Molecular Therapy
Nucleic Acids
Original Article

Blood pressure reduction through brain delivery of nanoparticles loaded with plasmid DNA encoding angiotensin receptor shRNA

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Elevated brain angiotensin II activity plays a key role in the development of neurogenic hypertension. While blood pressure (BP) control in neurogenic hypertension has been successfully demonstrated by regulating central angiotensin II activity, current techniques involving cerebrovascular injections of potential therapeutic agents are not suitable for clinical translation. To address this gap, we present the synthesis of dual-functionalized liposomes functionalized with targeting ligand and cell-penetrating peptide. Functionalized liposomes were synthesized using the thin film hydration technique and loaded with plasmid DNA encoding short hairpin RNA targeted toward angiotensin II receptors (PEAS), via the post-insertion method. The synthesized liposomes had a cationic surface charge, an average size of 150 nm, and effectively entrapped more than 89% of loaded PEAS. These liposomes loaded with PEAS demonstrated biocompatibility and efficient delivery to brain-derived cell lines, resulting in a remarkable reduction of more than 70% in receptor expression within 7 days. To assess the therapeutic potential, spontaneously hypertensive rats were administered intravenous injections of functionalized liposomes loaded with PEAS, and the changes in mean arterial pressure were monitored for 45 days. Remarkably, this treatment led to a significant ($p < 0.001$) decrease in BP of more than 30 mm Hg compared with saline-treated rats.

INTRODUCTION

Neurogenic hypertension is characterized by a significant increase in angiotensin II-mediated sympathetic nervous system activity within the brain.[1,2] Central to neurogenic hypertension progression is the significantly elevated expression in levels of angiotensin II type 1 receptors (AT1rs) within the brain.[3] Based on this, several studies have attempted to control blood pressure (BP) in neurogenic hypertension by limiting central angiotensin II activity using AT1r gene silencing nucleic acids. Since nucleic acids are generally unstable and rarely cross the blood-brain barrier, these treatments have been introduced into the brain via invasive means such as intracerebrovascular injections. Albeit successful, these invasive strategies employed limit clinical translation. Therefore, a gene delivery strategy that is less invasive and specific in delivering therapeutics to the brain could provide an effective treatment strategy for neurogenic hypertension.

Liposomes have great potential for performing this function since they can entrap nucleic acids, protect them *in vivo*, and deliver them to target regions, given the appropriate modifications.[4] Liposomes have a desirable nanosize advantage and the ability to interact with cellular membranes, leading to significant gene transfections.[5,6] Conversely, simple liposomes can be readily cleared from the body by the reticuloendothelial system, and their distribution is often nonspecific into highly vascularized tissues.[7] Nonetheless, liposomes can be readily functionalized to evade this clearance mechanism and promote their biased distribution into specific organs, including the brain.[8–11] Accordingly, we hypothesize that liposomes surface modified with transport proteins and cell-penetrating peptides can serve as a potent gene-delivery tool to the brain. Transferrin (Trans) is a serum glycoprotein that facilitates the transport of iron into brain via receptor-mediated transcytosis across the brain endothelial cells.[12] Penetratin (Pen) has been used for intracellular delivery of a variety of molecules and presents the advantage of the absence of saturable transport.[13] A non-toxic fragment of tetanus toxin, known as tetanus toxin C fragment (TTC) has previously been reported to enhance permeability through the blood-brain barrier.[14]

In this study, we develop dual surface-functionalized liposomes as a therapeutic tool to deliver plasmid DNA encoding angiotensin II receptor short hairpin RNA (shRNA) (PEAS) into the brain of spontaneously hypertensive (SHR) rats. Herein, we describe the synthesis and characterization of Trans, Pen, and TTC surface functionalized liposomal nanoparticles. Further, we investigated the ability of these nanoparticles to encapsulate and protect PEAS from nucleases, along with their *in vitro* biocompatibility and efficiency. Finally, we investigated the impact of liposomal gene delivery on the expression levels of AT1r in the SHR as well as the associated effect on BP.

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Table 1. Characterization of PEAS loaded liposomes for particle size, polydispersity index, and surface charge (zeta potential)

Liposomes	Particle size (nm)	Poly dispersity index	Zeta potential (mV)
Plain	152.4 ± 3.89	0.268 ± 0.02	26.9 ± 1.18
Trans	141.5 ± 3.03	0.273 ± 0.01	14.5 ± 3.40
Pen	149.3 ± 6.63	0.230 ± 0.04	23.4 ± 2.37
TTC	135.7 ± 2.99	0.280 ± 0.04	19.4 ± 1.85
Trans - Pen	150.8 ± 4.86	0.220 ± 0.01	19.9 ± 2.11
Trans - TTC	144.7 ± 3.75	0.220 ± 0.03	17.4 ± 2.75

Data are presented as mean ± SD (n = four independent preparations).

RESULTS

Preparation and characterization of surface functionalized liposomes

To improve uptake into brain, the surface of liposomes was modified with either Trans, TTC, Pen, or both Trans and either Pen or TTC. All active ligands were introduced into the formulation conjugated to DSPE-Peg_{2000}. A carbodiimide crosslinker reaction was used to introduce the active ligands, Pen, TTC, and Trans, to the liposomes as DSPE-Peg conjugates. The conjugation reaction is based on coupling the primary amine terminal on the peptide to the activated NHS ester group. The NHS group is released, and the peptide is attached to the DSPE-Peg. A micro bicinchoninic acid assay was employed to investigate conjugation efficiencies for conjugation reactions. The conjugation efficiencies of all reactions were found to be 84%, 87%, and 88% for Pen, TTC, and Trans, respectively. Dialysis of the resultant product aided in removing all unconjugated products and exchanging the organic reaction conditions with water. In all, six different liposomal nanoparticles were formulated with various surface modifications, as presented in Table 1.

The synthesized conjugates were incorporated into the liposome formulations and physically characterized using dynamic light scattering techniques. The average hydrodynamic diameters of liposomes were found to be approximately 150 nm, as seen from Table 1. Despite the conjugation of different active ligands on liposome surface, the particle size was not significantly impacted. Nanoparticle polydispersity was also found to be less than 0.3 in all cases, and the uniformity of size is also supported by the size distribution by intensity plot (Figure S1). All nanoparticles exhibited a positive surface charge ranging from 14 mV to 26.9 mV, as seen in Table 1.

PEAS polyplex formation, encapsulation, and protection from DNase

PEAS was introduced into the formulations polyplexed with chitosan. Before insertion of PEAS into liposomes, the optimum chitosan-PEAS nitrogen group to phosphate group (N/P) ratio was investigated using gel electrophoresis, as shown in Figure 1A. Forming an initial polyplex with chitosan was to improve the stability and retention of PEAS within the liposome. Therefore, it was crucial to determine the amount of chitosan that is just sufficient to allow binding and release of PEAS under the right conditions.

The binding of chitosan to pDNA produces a polyplex that prevents the migration of the plasmid under electrophoresis. A weak polyplex allows the migration of the plasmid as observed with the migration at an N/P ratio 0.5/1. At an N/P ratio of 10/1, PEAS was well retained on the gel. Following the outcome of the N/P ratio determination, chitosan-PEAS at an N/P ratio of 10:1 was prepared and incorporated into all the different formulations.

The encapsulation efficiencies were investigated using Hoechst dye. Hoechst dye binds to adenine-thymine-rich regions of DNA in the minor groove, resulting in the emission of a measurable fluorescent signal.[15] The intensity of the signal depends on how much binding occurs. From the observed fluorescence, the encapsulation efficiencies were determined. Encapsulation efficiencies of more than 89% were observed for all formulations, regardless of conjugation (Figure 1B). No significant differences were observed in the encapsulation efficiencies of different formulations regardless of conjugation.

Free DNA is liable to degradation by DNase. Due to this, a critical parameter of a gene delivery system is its ability to protect the gene from enzyme degradation. The ability of liposomal formulations to protect PEAS from enzymatic degradation was investigated using gel electrophoresis with naked PEAS as a control (Figure 1C). Naked PEAS and liposome-encapsulated PEAS were incubated with DNase. After 1 hour, the reaction was stopped, and the PEAS was liberated. The liberated PEAS was mixed with Safegreen dye and run on a gel. Safegreen dye forms a fluorescent molecule upon intercalation with double-stranded DNA, making it an excellent indicator for observing the DNA integrity visually. As seen in Figure 1C, all formulations (present in lanes C–H) provided sufficient protection to the encapsulated PEAS. Conversely, naked PEAS was hydrolyzed, resulting in no fluorescent bands in lane B.

In vitro cytocompatibility evaluation of PEAS-loaded liposomes

A crucial parameter for every delivery system is biological safety. To this end, we investigated the cytocompatibility of our formulations using the 3-[4,5-dimethylthiazol-2-yl]-2,5 diphenyl tetrazolium bromide (MTT) assay. The *in vitro* biocompatibility of liposomes was evaluated by testing different phospholipid concentrations in primary rat neurons, primary rat astrocytes, and mouse brain endothelial (bEnd.3) cells. As depicted in Figures 2A–2C, cytotoxicity was concentration dependent in all cell lines. In all cell lines, cell survival was lowest at 400 nM.

Effect of PEAS-encapsulated liposomal nanoparticles on *in vitro* AT1r gene expression

The day required to cause significant gene knockdown was investigated in rat primary neurons using PEAS-loaded plain liposomes. After introducing liposomes to rat primary neuron cultures, cells were

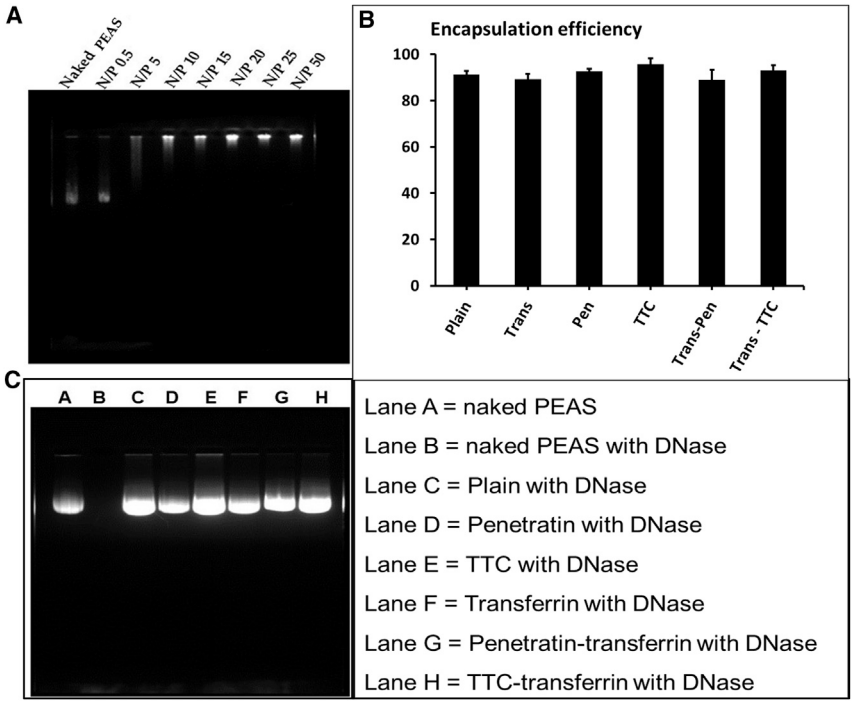

Figure 1. Polyplex formation, encapsulation, and protection from DNase

(A) Agarose gel electrophoresis of the chitosan-PEAS complex at different N/P ratios to determine the binding affinity of PEAS to chitosan. Naked PEAS was used as a positive control. (B) Encapsulation efficiency of different liposomal nanoparticles. (C) DNase protective effect of chitosan-PEAS (N/P 1: 10) loaded liposomal formulation. All data is represented as mean ± SD for n = 4 independent samples.

lysed, and AT1r gene expressions were analyzed. We performed qRT-PCR analysis of the AT1r gene to determine the mRNA expression at the cellular level. Commercially available primers were used to determine AT1r gene expression levels. Prior to use the primer, amplification efficiency was investigated and found to have a mean of 2.017 (Figure S2). GAPDH was used as an in-house gene for the qRT-PCR analysis. AT1r gene expression was decrease approximately 50% by day 3, a decrease that was seen to be significant ($p < 0.05$) when compared with the untreated group. Gene knockdown continued to day 9 and was not significantly different beyond this point (Figures 3A and 3B).

After the success in transfecting and knocking down gene expression in rat primary neurons using unfunctionalized liposomes. The ability of the functionalized formulations to deliver PEAS and reduce the AT1r gene expression was investigated in three cell lines: mouse brain endothelial cells, rat primary astrocytes, and rat primary neurons. Scrambled gene as a control was loaded into *trans*-TTC conjugated liposomes. Although all formulations successfully led to plasmid transfection, the gene knockdown was observed to be greater with dual functionalized liposomes compared with single functionalized liposomes. PEAS delivery with dual functionalized liposomes led to a more than 80% decrease in AT1r expression in all cell lines used, as seen from Figures 3C–3E.

In vitro blood compatibility

Investigation of the interaction of liposomal formulations with erythrocytes is important to help predict and prevent possible untoward effects upon systemic administration. Hemoglobin release because of erythrocyte lysis was dependent on concentration, as can be seen in Figure 2D. At lower phospholipid concentrations, only very minimal hemolysis was detected; however, the hemolysis increased as the concentration increased toward 500 nM.

PEAS delivery to SHR led to a decrease in AT1r gene expression

The goal of our study was to investigate the ability of PEAS-loaded liposomes to penetrate the blood-brain barrier and subsequently affect AT1r gene expression. To investigate this, we administered the different formulations to SHR rats. SHR rats develop are genetic hypertensive models whose elevated BP is mediated by the sympathetic nervous system.[16] After the injection of the PEAS-loaded liposomes, a decrease in gene expression was observed by the third day of administration, leading unto day 7 where a more than 50% decrease in gene expression was observed. In general, mRNA expression decreased with time upon the introduction of PEAS, leading to more than 70% gene expression.

Since the liposomes were injected into the blood stream, it was crucial to investigate the effect of its distribution in highly perfused organs. To study this, highly perfused organs were harvested, and the gene expression was analyzed. A decrease in gene expression was also observed in other highly perfused organs (Figure 4B). Within the liver and kidneys, no significant differences ($p < 0.05$) in gene expression were observed, although there was a decline in gene expression by day 35. In the lungs and heart, gene expression was found to have decreased significantly when compared with the untreated group with only the trans-pen formulation showing a significant reduction when compared with the effect of naked gene. Similar patterns of protein expression were observed from the ELISA used to quantify AT1r protein obtained from the tissues (Figure S3).

Dual functionalized PEAS-loaded liposomes cause a better decrease in BP

An elevated BP is the hallmark feature of hypertension; thus, to investigate whether our treatments posed any benefits, it was crucial to follow the changes in BP after the administration of the treatments. BP changes in SHR were monitored over 45 days using a CODA BP monitor, which provides a noninvasive strategy to monitor BP.

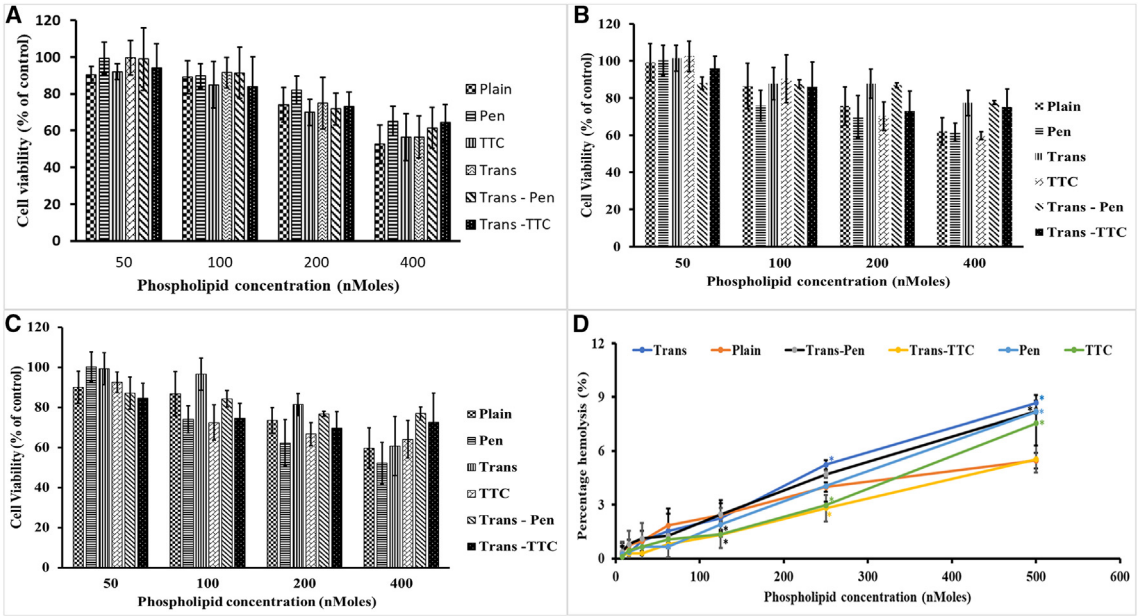

Figure 2. *In vitro* cytocompatibility and hemocompatibility of liposomal nanoparticles

Cellular viability of (A) b.End3 cells, (B) primary neurons, and (C) primary astrocytes after exposure to PEAS-loaded liposomes at different phospholipid concentrations (50, 100, 200, and 400 nM). MTT assay performed after 48 h of cell incubation and viability of untreated cells was considered as a control (100% cell survival). Cellular viability data is expressed as mean ± standard deviation for n = 4 independent samples. (D) Percentage hemolytic activity of PEAS-loaded liposomal formulations. The hemolysis observed with PBS and 1% v/v Triton X-100 was considered as no hemolysis, and 100% hemolysis, respectively. Data are expressed as mean ± SD (n = 4). Statistically significant ($p < 0.05$) differences with PEAS-loaded plain liposomes are shown as (*).

After treatment, BP was generally decreased in groups that received PEAS treatment, whether in PBS or encapsulated in liposomal formulations. Dual functionalized liposomes (Trans-TTC and trans-pen) loaded with PEAS caused the most reduction in BP, which was maintained until day 45 (Figure 5). No significant changes ($p < 0.05$) were observed in heart rate between all treatment groups (Figure S4). Trans-TTC and trans-Pen loaded with scrambled gene was used as formulation controls to investigate the influence of the nanoparticles on the observed outcomes. BP of rats that received scrambled gene loaded within nanoparticles were similar to the saline-treated groups.

DISCUSSION

Over the past few decades, surface-functionalized lipid nanoparticles have become an attractive platform for the targeted delivery of genetic materials. In this study, we synthesized cationic dual-functionalized liposomes loaded with PEAS. In the initial conjugation reactions, more than 80% of peptides were successfully conjugated to the DSPE-Peg in all cases. After the coupling of the peptides to the DSPE-Peg, the purified conjugates were introduced into the liposomal formulations. The thin film hydration technique was used to synthesize liposomes. Compared with other small-scale liposome production techniques, this technique was suitable for preserving the integrity of the conjugated active ligands. All liposomes were successfully formed upon hydration with HEPES buffer (pH 7.4). PEAS was electrostatically polyplexed to a 30-kDa chitosan and incorporated into the liposomal formulation.

Liposome particle size and size distribution are crucial to performance.[17,18] In a previous study, larger particles (>200 nm) were found to be rapidly mopped up by the spleen, and very small (<70 nm) particles were taken up into the liver.[19] As evident from Table 1, the hydrodynamic diameter of the synthesized liposomes was an average of 150 nm. This size is desirable, since it suggests that the synthesized liposomes would have a relatively longer circulation time, thus, large enough to avoid rapid clearance and small enough to not penetrate tissues. No significant differences in liposomal size were observed, regardless of conjugation. As shown in Table 1 and Figure S1, the size distribution was symmetrical about the individual averages, indicating that liposomal formulations were uniformly distributed in size. Homogeneity in particle size distribution was further confirmed by the observed polydispersity index values, which were less than 0.3.[20]

The surface charge of nanoparticles is a critical parameter, that can determine the stability of formulations. Close to neutral surface charged nanoparticles are known to make less stable formulations due to the high tendency to aggregate and phase separate.[21] All formulations presented with a positive surface charge, which can be directly attributed to the presence of the cationic phospholipid dioleoyl-3-trimethylammonium-propane chloride (DOTAP). In addition to DOTAP, incorporating dioleoyl-sn-glycerol-3-phosphoethanolamine (DOPE) as a helper lipid promotes fusion with cells and endosomal membranes, while decreasing the potential toxicity of the cationic lipid. Polyethylene glycol incorporation into liposomes

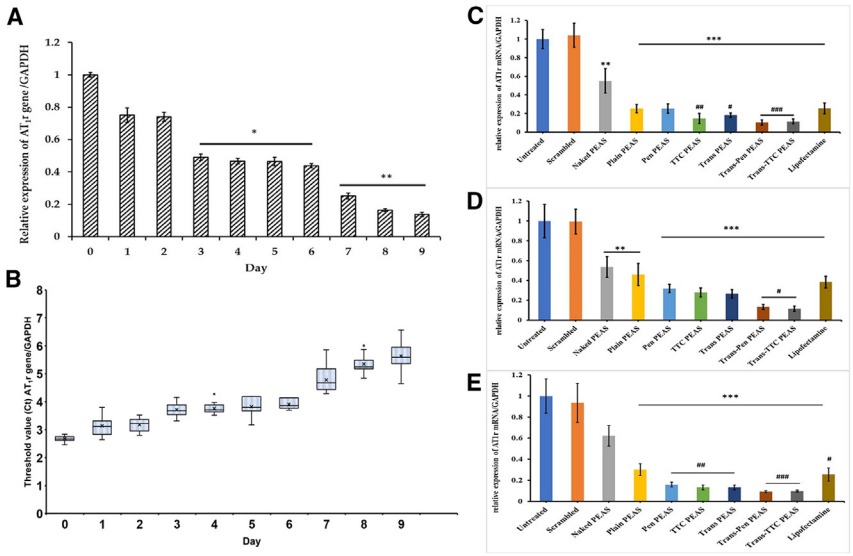

Figure 3. *In vitro* transfection efficiencies of liposomal nanoparticles

(A) AT1r gene expression in primary neurons at different days after transfection of Plain liposomes loaded with PEAS. (B) Threshold Cycling time data showing changes in cycling time for fluorescence detection. The data are expressed as mean relative expression ofAT1r gene/GAPDH ± SEM (n = 8 independent samples). *p < 0.05, ** p < 0.01 decrease in gene expression from day 0. AT1r gene expression in (C) primary neurons, (D) primary astrocytes, and (E) b.End3 cells at day 7 after transfection of different PEAS-loaded liposomes. Trans-TTC liposomes were used to deliver scrambled gene as a control. The data are expressed as mean relative expression of AT1r gene/GAPDH ± SEM with n = 4 independent samples. Statistically significant (p < 0.05, p < 0.01, p < 0.001) differences with naked PEAS and untreated groups are represented with (#, ##, ###) and (*, **, ***), respectively.

was to improve colloidal stability and decrease reticuloendothelial clearance. Adding cholesterol to the nanoparticle helped to improve bilayer fluidity and stability.

To investigate the optimal concentration of chitosan required to form reasonable polyplexes with PEAS, a nitrogen/phosphorus binding assay was performed. It is essential that PEAS remains bound until the formulation was within the cells. As can be seen from Figure 1A, beyond the N/P ratio of 10:1, optimum binding was observed. A good complex should also permit the release of PEAS when the formulation is within the cells, and thus a stronger bind will not be suitable since it will retard the release of PEAS. To this effect, the lowest concentration that showed reasonable binding was considered optimum for our studies. In this case, it was an N/P ratio of 10:1. With this, chitosan-PEAS polyplexes were prepared and incorporated into the liposome formulations at 1 μg pDNA/100 nM phospholipid. High encapsulation efficiencies (>89%) of PEAS were observed for all liposomal formulations, as seen in Figure 1B. The cationic nature of the liposomes promoted sufficient binding of PEAS into itself.

Nucleases and lysosomal enzymes in the plasma rapidly hydrolyze free plasmid DNA. Thus, liposomal nanoparticles should be able to protect and deliver PEAS wholly to the target site. To confirm this, the DNase protection assay was used to investigate the ability of liposomes to protect encapsulated PEAS and the outcome is depicted in Figure 1C. Naked PEAS was completely hydrolyzed upon exposure to DNase, as evident from the lack of a fluorescence band under lane B from Figure 1G. Conversely, all formulations could protect PEAS from degradation by DNase, as evident from the fluorescent bands observed in lanes C–H. Naked PEAS without DNase was kept in lane A as a control group. With the absence of DNase, naked PEAS was untampered.

Cationic nanoparticles are known to be particularly toxic to cells, especially at higher concentrations.[22] The liposomal formulations were assessed for their biocompatibility in mouse bEnd.3 cells, rat primary astrocytes, and rat primary neurons. The effect of increasing phospholipid concentrations on cellular viability was investigated using an MTT assay. Cellular viability generally decreased as the phospholipid concentration was increased, as seen in Figures 2A–2C. The observed decrease in cellular viability can be attributed to the positive surface charges on the nanoparticles. Cationic nanoparticles are known to disrupt cellular membranes and cause the death of cells, a situation that is heightened at higher concentrations.[23] As seen from the cytocompatibility investigation, at lower concentrations, cellular viability was relatively higher. Treatment with 100 nM phospholipid resulted in an average cellular survival of approximately 80%. Therefore, a phospholipid concentration of 100 nM was used in the subsequent cellular transfection studies.

To effectively control BP in neurogenic hypertension, decreasing the impact of the overactivated angiotensin II pathway within the brain is essential. One way to achieve this is to decrease the expression of angiotensin II receptors in the brain. To this effect, liposomes were designed to facilitate the wholesome and efficient transport of PEAS to brain cells. Prior to the *in vivo* investigation of this potential, the ability of liposomal encapsulated PEAS to decrease gene expression was investigated in rat primary astrocytes, rat primary neurons, and mouse bEnd.3 cells.

shRNA decrease protein production by knocking down gene expression.[24] Gene effects produced with shRNAs are longer lasting due to their unique ability to get integrated into the host DNA.[25] Thus, the successful delivery of PEAS should result in the knockdown of AT1r gene expression. An initial study was performed with plain liposomes encapsulated PEAS in rat primary neuron culture to investigate how long it took to achieve significant gene knockdown. Phospholipid concentrations were

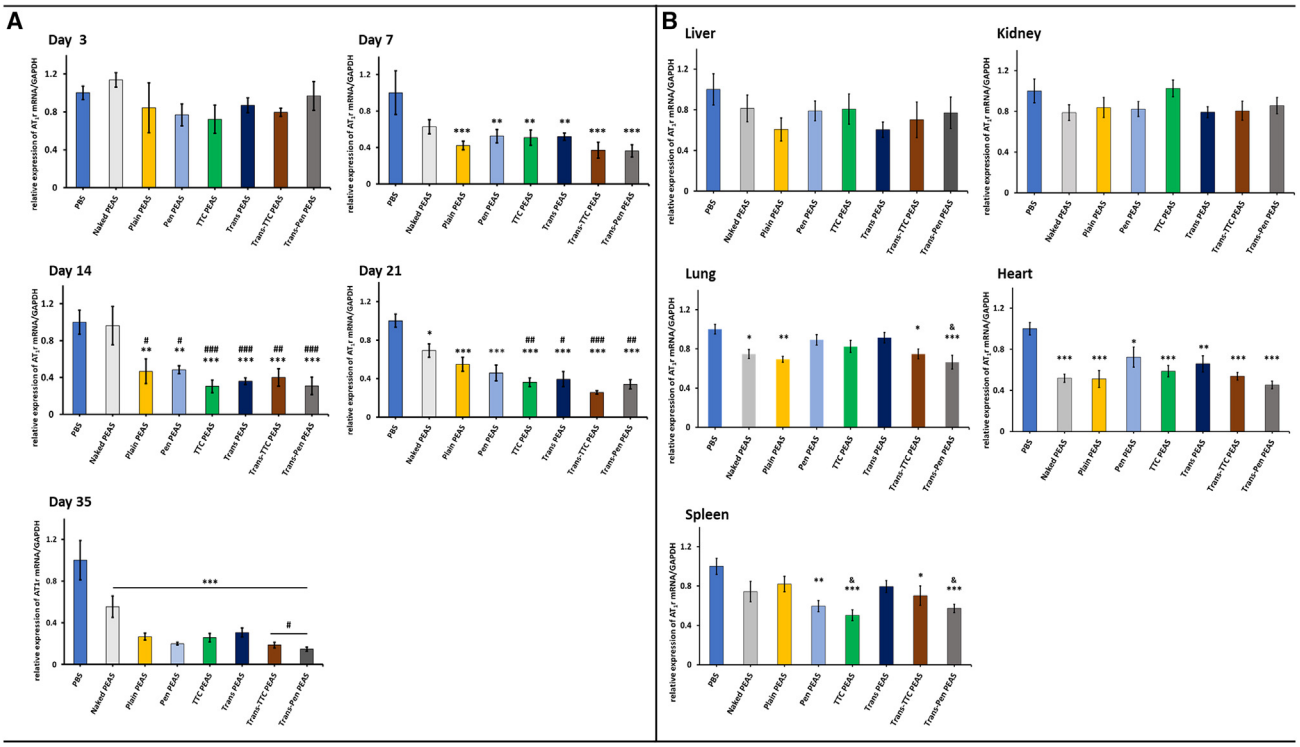

Figure 4. AT1R mRNA expression following administration of PEAS with Liposomal nanoparticles

(A) AT1r mRNA levels in the SHR brain at different time points following PEAS administration with different formulations. Data expressed as mean ± SEM (n = 6 biologically independent samples). Statistically significant differences ($p < 0.05$, $p < 0.01$, $p < 0.001$) differences with PBS-treated and naked PEAS treated are shown as (*, **, ***), and (#, ##, ###), respectively. (B) AT1r mRNA levels in the SHR brain at different time points following PEAS administration with different formulations. Data expressed as mean ± SEM (n = 6 biologically independent samples). Statistically significant differences ($p < 0.05$, $p < 0.01$, $p < 0.001$) differences with PBS-treated and plain PEAS treated are shown as (*, **, ***), and (&, &&, &&&), respectively. See also Figure S3.

maintained at the previously optimized 100 nM in this study, and the AT1r gene expression was evaluated on different days. As seen in Figure 3A, successful transfection with PEAS resulted in a significant decrease ($p < 0.05$) in AT1r gene expression by the day 3. The decrease in gene expression was observed to have peaked by 9 days, beyond which gene expression was not significantly different. AT1r gene expression by day 7 was significantly decrease ($p < 0.01$) compared with approximately one-third of the gene expression observed in the untreated cells used as control (day 0). Consequently, different formulations were tested, and gene expression was evaluated on day 7.

After this outcome, the ability of different formulations to transfect different cells and subsequently impact AT1r expression was further investigated. In all PEAS-treated groups, AT1r gene expression was reduced, however, differences in the extent of gene knockdown due to treatment type were observed (Figures 3C–3E). Generally, PEAS delivery with dual functionalized liposomes resulted in the greatest decrease in gene expression regardless of cell lines. This observed effect can be attributed to the improved uptake of the formulation with dual functionalization when compared with the other formulations. Previously, dual functionalization of liposomes has been identified to lead to far better formulation uptake.[8,26] In this study, liposomes were functionalized with Trans with TTC/Pen; these active ligands are known to improve nanoparticle uptake. Trans binds to the C terminus of the Trans receptor, a receptor that is abundantly expressed in blood-brain barrier phenotype cells. Pen is a commonly used cell-penetrating peptide with selective accumulation in the brain.[27] TTC peptide is a non-toxic fragment of the native tetanus toxin that lacks the toxic effects yet maintains the ability to bind membranes, get internalized, and enhance retrograde transport. TTC is known to facilitate retrograde axonal transport to the CNS by binding to pre-synaptic motor neuron terminals.[28] TTC conjugation is known to improve neuronal targeting and internalization; in addition, it is known to provide protective benefits to neurons.[29–32]

Cationic surface charged nanoparticles can potentially cause lysis of erythrocytes resulting in the release of hemoglobin and consequently loss of blood cells. Also, since PEAS-loaded nanoparticles will be exposed to erythrocytes upon administration, it is essential to determine their biocompatibility for *in vivo* administration. From our study, all formulations exhibited a concentration dependent toxicity, as seen in Figure 2D. Hemolysis activity remained

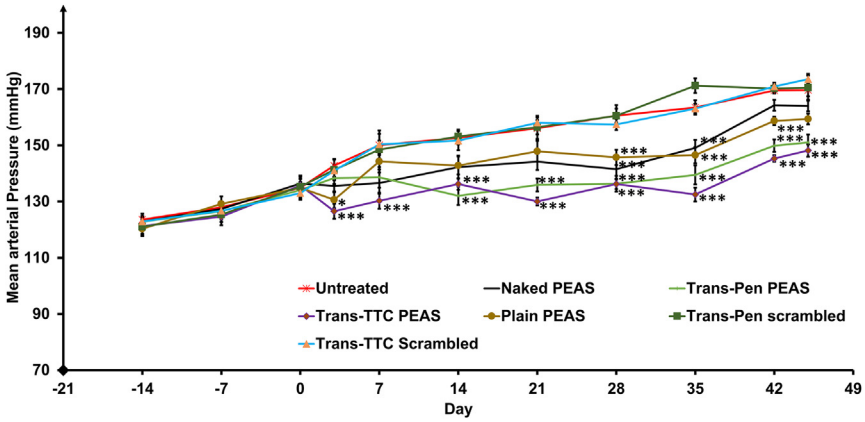

Figure 5. Mean arterial pressure is decreased in SHR
Data are expressed as mean ± SEM (n = 6). Significant differences *p < 0.05 and ***p < 0.001 in BP compared with the untreated group, respectively. See also Figure S4.

below 3%, even at 125 nM phospholipid. The increase in hemolytic activity due to the concentration increase can be attributed to the increase in surface interaction between the nanoparticles and the cell membrane of the erythrocytes.

Angiotensin II activity within the brain is crucial to the elevated BP observed in neurogenic hypertension.[2] Thus, to achieve proper control of BP in neurogenic hypertension, the proposed treatment must reach the brain at therapeutic levels. In this study, we investigated the efficacy of our formulations in knocking down the brain AT1r. Although the effect of knocking down central AT1r expression has been previously studied, most studies were carried out with intracerebrally injected treatments. Here we demonstrate the systemic delivery of liposomes that selectively target and deliver treatment to the brain, resulting in the significant knockdown of the AT1r gene and subsequent decrease in protein levels. As seen in Figure 4A, a decrease in AT1r was apparent by 7 days and reached peak values by 35 days for all PEAS-treated groups. Compared with other treatment kinds, treatment with dual-functionalized liposomes showed the lowest reduction in gene expression. Compared with the plain PEAS-loaded formulation, dual functionalized PEAS-loaded liposomes showed a significant gene knockdown beginning from day 14. This effect can be the reason for the differences in BP decrease observed using both treatments in Figure 5.

In SHRs, an increase in central angiotensin II is strongly associated with an elevation in BP as the rat ages.[3] This characteristic feature made the SHR a suitable candidate for observing the effects of AT1r knockdown on BP. Changes in AT1r expression levels were found to correlate with BP measurements. While control (saline/scrambled-treated) groups had BP values that showed a steady increase over time, the PEAS-treated groups showed significant retardation in BP increase over time. BP in the untreated and control groups reached as high as approximately 170 mm Hg, while dual functionalized liposome-treated groups measured approximately 140 mm Hg. Among the two different dual-functionalized liposomal nanoparticles, Trans-TTC seemed to have caused the earliest decrease in mean arterial BP. Also, it was crucial to neglect the impact of the nanoparticle formulation on the changes observed in BP. To this effect, plasmid DNA with the AT1r shRNA replaced with a scrambled RNA was used in place of PEAS. As can be seen from Figure 5, rats that received this treatment had similar BP recordings as the PBS-treated groups. In contrast, the knockdown of AT1r did not significantly impact the mean heart rate, as seen in Figure S4. Although we did observe quantitative evidence of our formulation reducing brain AT1r expression, we do not have visual proof at this point.

Throughout the studies, no major behavioral changes were observed in all rats that received the treatments. Also, tissue sections that were stained with hematoxylin and eosin did not show any evidence of necrosis or tissue damage. Heart sections were critically assessed for any signs of diffuse fibrosis or myofibrillar loss. Brain, lung, kidney, and liver sections also showed no signs of toxicity, as can be seen in Figure S5.

In summary, this study presents the synthesis and characterization of cationic dual functionalized liposomes loaded with PEAS for brain targeted gene delivery. Systemic administration resulted in the substantial reduction of AT1r gene expression in the brain of SHR rats, which was correlated with BP reduction. Overall, nanoparticle formulations exhibited promising therapeutic potential with no apparent toxicity in the rats.

MATERIALS AND METHODS

DOTAP, lissamine rhodamine, and DOPE were bought from Avanti Polar Lipids. DSPE-PEG2000-NHS was purchased from Biochempeg Scientific Inc. The bicinchoninic acid assay protein assay kit and penicillin-streptomycin (p-s) solution were bought from Thermo Fisher Scientific. Chitosan with a molar mass of 30 kDa was obtained from Glentham Life Sciences. Non-toxic TTC, Holo-transferrin (Trans), cholesterol, Hoechst 33342, EDTA, 4-(2-hydroxyethyl)-1- piperazineethanesulfonic acid (HEPES), and Triton X-100 were procured from Sigma-Aldrich. PBS, fetal bovine serum, and DMEM were acquired from Corning Incorporated. Pen peptide (RQIKIWFQNRRMKW KKGG) (Pen) was purchased from Zhejiang Ontores Biotechnologies Co., Ltd. PEAS (Figure S6) was designed and custom-made at Life Scientists' service center and amplified at Aldevron.

Preparation of Pen, TTC, and Trans conjugated DSPE-Peg$_{2000}$

As previously described, functionalized liposomes were prepared by initially conjugating Pen, TTC, and Trans to activated NHS-terminated DSPE-EG$_{2000}$.[9,33] For Pen and TTC, either was dissolved with

DSPE-Peg_{2000}-NHS in anhydrous dimethylformamide at a ratio of 1:5. Triethylamine was used to make the reaction mixture alkaline to achieve a pH of 8–8.5. The reaction was continually stirred at room temperature for 120 h. The uncoupled starting material was removed by dialysis (molecular weight cut off 3.5 kDa) against deionized water for 48 h. The final product was lyophilized and used once or stored at −20°C until use.

Similarly, Trans was dissolved in anhydrous dimethylformamide and conjugated with DSPE-Peg_{2000}-NHS at a ratio of 1:3. Triethylamine was added to the mixture to aid rapid deprotonation and stirred at room temperature for 24 h. The product was purified by dialysis (molecular weight cut off 3.5 kDa) for 48 h against deionized water. The final product was lyophilized and used once or stored at −20°C until use. The micro bicinchoninic acid protein assay was used to determine the coupling efficiency of all conjugation products.[34]

Synthesis of functionalized liposomes

As previously reported,[8,33] Pen- or TTC-conjugated liposomes were prepared using the thin film hydration technique. Briefly, DOPE/DOTAP/cholesterol (45:45:2 mol %) and DSPE-Peg_{2000}-Pen or DSPE-Peg_{2000}-TTC were dissolved in chloroform: methanol (2:1, v/v) solvent mixture. The organic solvent was evaporated with a rotavapor (Buchi Rotavapor RII) to produce a dry lipid film. HEPES buffer pH 7.4 was added to the dry lipid and stirred overnight to form TTC or Pen liposomes. To create Trans-liposomes, DSPE-Peg_{2000}-trans was added to the hydration stage of the liposome formation. Trans-pen or Trans-TTC liposomes were formed by adding DSPE-Peg_{2000}-trans to the hydration stage of DSPE-PEG_{2000}-Pen or DSPE-Peg_{2000}-TTC liposomes, respectively. Unentrapped DSPEPEG micelles were removed by purification through a G-100 Sephadex column lissamine rhodamine coupled to DOPE 0.5 mol % was added to the formulation as a liposome membrane marker to aid visualization.

Characterization of liposomes

The synthesized liposomes were characterized for hydrodynamic size, polydispersity index, and zeta potential by the dynamic light scattering method. Liposomes were diluted with PBS and held in a cuvette placed in a laser path to determine particle size, polydispersity, and zeta potential. Size, zeta potential, and polydispersity index were measured on a Malvern Instrument Zetasizer Nano ZR (Malvern Instruments) equipped with a 5-mW He-Ne laser of wavelength 633 nm at 25°C.[5] Data were collected at an angle of 90°.

Chitosan pDNA binding ability

As previously described, the optimal chitosan-plasmid DNA N/P ratio was investigated by agarose gel electrophoresis.[5] The complexes containing 1 μg PEAS at different chitosan weight ratios were stained with safegreen dye for 5 min. The complex was loaded on 0.8% w/v agarose gel and electrophoresed at 80 V in 0.5× Tris-acetate-EDTA (TAE) buffer for 80 min. PEAS migration was recorded as per published reports, with naked PEAS used as a positive control.[5]

Encapsulation efficiency of liposomes

The amount of PEAS encapsulated into each liposome was evaluated using DNA intercalating dye Hoechst 33,342 (0.15 μg/mL). Briefly, 100 μL of the liposomal formulation was taken and diluted with 1:3 HEPES buffered saline (A). Triton X- was added to a second 100-μL sample and also diluted to make up the same volume as (A) with HEPES buffered saline (T). The fluorescence intensities (354/458 nm emission/excitation wavelengths, respectively) of (A) and (T) were measured using a spectrophotometer (SpectraMax M5, Molecular Devices). Percent encapsulation was calculated considering the absorbance in the presence of Triton X-100 at 0.5% v/v as 100%. Encapsulation efficiency (%) = $[((T) - (A))/(T)] \times 100$.

DNase protection assay

DNase I protection assay was employed to investigate the stability of liposomal encapsulated PEAS against nuclease degradation. Liposomes loaded with 1 μg PEAS were incubated with 1 unit of DNase I in a DNase reaction buffer at 37°C with shaking at 50 rpm for 60 min. Naked PEAS incubated with DNase served as a positive control. After incubation, 5 μL of an aqueous EDTA (100 mM) solution was added to stop the DNase reaction. Subsequently, 20 μL of heparin solution (5 mg/mL) was added to each sample and incubated at 37°C for 2 h to release PEAS from the complex. To confirm the integrity, released PEAS was subjected to electrophoresis on 0.8% (w/v) agarose gel.

Cell culture and animal use and care

All Sprague Dawley, Wistar Kyoto (WKY), and SHR rats were purchased from Charles River Laboratories. Animal experiments, procedures, and handling were conducted per a protocol approved by the animal care and use committee of the North Dakota State University. Male and female rats were used for all experiments, maintained under standard housing conditions with free access to food and water, and exposed to a 12 h light-dark cycle.

Mouse brain endothelial (bEnd.3) cells, rat primary astrocytes, and rat primary neuron cell lines were used to perform *in vitro* assays. The isolation and preparation of primary neurons and astrocytes from the hypothalamus and brainstem of 1-day-old rat pups were performed as previously described.[8] Primary astrocytes were cultured in DMEM with 10% fetal bovine serum and 1% v/v p-s solution. Primary neuronal cells were cultured in neurobasal media with 10% v/v plasma-derived horse serum, B-27 supplements, L-glutamate (25 mM), and 1% v/v p-s solution. Cytosine arabinoside (10 μM) was added on day 3 to remove non-neuronal cells. Anti-MAP2 antibody immunostaining was used to confirm the purity of the culture. Cells were incubated in an atmosphere of 5% CO2 at 37°C bEnd.3 cells were purchased from the American Type Culture Collection and cultured in DMEM with 10% fetal bovine serum and 1% v/v p-s solution.

Cytocompatibility assessment of liposomal nanoparticles

Primary astrocytes, primary neurons, and bEnd.3 cell lines were used to study the cytotoxic potential of the liposomes. *In vitro* cellular viability was evaluated after treatment with 50, 100, 200, and

Table 2. qRT-PCR primer information

Gene	GeneBank accession	Sequence
AT_1r forward	NM_030985.4	TCTCAATCTCGCCTTGGCTG
AT_1r reverse	NM_030985.4	ACACACTGGCGTAGAGGTTG
GAPDH forward	NM_017008.4	CAGCCGCATCTTCTTGTGC
GAPDH reverse	NM_017008.4	GGTAACCAGGCGTCCGATA

400 nM liposomal formulations. Briefly, 1,000 cells/well density were seeded into 96-well plates and supplemented with 200 μL suitable media. After 24 h, the media was replaced with serum-free media, and different phospholipid concentrations were introduced into the cultured cells. Cells were incubated for 4 h and then replenished with fresh serum-containing media. The cells were incubated, and the cellular viability was determined after 48 h using the MTT assay considering the untreated cells as the control group under the same conditions. All cell incubation was at 37°C under a 5% CO_2 atmosphere.

Effect of PEAS-encapsulated liposomal nanoparticles on *in vitro* AT1r gene expression

PEAS-encapsulated liposomes were evaluated for their transfection efficiency in bEnd.3, primary astrocytes and primary neuronal cells. Initially, the time taken to achieve a desirable *in vitro* effect was investigated in primary neuronal culture using plain/unfunctionalized liposomes loaded with PEAS. Briefly, a primary rat hypothalamic neuronal culture was prepared from SHR pups. At 60%–70% confluency, PEAS-loaded plain liposomes were introduced to the cultured cells. Cells were harvested daily at the same time for AT1r mRNA analysis. The total RNA extraction, cDNA synthesis, and quantitative real-time PCR were performed, as described below.

After the determination of the day to achieve significant gene knockdown using PEAS. Approximately 1×10^5 cells/well were seeded in 12-well plates 24 h before transfection. Cells were treated with naked PEAS, Lipofectamine 3000, and liposomal formulations (100 nM) containing either scrambled gene or PEAS complexes, in a serum-free medium, for 4 h.[8,35] After that, cells were rinsed with PBS and incubated further for 48 h for GFP and 7 days for AT1r.

For PEAS transfection, angiotensin II receptor expression was quantified using quantitative RT-PCR (described below) and an Angiotensin-II type 1 receptor ELISA kit (MyBiosource).

Measurement of the AT1r mRNA levels by RT-PCR

Cells were homogenized in 1 mL of ice-cold RNA lysis reagent, and total RNA was isolated with affinity columns (Quick-RNA Microprep Kit) according to the instructions of the manufacturer. The quantity and quality of isolated total RNA were estimated using Varioskan Lux multimode microplate reader (Thermo Fischer Scientific). To synthesize single-stranded cDNA from total RNA, Reverse Transcription Kit (Applied Biosystems) with 300 ng total RNA per 20 μL reaction. Reverse transcription was performed on a ProFlex PCR system (Thermo Fischer Scientific) by warming at 55°C. The reaction was stopped by raising the temperature to 85°C, and the synthesized cDNA was cooled at 4°C.

The relative expressions of AT1r were quantified by using the delta-delta Ct method, following a RT-PCR. Commercially available primers were used for this study, as described in Table 1. All primers were designed to span an exon-exon boundary and melt curves 60–95 were monitored to verify that a single transcript was always amplified. Each 20-μL final PCR solution contained cDNA that corresponded with 300 ng total RNA with gene-specific primers at a final concentration of 200 nM. Amplification of cDNA was performed in a 96-well plate with an SYBR green master mix; Blastaq 2X qPCR Mastermix kit (Applied Biological Materials Inc). All cDNA samples and in-house gene amplifications were carried out in triplicate and duplicate, respectively, by using the standard shuttle PCR protocol (120-s enzyme activation for 1 cycle followed by 40 cycles of 95°C for 15 s [denaturation] and 60°C for 60 s [annealing]). All sample measurements were normalized against GAPDH values following a primer validation and selection assessment involving GAPDH and beta actin.

Commercially available primers were purchased for RT-PCR analysis. Before use, the primers were validated to determine their specificity toward the AT1r. The primer efficiency was found to be 2.017 and the standard curve is represented in Figure S2. Further details of all primers used in the study are provided in Table 2 below. The 5 μL PCR products were analyzed by 1.5% agarose gel electrophoresis. The PCR product for AT1R is 471 bp.[36]

AT1r ELISA technique

Supernatant and cell lysates were incubated for 1 h in 96-well plates pre-coated with primary anti-AT1r antibody. The lysate was removed, and a detection antibody was incubated for 1 h. The reaction was developed with 3,3′,5,5′-tetramethylbenzidine substrate and stopped with a stop solution followed by colorimetric measurement at 450 nm. Total protein levels were determined by Micro Bicinchoninic acid protein assay and used to normalize AT1r measured values in the samples.

In vitro blood compatibility

Prior to *in vivo* administration, a hemolysis study was performed as previously described.[35] Blood compatibility of liposomes was investigated using erythrocytes harvested from Sprague-Dawley rats. Erythrocytes were washed three times with sterile PBS, pH 7.4.

The erythrocytes (1.5×10^7) were incubated with increasing phospholipid concentration of liposomes at 37°C for 1 hour. The resultant was centrifuged at 2,000 rpm for 10 min, and the supernatant was collected. The absorbance of the supernatant was measured at 540 nm to quantify the released hemoglobin. Triton X-100 and PBS were used as positive (100% toxicity) and negative (0% toxicity) control, respectively. Relative hemolysis was calculated by considering hemolysis in the presence of Triton X-100 as 100% hemolysis.

In vivo AT1r knockdown and biocompatibility

In vivo biocompatibility was assessed to determine any signs of toxicity in various organs of the rats. WKY rats were injected with naked genes, optimized formulations, and PBS (negative control) via the tail vein. All treated animals were closely monitored for any sign of pain or distress such as weight loss of more than 10%, unresponsiveness, refusal to eat or drink, abnormal posture or movement, hiding, biting, and loss of body hair. Rat weight was monitored continuously throughout the study; a decrease of more than 10% in rat weight was categorized as toxic. In addition, the biocompatibility of the formulations was further assessed using a histological examination of tissue sections of different organs (liver, lungs, kidney, heart, brain, and spleen) after 35 days of injection. After the sections were stained with hematoxylin and eosin, they were assessed for necrosis, inflammation, fibrosis, and any lesions. Saline-treated rats were used as a negative control. To investigate the efficacy of our formulations, SHRs were injected with formulation via the tail vein. The AT1r expression levels in the brain were analyzed at different time points (3, 7, 14, 21, and 35 days) after intravenous injection. Quantitative analysis was performed using qRT-PCR and commercially available ELISA kits following the procedure described above. Rats injected with saline were used as a negative control.

Rat tail vein BP measurement

SHR and WKY rats, at 10 weeks of age, were subjected to weekly BP measurements via a noninvasive tail-cuff plethysmography method (CODA 6; Kent Scientific).[37,38] BP was measured as described by the manufacturer (Kent Scientific). Rats were acclimatized for 1 week before the measurement procedures. The first measurement week was to get the rats adapted to the measurement process. For a given session, animals were introduced into plexiglass restraint devices resting upon thermal heating pads set to 32 ± 2°C with the tail exposed. Both an occlusion cuff and a volume pressure-recording cuff were placed close to the base of the tail. The cuffs were inflated and deflated automatically within 90 s. Animals were acclimated to the measurement conditions for 2 min before a regimen of eight consecutive computer-automated inflation/deflation cycles of the balloon cuff (3 preliminary measurements and 5 test measurements). Unlike other tail-cuff systems, CODA uses volume pressure recordings to measure both systolic and diastolic BP, which is then used by the software to calculate the mean BP. Data from the preliminary measurements were discarded, and data from the test measurements was averaged. Signals were recorded and analyzed using Kent Scientific software.[39,40] To minimize stress-induced variations in BP, all measurements were taken by the same person in the same peaceful environment and at the same time of the day. All measurements were conducted during the light cycle between 8:00 AM and 2:00 PM.

Data analysis

Data analysis was performed using GraphPad Prism and JMP pro software. Statistical analysis between groups was performed by either a two-way ANOVA or a Student's paired t test. All quantitative data are represented as mean ± SD/SEM. A p value of less than 0.05 was considered statistically significant in all statistical tests.

DATA AND CODE AVAILABILITY

All study data are included in the article and/or in the supplemental information.

SUPPLEMENTAL INFORMATION

Supplemental information can be found online at https://doi.org/10.1016/j.omtn.2024.102210.

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AUTHOR CONTRIBUTIONS

Conceptualization: J.S.; methodology: R.N.L.L., C.S., and S.A.; formal analysis: R.N.L.L., C.S., and J.S.; investigation: R.N.L.L., C.S., and S.A.; resources: C.S. and J.S.; data curation: R.N.L.L., C.S., and J.S.; writing original draft preparation: R.N.L.L. and J.S.; writing review and editing: R.N.L.L., C.S., and J.S.; visualization: R.N.L.L. and J.S.; supervision: J.S.; funding acquisition: J.S. All authors have read and agreed to the published version of the manuscript.

DECLARATION OF INTERESTS

The authors declare no competing interests.

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