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Research Article

Preclinical Biodistribution and Safety Evaluation of a pbi-shRNA STMN1 Lipoplex after Subcutaneous Delivery

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ABSTRACT

Stathmin-1 (STMN1) is a microtubule-destabilizing protein which is overexpressed in cancer. Its overexpression is associated with poor prognosis and also serves as a predictive marker to taxane therapy. We have developed a proprietary bi-functional shRNA (bi-shRNA) platform to execute RNA interference (RNAi)-mediated gene silencing and a liposome–carrier complex to systemically deliver the pbi-shRNA plasmids. *In vitro* and *in vivo* testing demonstrated efficacy and specificity of pbi-shRNA plasmid in targeting STMN1 (Phadke, A. P., Jay, C. M., Wang, Z., Chen, S., Liu, S., Haddock, C., Kumar, P., Pappen, B. O., Rao, D. D., Templeton, N. S., *et al.* (2011). In vivo safety and antitumor efficacy of bifunctional small hairpin RNAs specific for the human Stathmin 1 oncoprotein. DNA Cell Biol. 30, 715–726.). Biodistribution and toxicology studies in bio-relevant Sprague Dawley rats with pbi-shRNA STMN1 lipoplex revealed that the plasmid DNA was delivered to a broad distribution of organs after a single subcutaneous injection. Specifically, plasmid was detected within the first week using QPCR (threshold 50 copies plasmid/1 μg genomic DNA) at the injection site, lung, spleen, blood, skin, ovary (limited), lymph nodes, and liver. It was not detected in the heart, testis or bone marrow. No plasmid was detected from any organ 30 days after injection. Treatment was well tolerated. Minimal inflammation/erythema was observed at the injection site. Circulating cytokine response was also examined by ELISA. The IL-6 levels were induced within 6 h then declined to the vehicle control level 72 h after the injection. TNFα induction was transiently observed 4 days after the DNA lipoplex treatment. In summary, the pbi-shRNA STMN1 lipoplex was well tolerated and displayed broad distribution after a single subcutaneous injection. The pre-clinical data has been filed to FDA and the pbi-shRNA STMN1 lipoplex is being investigated in a phase I clinical study.

Key words: nanoparticles; agents; dose–response; risk assessment; biomarkers; safety evaluation.

RNA interference (RNAi) is a mechanism of gene silencing produced by small RNAs, including microRNA (miRNA), small interfering RNA (siRNA) and small hairpin (shRNA) (Fire *et al.*, 1998). This evolutionarily conserved gene-silencing mechanism is highly dependent on complimentary or near-complimentary nucleotide sequences and is executed via the intracellular RNA-induced silencing complex (RISC). RNAi is considered a revolutionary therapeutic approach to target otherwise "undruggable" targets with specificity and robustness with superiority over small molecules, recombinant proteins, and monoclonal antibodies. Since the discovery of RNAi in 1998, RNAi has translated from lab to the clinic with an unprecedented pace. RNAi has been used to treat a wide array of diseases, including cancer, cardiovascular metabolic disease, HBV, hemophilia, Ebola virus, transthyretin amyloidosis, and ocular diseases. A dozen RNAi-based drugs are now undergoing clinical trial investigation

(Coelho *et al.*, 2013; Davis *et al.*, 2010; Ramanathan *et al.*, 2013; Senzer *et al.*, 2012; Tabernero *et al.*, 2013). RNAi-based therapies in development are increasingly exploring molecular target-specific delivery approaches.

Stathmin-1 (STMN1) belongs to a family of microtubule-destabilizing proteins. STMN1 regulates rapid microtubule remodeling of the cell skeleton and plays an important role in cell cycle progression and mitosis (Mistry and Atweh, 2002). It is overexpressed in a broad range of human malignancies, including leukemia, lymphoma, neuroblastoma, ovarian, prostate, breast, lung, and mesothelioma (Rana *et al.*, 2008). Overexpression of STMN1 has been correlated with poor prognosis in cancer patients (Kouzu *et al.*, 2006; Watanabe *et al.*, 2014). We have identified STMN1 as a candidate target gene in >80% of 30 paired tumor versus normal tissues analyzed by proteomics and microarray analysis (Nemunaitis *et al.*, 2007). The characterization of STMN1 as an oncogene is also corroborated by findings in various human cancers including colorectal cancer, melanoma and osteosarcoma (Alli *et al.*, 2007b; Iancu *et al.*, 2000; Zhang *et al.*, 2004).

The therapeutic potential of STMN1 has been examined as either a singlet therapeutic or in combination with chemotherapeutic agents. For instance, RNAi-based therapies have been employed to silence STMN1 gene expression and inhibit growth of tumor cells (Zhang *et al.*, 2006). Silencing STMN1 expression by siRNA in breast cancer cells sensitized STMN1-overexpessing cancer cells to anti-microtubule agents (Alli *et al.*, 2007a). In addition, antisense inhibition of STMN1 in leukemia cells demonstrated therapeutic synergy with taxol, thus raising the possibility of reduced dose taxol administration so as to maintain effectiveness whereas alleviating the severe toxicities leading to premature discontinuation of therapy (Iancu *et al.*, 2000).

We have developed a novel "bi-functional" shRNA expression platform to knockdown cancer molecular signal targets through cleavage-dependent-RISC mRNA cleavage and cleavage-independent-RISC mRNA degradation, sequestration and translational suppression (Rao *et al.*, 2013). Using bi-functional shRNA to target human STMN1, we have demonstrated effective STMN1 silencing in human colorectal cancer cells and human melanoma cells with lower dosage and prolonged silencing duration compared with RNA oligonucleotides duplex (Rao *et al.*, 2010). We also examined the effects of an intratumoral delivery of pbi-shRNA targeting STMN1 in several tumor xenograft models. The pbi-shRNA STMN1 was formulated with a proprietary cationic liposome delivery system to form a pbi-shRNA STMN1 lipoplex. pbi-shRNA STMN1 lipoplex was shown to knockdown STMN1 expression and inhibit the growth of tumorgrafts derived from colorectal cells, melanoma cells and primary osteosarcoma (Phadke *et al.*, 2011).

The present study evaluated the toxicology profile and biodistribution of a single subcutaneous administration of pbi-shRNA STMN1 lipoplex in a bio-relevant rat model. The results of the study had been filed to FDA for a phase I IND approval and pbi-shRNA STMN1 lipoplex is being tested in a phase I clinical trial (BB-IND 14938).

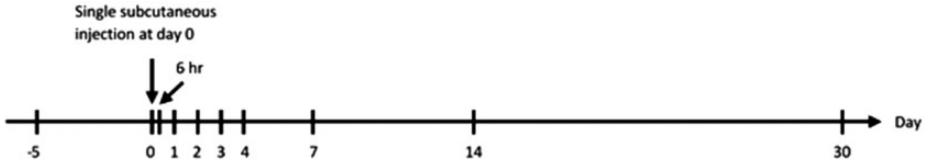
MATERIALS AND METHODS

Plasmid DNA and lipoplex formulation. The pbi-shRNA STMN1 plasmid was constructed as described previously (Rao *et al.*, 2010) and the GMP grade plasmid was manufactured by Gradalis (Carrollton, TX). pbi-shRNA STMN1 LPX was prepared as previously described (Phadke *et al.*, 2011). Briefly, Liposomes (LP) were made by the thin film method in our cGMP facility. DOTAP and Cholesterol powder (Avanti Polar Lipids, AL) were dissolved in chloroform and dried down to a thin film, which was then resuspended in 5% dextrose, USP (Baxter, IL). The crude Liposomes were sonicated and extruded through a series of filters to create the final LP product. The Liposomes were QC checked for particle size, zeta potential, residual chloroform, and sterility before proceeding to the next processing step. pbi-shRNA plasmid DNA (1 mg/ml) was mixed with 2 times DOTAP:Cholesterol Liposomes in our cGMP facility to form the final plasmid DNA-Lipoplex suspended in D5W (diluent consisting of 5% dextrose in water). The final pbi-shRNA STMN1 Lipoplex was at a concentration of 0.46 mg/ml and was stored in sterile vials at 2–8 °C in a dark container until ready for use. The DNA-Lipoplex product was tested for sterility, endotoxin, optical characteristics (OD 400, particle size and zeta potential), and payload (DNA extraction, quantitation, and identity) prior to final release. Empty liposomes without plasmid DNA and/or D5W were used as control articles in this study.

Biodistribution study. The subcutaneous route of administration was selected in preparation for initial testing in cancer patients as a single intra-tumor injection in order to demonstrate initial safety and molecular mechanism of action. The selected dose level (100 μg) for the test article is approximately double the highest dose that has been proposed to be administered in the phase I clinical trial. This dose has been shown to be safe in animals as described previously (Phadke *et al.*, 2011).

The in-life study was performed by Charles River Laboratories (Wilmington, MA). Two hundred twenty μl of either test article or control article were administered to Sprague Dawley rats via a single subcutaneous injection (Figure 1). Ten rats receiving no injections were used as naïve controls. The injection site (approximately 2 cm × 2 cm) was delineated with an indelible marker. A 1 ml syringe was used for dosing and the doses were administered slowly and evenly over at least 1 minute. The day of dosing was designated as day 0. At day 1, 2, 3, 4, 7, 14, and 30 after injection, blood and a panel of organs (12 total, including skin at injection site, liver, lung, heart, spleen, brain, kidney, bone marrow, diaphragm, testis or ovary, muscle, and lymph node) were harvested from 10 rats (5 females and 5 males) at each designated time point. Appropriate precautions were taken to minimize tissue cross-contamination. To minimize the influence of plasmid DNA in blood circulating through the tissues at the time of sampling, the heart and lung tissue samples were thoroughly washed with sterile phosphate buffered salt (PBS) after weighing and before freezing tissues.

From each animal, blood and 12 organs were collected and snap frozen immediately. The organs were homogenized using a RNA lysis buffer (Macherey-Nagel) and genomic DNA was isolated from each tissue (25 mg ± 5 mg, each) or 0.2 ml of blood by using the NucleoSpin RNA II kit followed by NucleoSpin RNA/DNA buffer set (Macherey-Nagel). Genomic DNA from the rat blood was isolated using the NucleoSpin Blood kit (Macherey-Nagel). The quality of extracted DNA was examined by amplifying rat genomic GAPDH using a QPCR assay. The plasmid DNA was quantified by QPCR using 1 μg of extracted DNA as template per reaction. The QPCR were performed in a CFX96 Real-Time PCR Detection System (Bio-Rad) in a final volume of 25 μl (12.5 μl of IQ Supermix, 300 nM of each primer, 150 nM of FAM-TaqMan Probe and 1 μg of DNA sample). The forward primer (5′-TTTATTCAACAAAGCCGCCGTCCC-3′) and reverse primer (5′-CTATGGAACTGCCTCGGTGAGTTT-3′) amplified a 199 bp region on the vector backbone and a Taqman probe labelled with both FAM and a quencher (5′-AGTCAGCGTAATGCTCTGCCAGTGTT-3′)



Collect blood and tissue samples at day -5, 1, 2, 3, 4, 7, 14 and 30 for plasmid biodistribution study.

Collect blood at day -5, 6 hr, and day 1, 2, 3, 4, 7 for immunoassays.

Group	Treatment	Dose	Injection volume	Total number of rats
1	pbi-shRNA™ STMN1 LP	100 μg	220 ul	50 females, 50 males
2	D5W (5% Dextrose in Water)	N/A	220 ul	50 females, 50 males
3	Non-treated	N/A	None	5 females, 5 males

FIG. 1. The study design of biodistribution and immunology. 100 rats (50 of each gender) received 220 μl of either pbi-shRNA STMN lipoplex or D5W, with blood and tissues harvested at designated time points. In addition, 10 rats (5 of each gender) were included as negative controls. The collected blood and tissues were evaluated for innate immune responses and biodistribution.

was used to increase the specificity of QPCR. The thermal cycling conditions for the QPCR were: 95 °C for 3 min, followed by 40 cycles of 95 °C for 15 s and 60 °C for 1 min. Amplification of rat GAPDH was used as quality control of the genomic DNA in one of the 3 replicates to rule out the disturbance of inhibitory substances. Samples with a Ct (cycle threshold) ≥ 38 were considered negative. One microgram of extracted DNA was tested in triplicate (when quantity allowed) and one of the triplicates for each control tissue was spiked with 50 copies of plasmid. Each QPCR plate contained a standard curve established with different concentrations of pbi-shRNA STMN1 plasmid ranged at least 6 Log concentrations. The QPCR parameter LOQ (limit of Quantification) was determined independently for each plate. The detection limit of this QPCR assay is established as 50 copy of plasmid for 1 μg of total DNA, which meets the assay requirements of FDA guidelines for non-viral vector quantification. The DNA extraction and QPCR quantification were performed by Harlan Laboratories (Switzerland).

Immune response study. Blood collected from the Sprague Dawley rats enrolled in the biodistribution study was analyzed for detection of IL-6, TNFα, and IL-1β. Rats were sacrificed at 6 h, day 1, day 2, day 3, day 4, and day 7 post-injection. Animals were not fasted prior to blood collection. Plasma was separated from the blood samples and then frozen for cytokine analysis using ELISA immunoassay. The Quantikine ELISA kits were purchased from R&D Systems (Minneapolis, MN) and then validated in house before cytokine analysis. The ELISA assays were performed in house following the manufacturer's instructions. Briefly, rat plasma samples were diluted 1:2 fold using the assay buffer provide in the ELISA kit. Diluted samples were loaded onto a pre-coated plate and then incubated for 2 h at room temperature. A substrate solution was added to each well after aspiration and washing for 4 times. After incubation for 30 min at room temperature, the plate was read using a microplate reader set at 450 and 550 nm. The cytokine concentration was calculated from a standard curve generated from the same run.

Toxicology study. Three groups of Sprague Dawley rats (45 males and 45 females in each group) were treated with 220 μl of pbi-shRNA STMN1 lipoplex, empty liposome or D5W (5% dextrose in water) as a single subcutaneous injection. Test animals were sacrificed and evaluated at one of the following time points: Day 1, 2, 3, 4, 7, 14, 30, 60, or 90 (Figure 2). Evaluated endpoint parameters include clinical signs, body weight, body weight change, clinical pathology parameters (hematology, coagulation, and clinical chemistry), gross necropsy findings, organ weight, and histopathological examination. The in-life study, clinical chemistry analysis and histopathology examinations were conducted by Charles River Laboratories (Wilmington, MA).

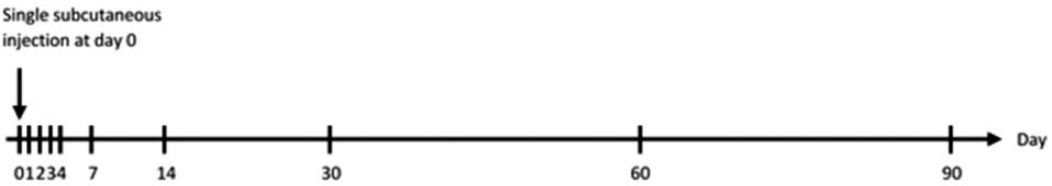
Blood samples were collected for hematology in tubes containing K_2EDTA anticoagulant and were analyzed for red blood cell count, white blood cell count, hemoglobin concentration, neutrophil count, hematocrit, lymphocyte count, mean corpuscular volume, monocyte count, mean corpuscular hemoglobin concentration, eosinophil count, mean corpuscular hemoglobin, basophil count, reticulocyte count, large unstained cells, platelet count, and red cell distribution width. Blood smear slides were prepared for all animals for possible RBC morphology evaluation. One slide per animal was prepared, stained, and archived. Slide review was only performed on samples that met flagging criteria to confirm accurate hematology data.

Blood samples were collected for coagulation assessment into tubes containing sodium citrate anticoagulant and were processed for plasma. The plasma samples were then analyzed for both activated partial thromboplastin time and prothrombin time.

Serum was analyzed for alanine aminotransferase [ALT], aspartate aminotransferase [AST], total protein, albumin, alkaline phosphatase, globulin, gamma-glutamyltransferase, albumin/globulin ratio, total bilirubin, glucose, urea nitrogen, cholesterol, creatinine, triglycerides, calcium, sodium, phosphorous, potassium, and chloride.

All animals were subjected to a complete necropsy examination, which included evaluation of the carcass and musculoskeletal system; all external surfaces and orifices; cranial cavity and external surfaces of the brain; and thoracic, abdominal, and pelvic cavities with their associated organs and tissues. Necropsy examinations were conducted under the supervision of a board certified veterinary pathologist.

The organs were weighed at necropsy. The weighed organs included brain, kidney, diaphragm, liver, epididymis, lung, ovary, spleen, testis, thymus, heart, uterus, adrenal gland,



Collect blood and tissue samples at day 1, 2, 3, 4, 7, 14, 30, 60 and 90 for blood chemistry, gross pathology and histopathology evaluations.

Group	Treatment	Dose	Injection volume	Total number of rats
1	pbi-shRNA™ STMN1 LP	100 μg	220 ul	45 females, 45 males
2	Empty Liposome	N/A	220 ul	45 females, 45 males
3	D5W (5% Dextrose in Water)	N/A	220 ul	45 females, 45 males

FIG. 2. The study design of toxicology. 90 rats (45 of each gender) received 220 μl of either pbi-shRNA STMN lipoplex, or empty liposome or D5W, with blood and tissues harvested at designated time points. The collected blood and tissues are examined for clinical pathology (including hematology, coagulation, and clinical chemistry), gross pathology, and histopathology.

TABLE 1. Tissue Distribution of Positively Detected pbi-shSTMN1 Plasmid DNA

Day	No.	Blood	Bone Marrow	Brain	Diaphragm	Heart	Kidney	Liver	Lymph Node	Lung	Muscle	Skin	Spleen	Ovaries*	Testes*
1	10	4	0	1	1	0	2	1	4	7	1	10	5	2	0
2	10	0	0	0	0	0	0	0	0	4	0	10	5	1	0
3	10	2	0	1	1	0	0	2	1	8	1	10	10	1	0
4	10	0	1	1	0	0	0	0	1	8	0	9	3	0	0
7	10	0	0	0	0	0	0	0	1	7	0	9	5	1	0
14	10	0	0	0	0	0	1	0	0	2	0	3	2	0	0
30	10	0	0	0	0	0	0	0	0	0	0	0	0	0	0

The detection limit is 50 copies for 1 μg of genomic DNA.
*5 rats for ovaries and testes.

pituitary gland, thyroid, and prostate. Paired organs were weighed together. Organ to body weight ratios (using the terminal body weight) and organ to brain weight ratios were calculated.

Tissues were processed for histopathology at Charles River Laboratories, Pathology Associates (Maryland). Tissues were trimmed, embedded in paraffin, sectioned, mounted on glass slides, and then stained with hematoxylin and eosin. Histopathological evaluation was performed by a board certified veterinary pathologist. The examined tissues included administration site, artery/aorta, lung, bone marrow, bone, brain, cervix, diaphragm, epididymis, esophagus, eye, heart, kidney, large intestine, liver, lung, lymph node, skeletal muscle, optical nerve, sciatic nerve, ovary, oviduct, pancreas, skin, small intestine, spinal cord, spleen, stomach, testis, thymus, tongue, trachea, ureter, urinary bladder, uterus, vagina, adrenal gland, harderian gland, mammary gland, parathyroid gland, pituitary gland, prostate, salivary gland, seminal vesicle gland, thyroid gland, gross lesions/masses, and gut-associated lymphoid tissue.

Statistical analysis. Means and standard deviations were calculated for body weight, body weight changes, hematology parameters, coagulation parameters, clinical chemistry parameters and organ weight. Student's *t*-test was used to analyze cytokine changes and the data was presented as mean ± SD.

RESULTS

DNA-Lipoplex Characterization

The pbi-shRNA STMN1 Lipoplex was manufactured in our cGMP facility, as described in the methods and materials section above. The final product had an optical density (OD400) of 0.821 and an average particle size (Dynamic Light scattering) of 357 nm. The zeta potential was +59 mV. The product was sterile, had less than 0.5 EU/mL endotoxin, and less than 0.3 ppm residual chloroform. QC vials of the final product were retained and checked according to our stability program throughout the duration of the animal study. The DNA-LPX product continued to pass all QC tests for the duration of the testing period (12 months).

Biodistribution Study

The pbi-shRNA STMN1 plasmid DNA was quantified in rat blood and in a panel of 12 rat organs using QPCR after a single subcutaneous injection of pbi-shRNA STMN1 lipoplex. The detection limit of this QPCR assay was 50 copies of plasmid for 1 μg of genomic DNA. Twenty-four hours after a single subcutaneous injection, the plasmid DNA could be found in all organs except testis, heart and bone marrow. As shown in Table 1, the plasmid DNA was mainly localized in blood (4/10), lung (7/10), spleen (5/10), lymph node (4/10), and injection site (10/10). Within 3–4 days after the single subcutaneous injection, the pbi-shRNA

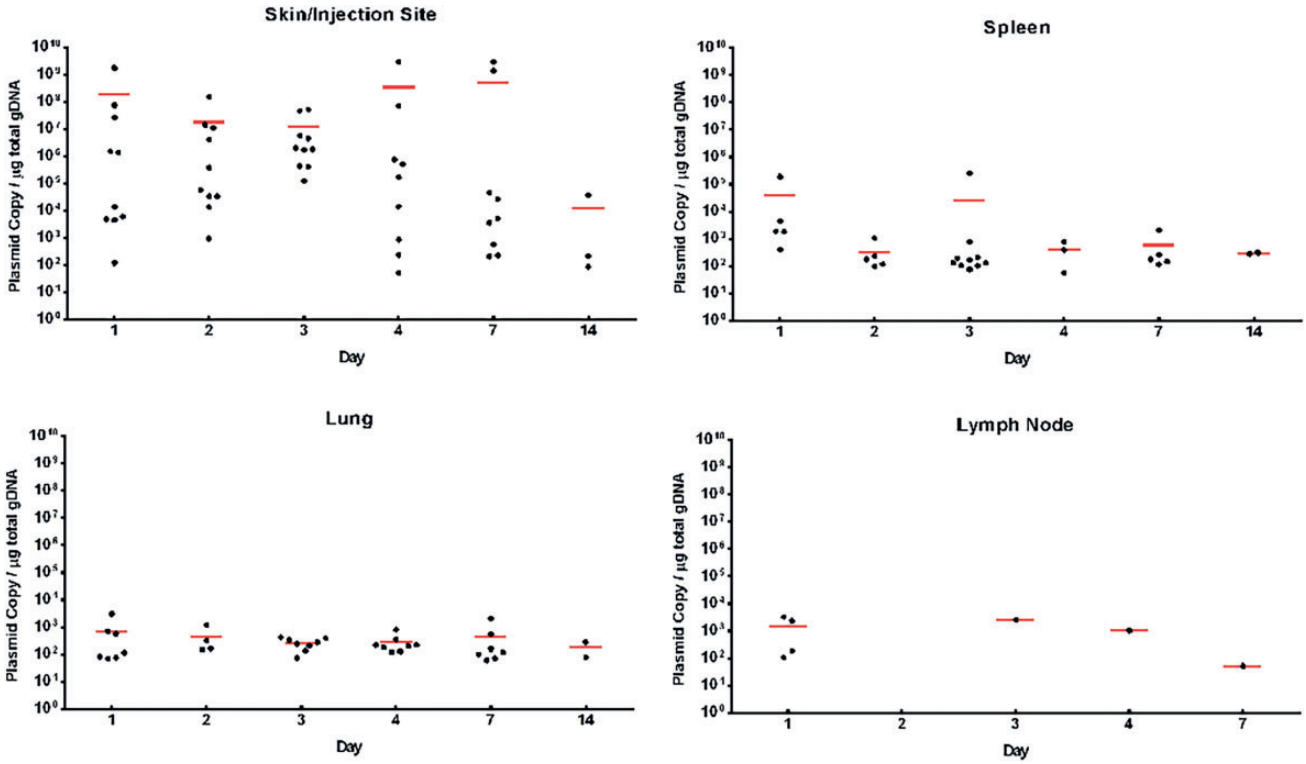


FIG. 3. Tissue distribution of plasmid DNA in major organs of rats that received a single-dose subcutaneous injection of pbi-shRNA STMN1 lipoplex. The plasmid copy number detected from each animal was presented as individual value ($n = 10$ each group). The red horizontal lines represent the average copy number of plasmid detected at designated time point from each major organ.

STMN1 plasmid was found in most of the organs consistent with systemic distribution. The plasmid DNA was mainly localized in spleen (10/10 on day 3; 3/10 on day 4), lung (8/10 on day 3; 8/10 on day 4), liver (2/10 on day 3; 0/10 on day 4), bone marrow (0/10 on day 3 and 1/10 on day 4), and injection site (10/10 on day 3 and 9/10 on day 4); there was no detectable plasmid DNA in testis, heart, and kidney. Seven days after subcutaneous injection, pbi-shRNA STMN1 plasmid was detected in spleen (5/10), lung (7/10), and skin (9/10), the ovary and draining lymph node in one animal each, and no plasmid DNA in other organs. Fourteen days after the subcutaneous administration, the plasmid DNA could be found in lymph node (1/10), spleen (2/10), lung (2/10), kidney (1/10), and skin (3/10). Thirty days after a single subcutaneous injection, all the tissues examined were found to be below detection limit of QPCR. Because there was no plasmid detected beyond the day 30 time point, tissues were not harvested 60 and 90 days after administration. Twenty-four hours after a single subcutaneous injection of pbi-shRNA STMN1 lipoplex, an average of 1.9×10^8 copies of plasmid DNA per µg of genomic DNA was detected from the injection sites and persisted at similar levels through day 7 as shown in Figure 3. The average copy of plasmid DNA declined to 1.27×10^4 copies per µg of genomic DNA 14 days after injection with only 3 samples testing positive. Furthermore, an average of either 3.39×10^2 or 4.08×10^4 copies of plasmid DNA per µg of genomic DNA was detected in the spleen either 1 or 3 days after the injection. The plasmid DNA level in spleen declined to 3.04×10^2 copies per µg of genomic DNA 14 days after injection and remained undetectable onwards. The presence of plasmid DNA in lung was consistent, with an average 6.87×10^2–1.81×10^2 copies of plasmid DNA per 1 µg of genomic DNA detected from days 1 to 14. There were only 2 animals found to have detectable plasmid DNA in the lung 14 days after the subcutaneous injection. The plasmid DNA in lymph node was only detected in a few animals 1–7 days post-injection and was found to be negative 14 days post-injection and afterwards. Detectable levels of plasmid DNA over a period of 14 days after a single subcutaneous injection were found in the greatest number of rats in the lung and spleen injection sites. There was no plasmid detected from the heart or testes at any time point examined. All animals treated with D5W (control group) showed no detectable plasmid DNA, as expected.

Cytokine Assessment

The temporal responses of cytokines in rats following a single subcutaneous administration of pbi-shRNA STMN1 lipoplex were examined using ELISA assays including TNFα, IL-6, and IL-1β. Blood was collected at designated time points following either pbi-shRNA STMN1 lipoplex or vehicle control (D5W).

As shown in Figure 4, the injection of pbi-shRNA STMN1 lipoplex led to a statistically significant elevation of IL-6 in rat blood at 6, 24, and 48 h post-injection. Beyond 48 h post-injection, the elevated IL-6 level in animals treated with lipoplex declined to that of the vehicle control group. There was no statistically significant elevation of TNFα detected in rat blood until the 4th day after treatment with pbi-shRNA STMN1 lipoplex. In addition, the TNFα level in both treatment group and vehicle control group was lower than that detected in the naïve rats sacrificed 5 days before injection. Six hours after receiving pbi-shRNA STMN1 lipoplex, the average TNFα level increased, but was not statistically significant ($P = 0.074$) in comparison with the TNFα level in the vehicle control group. The treatment group and vehicle control group presented comparable levels of TNFα at days 1, 2, and 3 post-injection. The TNFα level remained

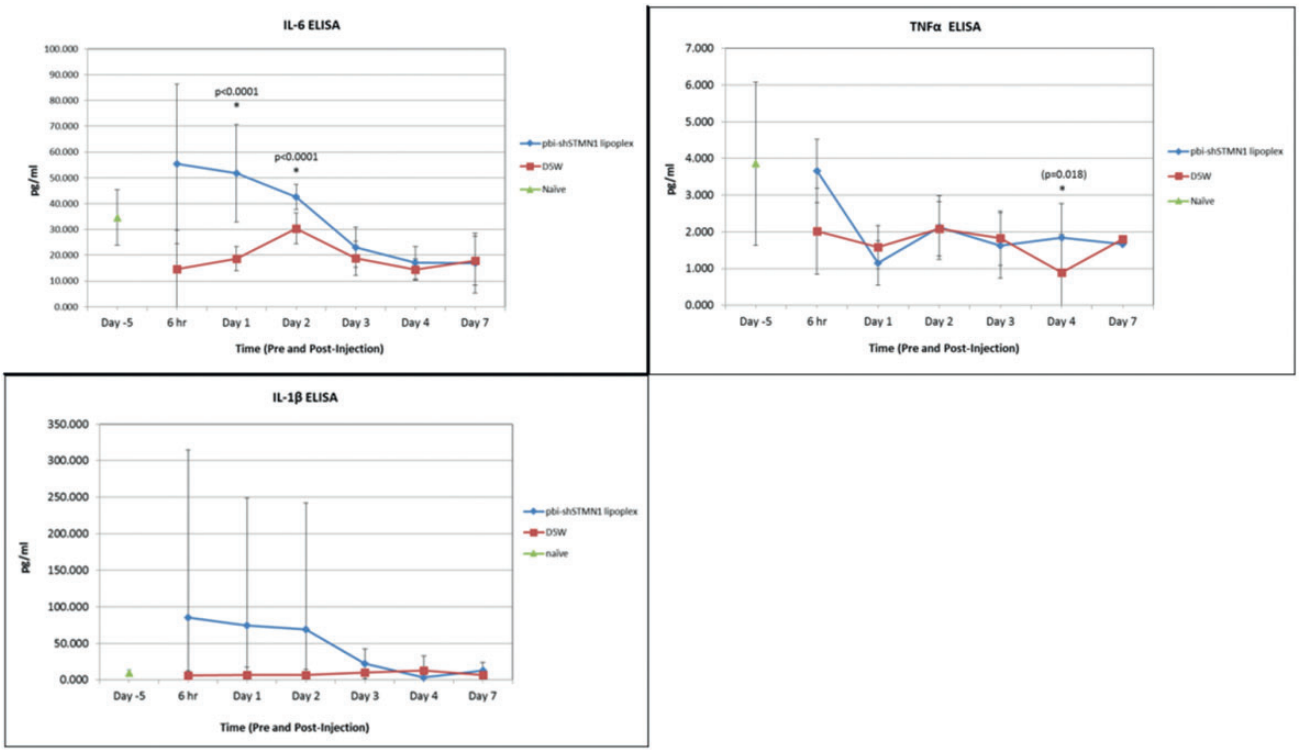


FIG. 4. Induction of cytokines after a single subcutaneous injection of pbi-shRNA STMN1 lipoplex. TNFα, IL-1β and IL-6 before and after the treatment were quantified using ELISA assays ($n = 10$ each group). Control group animals received a subcutaneous injection of D5W. Animals without any treatment were used as naïve controls. Data are presented as mean ± SD.

elevated 4 days after the injection of DNA lipoplex and then declined to vehicle control group levels at day 7. In addition, the subcutaneous injection of pbi-shRNA STMN1 lipoplex did not result in statistically significant changes of IL-1β in rats when compared with rats in the vehicle control group. At 6, 24, and 48 h post-injection, the pbi-shRNA STMN1 lipoplex group presented a higher average IL-1β level than both the vehicle control group and naïve group. At each of the 3 time points, the DNA lipoplex treatment group had a single animal with an unusually high concentration of IL-1β, which resulted in a large standard deviation skewing the data. Despite the apparent increase of average IL-1β concentration, there was no statistically significant difference between the 2 groups. From day 3 to 7 post-injection, the pbi-shRNA STMN1 LP and vehicle control group IL-1β levels were comparable.

In summary, treatment with pbi-shRNA STMN1 lipoplex resulted in a statistically significant increase of IL-6 in rat blood 6 h post-injection with normalization level by 72 h post-injection. The results also show that the subcutaneous treatment with pbi-shRNA STMN1 lipoplex induces a limited but significant increase in the concentration of TNFα in rat blood 4 days after administration. Finally comparable, the analysis detects some variable non-significant increases of IL-1β level from 6 to 48 h post-injection.

Toxicology Study

The toxicological profile of subcutaneously administered pbi-shRNA STMN1 lipoplex was evaluated in rats, a bio-relevant animal with a STMN1 sequence highly homologous to the human. Rats receiving the same dosage of either empty liposome or D5W were employed as controls in this study.

There was no mortality in any of the animals during the study. Treatment with neither pbi-shRNA STMN1 lipoplex nor empty liposome affected body or organ weights during the study. Animals gained weight throughout the study.

The subcutaneous administration of pbi-shRNA STMN1 lipoplex did not exert significant effects on hematology and coagulation parameters during the study. A slight increase in segmented neutrophils was noted in the DNA lipoplex-treated males and empty liposome-treated males on day 1 to day 2 and in the DNA lipoplex-treated females and empty liposome treated females on day 1 to day 3. However, the values were generally well within the historical control range and the apparent changes were transient. None of the other differences in hematology and coagulation parameters were considered to be toxicologically meaningful based on the slight magnitude of the changes because the values typically were within the historical control ranges. Neither were there notable effects on clinical chemistry parameters during the study. None of the apparent differences noted in clinical chemistry parameters between the groups were considered to be toxicologically meaningful based on the minimal variance from the control-treated animals and because the values were typically within the historical control ranges.

There were no observed adverse effects during the study. Sporadic incidences of hair loss and/or scabs were noted in both the treatment and control groups which were considered common observations in this strain and at this age of laboratory rat. The most common finding during the study was that raised areas developed at the location of administration of the pbi-shRNA STMN1 lipoplex. In males, raised areas were noted beginning on day 1 (4/5 rats being observed) and continued

through day 90 (2/5 rats), whereas in the females, the raised areas were not noted until day 2 (3/5 rats). Additionally, raised areas at the administration sites were noted in the empty liposome-treated females beginning on day 2 (5/5 rats), and in empty liposome-treated males beginning on day 3 (1/5 rats). Once the raised areas developed in the pbi-shRNA STMN1 lipoplex-treated animals, they persisted, whereas the raised areas noted in the empty liposome-treated animals resolved by day 30. The specific cause of the raised areas seemed to be related to the liposome, because no raised areas were found in the animals receiving the same volume of D5W.

Liposome-related changes were also observed at the injection site during gross necropsy with a slight increase in the total number of animals affected in the group treated with pbi-shRNA STMN1 lipoplex. These changes initially consisted of thickening, pale areas, dark areas and mottled areas in animals treated with pbi-shRNA STMN1 lipoplex or empty liposome. Thickening was no longer observed by day 7 and by day 14 only pale areas were observed in animals treated with either pbi-shRNA STMN1 lipoplex or empty liposome. Pale areas were observed only in pbi-shRNA STMN1 lipoplex-treated animals on days 30, 60, and 90. All other gross changes observed during necropsy examination were considered incidental findings based on the isolated nature of the findings or the findings occurring across the dose groups indicating that the findings were not related to the treatment.

The only test article-related tissue changes were found at the injection site where most of the pbi-shRNA STMN1 lipoplex-treated animals and many of the empty liposome-treated animals had minimal to moderate subcutaneous abscesses consistent with sterile necrotic nodules characterized by a central focus of fibrin, degenerating neutrophils and basophilic material presumed to be test article often surrounded by a zone of necrotic connective tissue. Many of these animals also had a mild to moderate acute inflammation of the subcutaneous tissue surrounding the abscess. A minimal number of D5W-treated animals also showed subcutaneous inflammation that was never graded above minimal. Two of the empty liposome-treated females also had mild acute inflammation of the skeletal muscle beneath the affected subcutis. This pattern of change was noted throughout the study for both the pbi-shRNA STMN1 lipoplex-treated and empty liposome-treated animals until day 7 when the inflammation in the subcutaneous and adjacent tissues changed from acute to more chronic-active in nature. The abscesses resolved by day 14, except edema, and the chronic-active or sub-acute inflammation remained. Even though the lesions at injection sites had resolved by day 30 in a number of animals, several pbi-shRNA STMN1 lipoplex-treated and empty liposome-treated animals still had minimal chronic inflammation or chronic granulomas in the subcutis which correlated with gross observations of pale discoloration at the injection site. Other microscopic findings observed were considered incidental of the nature commonly observed in this strain and age of rats and/or were of similar incidence and severity in control and treated animals and therefore were considered unrelated to the administration of pbi-shRNA STMN1 lipoplex. There was no microscopic examination occurred on animals euthanized on days 60 and 90.

In summary, a single subcutaneous injection of pbi-shRNA STMN1 lipoplex was well tolerated by the rats. The most notable changes were related to the injection site at gross pathology, based on the presence of thickening of the tissue at the injection site and pale areas within the injection site. However, these changes seem to be related to the liposome rather than due to the injection of pbi-shRNA STMN1 lipoplex because similar changes were found in the empty liposome treated animals through day 30. Thickening or pale areas related to the injection site were present at days 60 and 90 in some of the pbi-shRNA STMN1 lipoplex treated animals. Histopathological evaluation through day 30 indicated injection site changes for the pbi-shRNA STMN1 lipoplex treated rats and empty liposome treated rats consisting of subcutaneous inflammation, abscesses, and seromas of progressive chronicity.

DISCUSSIONS

RNAi therapy exerts its effects by recognizing and cleaving sequence-specific mRNA targets. The pbi-shRNA STMN1 lipoplex was designed to target human STMN1. Therefore, a species with an identical or homologous mRNA sequence to human STMN1 is required by FDA to evaluate toxicology profile in the pre-clinical animal study. In RNAi design, the seed region of shRNA or siRNA is more critical because it mediates the recognition and binding of RNAi effectors to target mRNA. Base mismatches within the seed region may reduce or even abrogate RNAi activity (Jackson *et al.*, 2006) and are therefore less tolerable than those in the non-seed region. The mouse STMN1 transcript is discrepant from human STMN1 in the seed region and therefore not an ideal bio-relevant animal. The rat STMN1 transcript has a 2 base-pair mismatch with the human STMN1 in the non-seed region and bio-relevance was evaluated *in vitro*. We demonstrated that pbi-shRNA STMN1 can knock down rat STMN1 protein in multiple rat cell lines (data not shown). Therefore, the rat was chosen as the bio-relevant animal model of human STMN1 to examine the safety profile of pbi-shRNA STMN1 lipoplex in this study.

The biodistribution of pbi-shRNA STMN1 lipoplex was examined in this study. We found that the majority of plasmid was restricted to the injection site and that the presence of plasmid DNA declined with time. In addition, there was no gender difference in biodistribution profile and clearance pattern. By day 14, only 3 out of 10 rats displayed low-levels of plasmid at the injection site and the plasmid was not detected in any of the examined organs 30 days after a single subcutaneous injection. No evidence of host DNA integration was observed. Besides the injection site, the plasmid also distributed to lung, spleen, blood, skin, liver, ovary, and draining lymph nodes. The presence of plasmid in blood circulation is temporary at low levels and not found in blood 24 h after a subcutaneous administration. The detection of plasmid in ovaries was limited to a few animals and the level was only slightly above threshold of the assay. In addition, there are greater amount of plasmid detected from both lung and spleen than from other organs. For example, about 100–1000 copy number of plasmid per μg of genomic DNA was detected in the lung from day 1 (7 out 10 rats) to day 7 (7 out of 10 rats) before the number of positive lungs decreased to 2 (2 out of 10 rats) at day 14 and then the plasmid was completely cleared 30 days after the injection. A similar distribution pattern was also found in the spleen. We speculate that lung takes up most of the lipoplex that reaches into the circulation and hence the distribution to other major orangs is lesser and below detection limit of QPCR assay. In addition, the lipoplex that reaches into the regional lymph node underneath the injection site may travel to the spleen because lymphatic vessels may connect the regional lymph nodes to the spleen. Furthermore, the biodistribution of liposome-encapsulated plasmid DNA after a subcutaneous delivery displayed a different pattern than an intravenous delivery. In studies with an intravenous delivery of

liposome/DNA complexes, high levels of either plasmid DNA or transgene expression were detected from lung, heart, liver, and spleen (Liu *et al.*, 1997; Nabel *et al.*, 1992; Thierry *et al.*, 1995). However, after a subcutaneous delivery of either naked DNA or formulated DNA lipoplex, plasmid DNA was confined to the injections site with lower levels of plasmid DNA distributed to other organs as would be expected (Imboden *et al.*, 2003; Meuli *et al.*, 2001). The lipoplex distribution pattern found in our study supports the use of pbi-shRNA STMN1 DNA lipoplex as an intratumoral route of administration in the clinic to potentially explore further in limited patients with superficially accessible solid tumor. The intratumoral route circumvents the delivery challenges that the systemic administration has faced and provides acceptable delivery efficiency because of its local delivery nature, but effect with STMN1 target would not be applicable for systemic management. In addition, the toxicity on the major organs is minimal as a result of the limited distribution to the major organs and a fast clearance from the body. In addition, we had developed a systemic delivery platform of DNA lipoplex to extend the use of the bi-shRNA technology to patients with inaccessible tumor (Rao et al., 2016). Non-viral vectors have been reported to induce innate immune responses. In this study, we examined several cytokines, including IL-6, TNFα, and IL-1β, which have been used to evaluate innate immune responses induced by plasmid vectors (Li *et al.*, 1999; Sakurai *et al.*, 2007; Zhao *et al.*, 2004). We found that test product induction of IL-6, TNFα and IL-1β was transient and resolved 3 days after a single subcutaneous injection. We detected a statistically significant increase of IL-6 6 h after injection of pbi-shRNA lipoplex with return to baseline 72 h after administration. We also detected a trend of IL-1β upregulation 6 hours after the administration of the DNA lipoplex with return to baseline at 72 h. Similar results were reported in other studies after a systemic administration of cationic lipid vectors (Li *et al.*, 1999; Zhao *et al.*, 2004). We did not detect any statistical changes of TNFα until 4 days after the DNA lipoplex treatment. It is thought that these cytokine responses are triggered by the bacterial origin plasmid DNA and are not specifically related to the gene target (ie, in this case with STMN1). Bacterial DNA contains a higher frequency of unmethlyated CpG compared with mammalian DNA and the recognition of CpG motifs by TLR-9 induces innate immune responses (Zhao *et al.*, 2004). CpG-containing DNA exerts its immunostimulatory effects by binding TLR-9, activating nuclear transcription factors including NF-κB and AP-1 and thus promoting production of TNFα, IL-6, and activation of NK cells. Therefore, reducing CpG motif in plasmid DNA can greatly alleviate cytokine production (Sakurai *et al.*, 2007). Alternatively, methylation of plasmid DNA can also circumvent innate immune responses and extend transgene expression in animal models (Whitmore *et al.*, 1999). This innate immune responses can be minimized by pre-medication with anti-inflammatory suppressors including glucocorticoids, non-steroidal anti-inflammatory drugs and NF-κB inhibitors (Liu *et al.*, 2004). In addition, co-administration of vertebrate DNA, such as either calf thymus DNA or human placenta DNA, has been shown to limit immunological responses induced by bacterial DNA due to the fact that methylated mammalian DNA inhibits the activation of both NF-κB and AP-1 pathways (Chen *et al.*, 2001; Pisetsky and Reich, 2000).

The primary toxicological findings were observed at the sites of injection. No other target organs demonstrated significant gross or microscopic findings. The adverse effects observed at injection sites were most likely the result of inflammation due to the local administration. This observation also correlated with the biodistribution study, in which the majority of plasmid remained at injection sites and was cleared over time.

In conclusion, these results support an initial phase I proof of principle testing of pbi-shRNA STMN1 lipoplex in patients with advanced cancer.

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