising RNA and polysaccharides.

31. The nanoparticle of claim 30, wherein the RNA is 17-30 nucleotides in length.

32. The nanoparticle of claim 31, wherein the stranded RNA comprises two separate but hybridized strands.

33. The nanoparticle of claim 30, wherein the RNA is blunt-ended.

34. The nanoparticle of claim 30, wherein the RNA is a hairpin.

35. The nanoparticle of claim 30, wherein the RNA has a 5' and/or 3' overhang.

36. The nanoparticle of claim 30, wherein the RNA comprises one or more modified nucleotides.

37. The nanoparticle of claim 30, wherein the polysaccharide comprises a galactomannan moiety.

38. The pharmaceutical composition of claim 1, wherein the ratio of RNA:polysaccharide is from about 1:20 to about 10:1.

39. A method for treating a patient for a disease or condition comprising administering to the patient an effective amount of the pharmaceutical formulation of any of claims 1-38.

40. The method of claim 39, wherein the disease or condition is cancer, a bacterial or viral infection, inflammation, or toxin exposure.

41. The method of claim 40, wherein the disease is cancer.

42. The method of claim 41, wherein the cancer is breast cancer, lung cancer, head & neck cancer, esophageal cancer, prostate cancer, ovarian cancer, uterine cancer, cervical cancer, colon cancer, stomach cancer, liver cancer, testicular cancer, skin cancer, pancreatic cancer, rectal cancer, melanoma, glioma, neuroblastoma or sarcoma.

43. The method of claim 42, wherein the cancer is breast cancer.
44. The method of claim 41, further comprising treating the patient with an additional cancer therapy, wherein the additional therapy is tumor resection, chemotherapy, or radiation.

45. The method of claim 44, wherein the patient is treated with the additional cancer therapy prior to treatment with the pharmaceutical composition.

46. The method of claim 45, wherein the patient is treated with the additional cancer therapy concurrently with the pharmaceutical composition.

47. The method of claim 39, further comprising identifying a patient in need of the pharmaceutical composition.

48. The method of claim 39, wherein the pharmaceutical composition is administered intravenously, intratumorally, intraperitoneally, intraarterially, subcutaneously, orally, topically, by lavage, or by direct injection.

49. A method for administering a therapeutic agent to a cell comprising contacting the cell with the pharmaceutical composition of any of claims 1-38.

50. The method of claim 49, wherein the cell is a cancer cell.

51. The method of claim 50, wherein the cancer cell overexpresses a heat shock protein, and the pharmaceutical composition inhibits expression of the heat shock protein.

52. A method for preparing a pharmaceutical composition comprising combining a reactive polysaccharide comprising galactomannan or galacturonic acid with a nucleic acid.

53. The method of claim 52, wherein the reactive polysaccharide is a free radical formed by contacting the polysaccharide with a composition comprising eerie ion, nitric acid, and/or potassium persulfate.

54. The method of claim 52, wherein the nucleic acid is an RNA molecule.

55. The method of claim 54, wherein the RNA molecule is fully or partially double stranded.

56. The method of claim 55, wherein the RNA molecule is fully double stranded.
57. The method of claim 56, wherein each strand of the RNA molecule is 17-30 nucleotides in length.

58. The method of claim 52, wherein the nucleic acid comprises one strand with a sequence that is at least 85% complementary to a mammalian heat shock protein coding sequence.

59. The method of claim 52, wherein the nucleic acid comprises one strand with a sequence that is at least 90% complementary to a mammalian heat shock protein coding sequence.

60. A composition comprising (i) a water soluble polysaccharide comprising galactomannan or galacturonic acid, and (ii) a nucleic acid made by a method comprising:

(a) obtaining purified polysaccharide;

(b) creating a reactive polysaccharide through free radical polymerization; and

(c) contacting the reactive polysaccharide with the nucleic acid to form a polysaccharide grafted with the nucleic acid.
**FIG. 1A-C**

A

5'LTR → EF-1α → GFP → WPRE → Hsp25 shRNA → SIN

4T1-Hsp25shRNA hairpin loop sequence

AGCCCGAGCUUGGAACCAU
UUUCGGGCUCCAGCCAUUGUA

4T1-controlshRNA hairpin loop sequence

GCUCUAUCGAGAGGAUAUA
UUCGAGUUGCCUCUCCUUAUA

B

4T1-Hsp25shRNA

C

4T1-controlshRNA  4T1-Hsp25shRNA

Hsp25

β-actin
FIG. 2A-B
FIG. 3A-B
FIG. 4A-B
FIG. 4C
FIG. 5A-B
FIG. 6A
FIG 6B
FIG. 7