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Efficient Gene Silencing in Metastatic Tumor by siRNA Formulated in Surface-modified Nanoparticles

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Abstract

We have developed a nanoparticle (NP) formulation for systemically delivering siRNA into metastatic tumors. The NP, composed of nucleic acids, a polycationic peptide and cationic liposome, was prepared in a self-assembling process. The NP was then modified by PEG-lipid containing a targeting ligand, anisamide, and thus was decorated for targeting sigma receptor expressing B16F10 tumor. The activity of the targeted NP was compared with the naked NP (no PEGylation) and non-targeted NP (no ligand). The delivery efficiency of the targeted NP was 4-fold higher than the non-targeted NP and could be competed by excess free ligand. Luciferase siRNA was used to evaluate the gene silencing activity in the B16F10 cells, which were stably transduced with a luciferase gene, in a lung metastasis model. The gene silencing activity of the targeted NP was significantly higher than the other formulations and lasted for 4 days. While confocal microscopy showed the naked NP provided no tissue selectivity and non-targeted NP was ineffective for tumor uptake, the targeted NP effectively penetrated the lung metastasis, but not the liver. It resulted in 70-80% gene silencing in the metastasis model after a single i.v. injection (150 μg siRNA/kg). This effective formulation also showed very little immunotoxicity.

Keywords

siRNA; tumor; metastasis; tumor targeted delivery; nanoparticles

1. Introduction

siRNA has become a potential alternative for treating multi-drug resistant metastasis, which is the major cause of death in cancer patients [1]. Selective delivery of siRNA to metastatic tumors remains a major obstacle for siRNA based therapy. Although various delivery systems for siRNA have been developed, only a few successful cases have been reported on delivering siRNA into metastatic tumor [2-6]. Between viral and non-viral vectors, non-viral siRNA delivery offers several advantages, such as high tissue selectivity and low immunotoxicity [7]. Our lab has developed a self-assembled non-viral nanoparticle (NP) formulation, which was prepared by condensing the siRNA and calf thymus DNA with protamine into a compact complex, followed by coating with cationic liposomes [8,9]. To further stabilize the

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formulation, PEG conjugated lipids were post-inserted into the outer lipid membrane. A targeting ligand (anisamide) was conjugated to the distal end of PEG for targeting sigma receptor expressing tumor cells. The targeted NP formulation was shown to selectively deliver siRNA to receptor positive tumor cells *in vitro* [8,9] and *in vivo* [10]. Here, we investigated the efficiency of our targeted NP for delivering siRNA into an experimental metastatic tumor model, B16F10 lung metastasis in C57BL/6 mice. The cells were stably transduced with luciferase gene by using a retroviral vector before introduction to the animals. siRNA against luciferase was used in this study for the assessment of the gene silencing effect. We also compared the activities of different siRNA formulations, including free siRNA, naked NP (no PEGylation), non-targeted NP (PEGylated but without ligand) and targeted NP (PEGylated with ligand).

2. Materials and Methods

2.1. Materials

DOTAP, cholesterol, and DSPE-PEG_{2000} (figure 1A) were purchased from Avanti Polar Lipids, Inc. (Alabaster, AL). Protamine sulfate (fraction X from salmon) and calf thymus DNA (for hybridization, phenol-chloroform extracted and ethanol precipitated) were from Sigma-Aldrich (St. Louis, MO). DSPE-PEG_{2000}-anisamide (DSPE-PEG-AA) was synthesized in our lab using the methods described previously [11] and the structure is shown in figure 1B.

Anti-luciferase siRNA (GL3) (target sequence 5′- CTT ACG CTG AGT ACT TCG A -3′) was purchased from Dharmacon (Lafayette, CO) in deprotected, desalted, annealed form. For *in vitro* quantitative and tissue distribution studies, fluorescein (FAM) and cy3 labeled siRNA (3′ end of the sense strand) provided by Dharmacon were used.

B16F10 cells, murine melanoma cells, were obtained from the American Type Cell Collection and were stably transduced with GL3 firefly luciferase gene using a retroviral vector in Dr. Pilar Blancafort's lab at the University of North Carolina at Chapel Hill (UNC). The cells were maintained in DMEM medium (Invitrogen, Carlsbad, CA) supplemented with 10% fetal bovine serum (FBS) (Invitrogen, Carlsbad, CA). B16F10 cells expressing sigma receptors [12] were used as model cells in our study.

2.2. Experimental animals

Female C57BL/6 mice of age 6-8 week (16-18 g) were purchased from Charles River Laboratories (Wilmington, MA). All work performed on animals was in accordance with and approved by the IACUC committee at UNC.

2.3. Preparation of siRNA containing nanoparticles

Nanoparticles (NP) were prepared as previously described with slight modifications [8,9]. Briefly, naked NP were obtained by quickly mixing suspension A (8.3 mM liposome (DOTAP: cholesterol = 1: 1, molar ratio) and 0.2 mg/ml protamine in 150 μl nuclease free water) with solution B (0.16 mg/ml siRNA and 0.16 mg/ml calf thymus DNA in 150 μl nuclease free water) followed by incubation at room temperature for 10-15 min. Non-targeted and targeted NP were prepared by incubating the naked NP suspension (300 μl) with 37.8 μl micelle solution of DSPE-PEG or DSPE-PEG-AA (10 mg/ml) at 50°C for 10-15 min, respectively. NP was allowed to stand at room temperature for 10 min. The charge ratio of the formulation was about 1:5 (-:+). The particle size was measured using the submicron particle sizer (NICOMP particle sizing systems, Autodilute^{PAT} Model 370, Santa Babra, CA) in the NICOMP mode. The zeta potential of the NP diluted in 1 mM KCl was determined by the Zeta Plus zeta potential analyzer (Brookhaven Instruments Corporation, Holtsville, NY). NP was freshly prepared and used within 20 min for the following experiments.

2.4. *In vitro* cellular uptake study

B16F10 cells (1×10^5 cells/0.5 ml/well) were seeded in 24-well plates (Corning Inc., Corning, NY) 20 h before experiments. Cells were treated with different formulations containing 500 nM FAM-siRNA in the culture medium at 37°C for 4 h. Cells were washed three times with PBS followed by incubation with lysis buffer (0.3% Triton X-100 in PBS) at 37°C for 0.5 h. Fluorescence intensity of cell lysate was determined by a plate reader (λex: 485 nm, λem: 535 nm) (PLATE CHAMELEON Multilabel Detection Platform, Bioscan Inc., Washington, DC). For free ligand competition study, cells were co-incubated with 50 μM haloperidol with different formulations.

2.5. *In vitro* luciferase gene silencing study

B16F10 cells (5×10^5 cells/5 ml/flask) were seeded in 25T culture flasks (Becton Dickinson Co., Franklin Lakes, NJ) 20 h before experiments. Cells were treated with different formulations at a concentration of 250 nM siRNA in DMEM containing 10% FBS at 37°C for 24 h. Cells were trypsinized and 2×10^5 cells were collected for luciferase activity assay and the rest of cells were re-cultured in a 25T culture flask for the assay of the next time point. Collected cell pellets were incubated with the lysis buffer (0.05% Triton X-100 and 2 mM EDTA in 0.1 M Tris-HCl) at room temperature for 10 min. Ten μl lysate was mixed with 100 μl substrate (Luciferase Assay System, Promega Co., Madison, WI) and the luminescence was measure by a plate reader. The protein concentrations of the samples were determined by using a protein assay kit (Micro BCA™ protein assay kit, Pierce). Luciferase activity of a sample was normalized with the protein content and expressed as percent luminescence intensity compared to the untreated control. Percent luciferase activity of cells treated with different formulations was plotted against the time. The AAC (area above the curve) of different formulations was calculated and compared with each other. AAC = AUC (area under the curve) $_{\text{untreated}} - \text{AUC}_{\text{treated}}$.

2.6. Tissue distribution study

C57BL/6 mice were injected with 2×10^5 B16F10 cells via the tail vein. Seventeen days later, mice received i.v. injections of cy3-siRNA at the dose of 1.2 mg/kg formulated in PBS, naked NP, non-targeted NP or targeted NP. After 4 h, mice were euthanized and major tissues were collected. Paraffin embedded tissue sections of 5 μm in thickness were prepared. Sections were re-hydrated, mounted with the DAPI containing medium (Vectashield®, Vector Laboratories Inc., Burlingame, CA) and imaged using a Leica SP2 confocal microscopy.

2.7. Immunohistochemistry

B16F10 metastasis loaded lungs and normal lungs from C57BL/6 mice were collected for the preparation of paraffin embedded sections (5 μm thick). Sigma receptor expression in the sections was stained by using the rabbit anti-mouse sigma receptor antibody (Santa Cruz Biotechnologies) and a kit (mouse-to-mouse detection system, Chemicon International, Temecula, CA). Tissue sections were counter stained with hematoxylin for nuclei, mounted and imaged using a Nikon phase contrast light microscopy.

2.8. *In vivo* gene silencing study

C57BL/6 mice were i.v. injected with 2×10^5 B16F10 cells via the tail vein. Seventeen days later, mice were given i.v. injections of anti-luciferase siRNA at the dose of 150 μg/kg formulated in PBS, naked NP, non-targeted NP or targeted NP. Control siRNA (target sequence: 5′-AATTCTCCGAACGTGTCACGT-3′) [13] formulated in targeted NP was also prepared to verify if the silencing effect was sequence dependent. For the dose-response study, tumor bearing mice were i.v. injected with siRNA in targeted NP at doses of 18.75, 37.5, 75, 150, 300, 600 and 1,200 μg/kg. After 48 h, mice were euthanized and the tumor loaded lungs

were collected. The lung was homogenized in 1 ml lysis buffer (0.05% Triton X-100 and 2 mM EDTA in 0.1 M Tris-HCl) followed by centrifugation at 5,000 rpm for 5 min. Ten μl supernatant was mixed with the luciferase substrate and the luciferase activity was measured by a plate reader.

2.9. Cytokine induction assay

C57BL/6 mice were i.v. injected with anti-luciferase siRNA formulated in PBS, naked NP, non-targeted NP and targeted NP at the dose of 150 μg/kg. Targeted NP formulated with plasmid DNA (pNGVL-Luc prepared by Bayou Biolabs) [14] instead of calf thymus DNA was prepared and used as a control for the evaluation of immunotoxicity of calf thymus DNA. Two h after the injections, blood samples were collected from the tail artery and allowed to stand at room temperature for 0.5 h for coagulation. Serum was obtained by centrifuging the clotted blood at 16,000 rpm for 20-40 min. Cytokine levels were determined by using ELISA kits for TNF, IL6, IL12 (BD Biosciences, San Diego, CA) and IFN-α (PBL Biomedical Laboratories, Piscataway, NJ).

2.10. Statistical analysis

Data are presented as the mean ± SD. The statistical significance was determined by using the analysis of variance (ANOVA). P values of <0.05 were considered significant.

3. Results

3.1. Characterization of NP

The characterizations of the NP are summarized in table 1. The particle sizes of the three NP formulations were similar to each other. DSPE-PEG modification significantly reduced the zeta potential of the NP. Targeted NP showed a slight increase in the zeta potential compared to the non-targeted NP.

3.2. *In vitro* cellular uptake

To investigate the delivery efficiency of our NP formulations, we performed the cellular uptake study using FAM labeled siRNA. The fluorescence intensity of the cell lysate represents the intracellular delivery efficiency for siRNA by a given formulation. As shown in figure 2, cell lysate from cells treated with free FAM-siRNA showed background fluorescence, while those from cells treated with NP formulations had significantly higher fluorescence intensities. After PEGylation, the delivery efficiency of non-targeted NP was significantly reduced to 1/10 compared to the naked NP. However, targeted NP further modified with a targeting ligand showed 4-fold increased delivery efficiency compared to non-targeted NP. The addition of free haloperidol, a known agonist for sigma receptors, significantly reduced the delivery efficiency of targeted NP but not other NP formulations.

3.3. *In vitro* luciferase gene silencing

We examined both the extent and the duration of the gene silencing effect by siRNA in different formulations. As shown in figure 3A and B, free siRNA showed minimal effect, while the NP formulations had moderate to strong gene silencing effect ($p<0.05$ compared to free siRNA). siRNA formulated in the naked NP and the targeted NP had 2-2.5 fold higher activity than in the non-targeted NP ($p<0.05$). The gene silencing effect decreased right after the dosing stopped for the naked NP, while the targeted NP showed a prolonged activity for 4 days. Therefore, the overall activity (AAC) of the targeted NP was significantly higher than the naked NP ($p<0.05$). Control siRNA formulated in the targeted NP showed moderate but very transient effect and the overall effect (AAC) was not significantly higher than that of the free siRNA ($p>0.05$).

3.4. Tissue distribution of cy3-siRNA

Tissue distribution study was performed to evaluate the *in vivo* tumor targeting by different NP formulations. Free cy3-siRNA showed poor tissue accumulation in both tumor free and tumor loaded lungs, as very little cy3 signal was detected in the sections (figure 4A). Naked NP effectively delivered cy3-siRNA to the lung alveolar cells, but showed relatively poor penetration into the tumor nodules. Non-targeted NP showed poor tissue uptake in both tumor free and tumor loaded lungs. On the other hand, targeted NP provided the highest tumor penetration and uptake among the four formulations. Other normal organs (heart, spleen and kidney) only showed minimal fluorescence signal except liver. As shown in figure 4B, naked NP showed significant liver accumulation at 4 h in both tumor free and tumor bearing mice. However, PEGylated NP (with or without ligand) only significantly accumulate in the liver of tumor free mice but not tumor bearing mice at 4 h. Liver uptake of free cy3-siRNA was very little in both tumor free and tumor bearing mice.

3.5. Immunohistochemistry of sigma receptor

Tissue distribution results indicated that the tissue surrounding the tumor significantly contributed to uptake of the targeted NP in the tumor loaded lung. To address if the uptake of targeted NP was associated with the presence of small B16F10 metastasis, we performed immunostaining of the sigma receptor on the lung tissues. Brown staining in the tissue section indicated the expression of sigma receptor, while nuclei were counter stained blue by hematoxylin. As shown in figure 4C, sigma receptor was expressed in a significantly higher level in the B16F10 tumor cells and the surrounding tissue than that in the normal lung, which only showed a basal level. The arrows indicate the presence of the B16F10 cells (figure 4C).

3.6. *In vivo* luciferase gene silencing

The *in vivo* activity of the NP formulations was assessed by the luciferase gene silencing effect in the whole B16F10 tumor loaded lungs. Figure 5A shows the activity of siRNA in different formulations. The luciferase activity was compared with the untreated control. siRNA in PBS, naked NP, non-targeted NP and targeted NP silenced 0%, 50%, 10% and 75% luciferase activity, respectively (figure 5A). Only the naked NP and the targeted NP showed a significant effect (figure 5A, $p<0.05$). Control siRNA formulated in the targeted NP had no effect. Figure 5B indicates that ED50 of the targeted NP formulation was 75 μg/kg. The optimal dose for the maximum gene silencing effect (70-80%) was 150 μg/kg, and further increase of the dose did not bring a higher gene silencing effect (figure 5B).

3.7. Cytokine induction study

The immunotoxicity of the NP formulations was monitored by measuring the serum concentrations of proinflammatory cytokines 2 h after injections (figure 6). None of the NP formulations composed of calf thymus DNA induced significant production of the proinflammatory cytokines (IL6, IL12, TNF and IFN-α). The targeted NP formulated with plasmid DNA, however, was very immunotoxic. All analyzed cytokines were significantly elevated except TNF.

4. Discussion

The NP was prepared by combining cationic liposomes, polycation and nucleic acids (calf thymus DNA and siRNA), which spontaneously assembled into nanoparticles. The calf thymus DNA, which contains limited amounts of immunostimulating CpG motifs [15], served as a carrier in this formulation to provide improved core compaction. This carrier DNA offers lower potential for inflammatory toxicity compared to the plasmid DNA, which was supported by the data shown in figure 6. This highly positively charged NP formulation (zeta potential ~40

mV) tends to aggregate when incubated with high salt buffer or serum [9] and thus had strong charge-charge interaction with the cells. Surface PEGylation offered steric hindrance for the NP, which stabilized the formulation in the serum containing medium [9] but also reduced the association with the cells (figure 2), which was supported by the zeta potential data (table 1). A targeting ligand (anisamide) tethered to the end of PEG retargeted the NP to the sigma receptor expressing cells (figure 2 and [9]), which was partially inhibited by the presence of free haloperidol, a known agonist for the receptor. Although the zeta potential slightly increased in the targeted NP compared to the non-targeted NP due to the positively charged anisamide ligand, the targeted NP did not show increased delivery efficiency for sigma receptor negative cells (CHO and CT26 cells, data not shown). Additionally, neither the non-targeted nor the targeted NP formed aggregates with the FBS [9]. The data suggest that the entry mechanism for the targeted NP was via a sigma receptor dependent pathway.

The *in vitro* gene silencing effect of different formulations showed high correlation with the siRNA sequence and the intracellular delivery efficiency (figure 3A and B). When anti-luciferase siRNA was formulated into NP formulations, the gene silencing activity was significantly improved ($p<0.05$, figure 3B). Naked NP and targeted NP showed 2-2.5 fold increased activity compared to the non-targeted NP due to the improved intracellular delivery (figure 2). Interestingly, targeted NP providing lower delivery efficiency (figure 2) showed higher gene silencing activity than the naked NP (figure 3B). It is also noted that the silencing activity of targeted NP was significantly prolonged for 4 days, while that of naked NP was relatively transient (figure 3A). First, the delivery efficiency was determined at 4 h, which favors the naked NP that entered into the cells via a strong charge-charge interaction. Targeted NP, on the other hand, delivered siRNA through a receptor mediated pathway, which is dependent on the receptor recycling/trafficking that usually takes longer time but is a continuous process. For a short half-life drug such as siRNA, continuous input of drugs often results in enhanced therapeutic effect. Second, naked NP formed aggregates before internalization and siRNA release from aggregate could be slow, which would result in poor bioavailability. Targeted NP did not form aggregates with serum and the intracellular release of siRNA should be faster and more complete, resulting in a prolonged gene silencing effect. Although the control sequence showed only very transient activity, which was probably due to an off-target effect, the overall activity was not significantly higher than that of the free siRNA (figure 3B). The result indicated that the gene silencing activity was highly sequence dependent.

The *in vivo* activity results are consistent with the *in vitro* observation, in which the targeted NP showed significantly higher activity (70-80% gene silencing) than other formulations (figure 5A). The enhanced activity of the targeted NP was mainly due to the significantly improved tumor uptake as shown in figure 4A (panel b). Naked NP having poor tumor tissue penetration (panel b of figure 4A) provided only 50% gene silencing (figure 5A), which was probably resulted from the formation of large aggregate when i.v. injected. Other formulations, including free siRNA, non-targeted NP and control siRNA in targeted NP, had little effect (figure 5A, $p>0.05$). Again, the results suggested that significant gene silencing activity was highly dependent on the correct siRNA sequence and sufficient tumor delivery. The ED50 of the targeted NP was 75 μg/kg (figure 5B), which was the lowest reported dose for i.v. delivery so far, to our knowledge. However, further increase of the dose did not result in increased activity (figure 5B).

Naked NP delivered siRNA via a non-specific charge-charge interaction mechanism. As shown in figure 4A and B, siRNA formulated in the naked NP accumulated in the lung, tumor and liver with little selectivity. The tissue uptake was particularly significant in the lung, the first pass organ for i.v. injection. Non-targeted NP, however, showed little lung uptake due to the steric hindrance provided by PEG (figure 4A). In the tumor free mice, the liver appeared to be

the major organ for eliminating the PEGylated NP (with or without ligand) (figure 4). Nevertheless, the liver uptake of the PEGylated NP (with or without ligand) was significantly reduced in the tumor bearing mice (panel b of figure 4B), which was probably due to the kinetic competition of the uptake by the tumor (panel b of figure 4A). In other words, in the tumor bearing mice, PEGylated NP effectively accumulated in the tumor by the EPR effect (enhanced permeability and retention) [16], resulting in a reduced liver uptake. It is noted that confocal microscopy only showed the intracellularly delivered and released cy3-siRNA signal. Signal of cy3-siRNA that were compactly formulated in NP and accumulated extracellularly was quenched and therefore, could not be shown in figure 4A (panel b). Our previous results suggest that PEGylated NP (with or without ligand) delivered comparable amount of siRNA to the tumor tissue but only targeted NP showed significant intracellular delivery [10]. As shown in figure 4C, normal lung expressed a basal level of sigma receptor and therefore, the targeted NP showed enhanced uptake in the lung compared to the non-targeted NP (figure 4A). Additionally, the surrounding tissue around the tumor contained small amounts of B16F10 cells (figure 4C, as indicated by the arrows), which also contributed to the significant uptake of the targeted NP. The enhanced uptake of the targeted NP in the surrounding tissue may be via these metastatic cells, which were closer to the blood supply and more accessible compared to the cells in the large tumor nodules.

The toxicity of the NP formulations was evaluated by their induction of the proinflammatory cytokines (IL6, IL12, TNF, IFN-α) (figure 6). At such a low dose (150 μg/kg), none of the NP formulations were immunotoxic. Additionally, no body weight decrease was observed for any of the mice treated with the formulations (data not shown).

To summarize, free siRNA could hardly penetrate through the cell membrane (figure 2) due to its highly hydrophilic nucleic acid backbone. Therefore, siRNA itself with low bioavailability showed no gene silencing activity (figures 3 and 5). Encapsulation of siRNA into the naked NP dramatically increased the intracellular delivery and the gene silencing activity (figures 2, 3 and 5) through the charge-charge interaction of the formulation with cells. However, the non-specific nature of the charge-charge interaction resulted in the formation of large aggregates when injected into the blood [17] and low tissue selectivity (figure 4). PEGylation of the naked NP abolished the non-specific interaction with negatively charged cells or proteins [9] but also reduced the cellular and tissue uptake (figures 2 and 4), resulting in low silencing activity (figures 3 and 5). Introduction of a targeting ligand at the distal end of PEG chain restored the intracellular delivery to the sigma receptor positive cells (figures 2 and 4), while the tissue selectivity was maintained (figure 4). This targeted NP formulation with improved tissue specificity and delivery efficiency silenced the luciferase gene in the lung metastasis effectively (figure 5) without any significant immunotoxicity (figure 6).

5. Conclusions

We have developed a surface-modified NP formulation that selectively delivered siRNA to the sigma receptor expressing B16F10 lung metastasis. With high delivery efficiency, a low dose (150 μg/kg) was required to achieve 70-80% gene silencing in the whole lung metastasis. This targeted NP formulation also showed little immunotoxicity, which promises its potential use for metastasis treatment.

Acknowledgments

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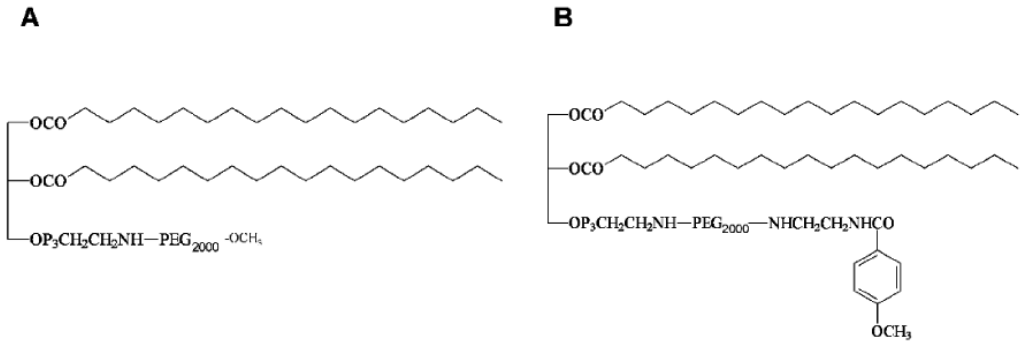

Figure 1.
Chemical structures of DSPE-PEG (A) and DSPE-PEG-anisamide (B).

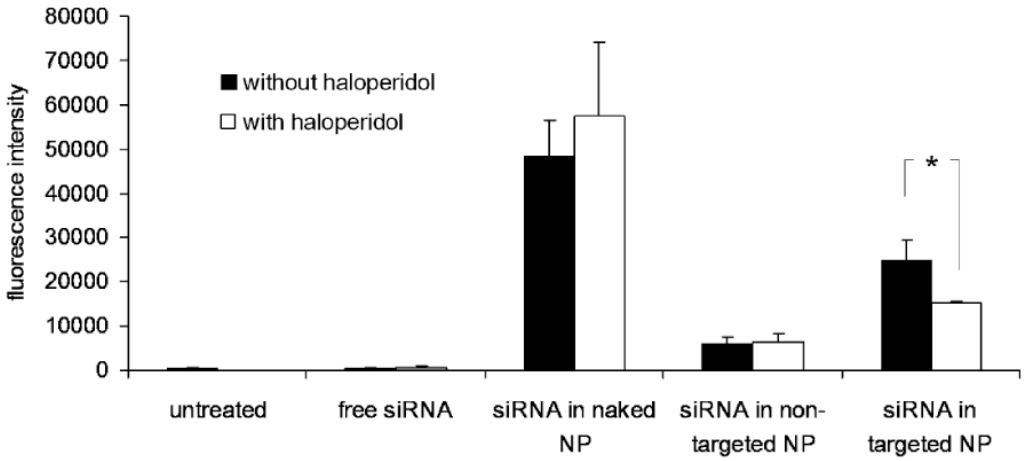

Figure 2.

Fluorescence intensities of cell lysate from cells treated with FAM-siRNA containing formulations. B16F10 cells were incubated with different formulations at 37°C for 4 h in the absence or presence of 50 μM haloperidol. Cells were washed and lysed. Cell lysate was analyzed for fluorescence intensity by a plate reader. Data = mean ± SD (n=3), * indicates $p<0.05$.

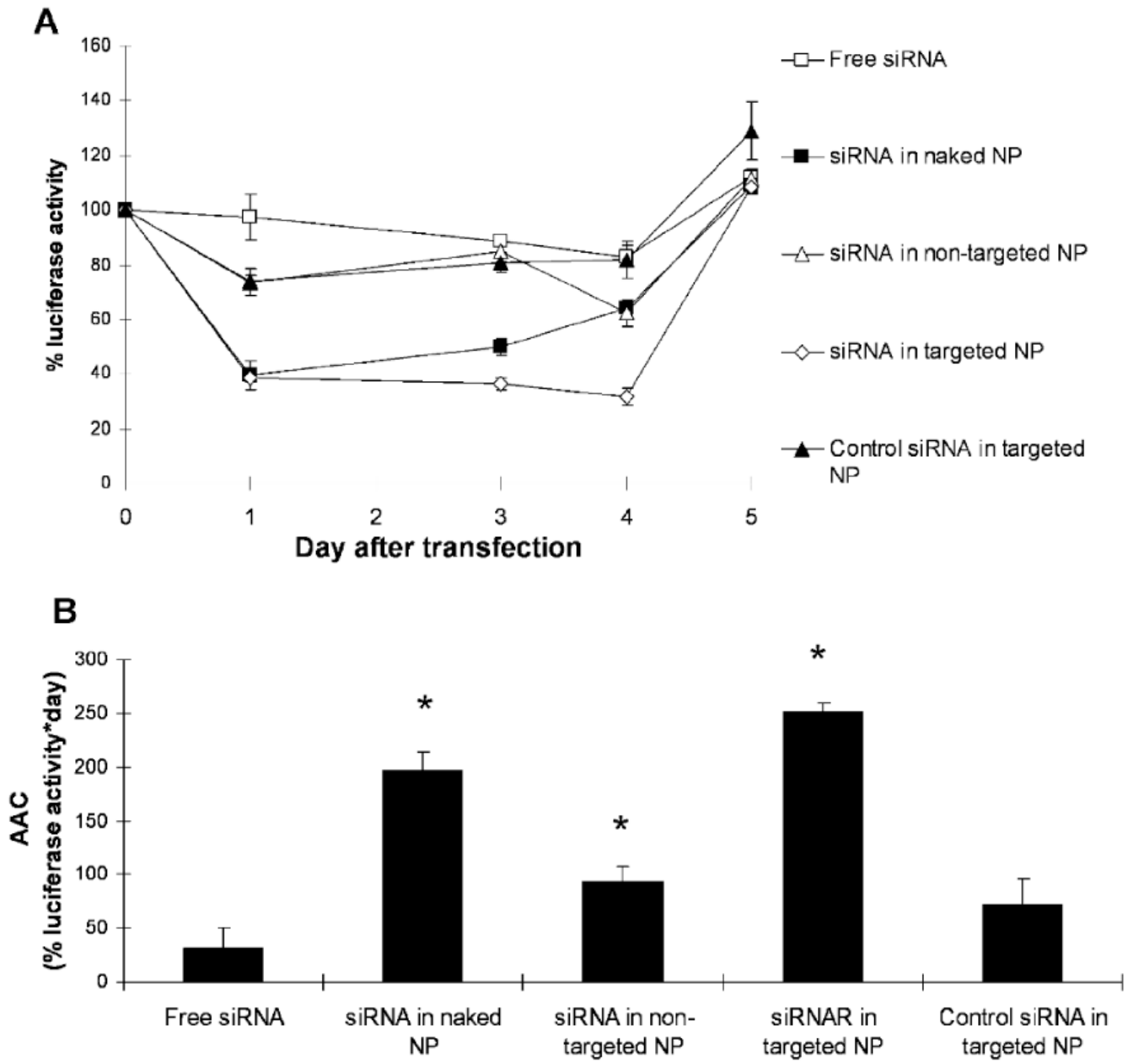

Figure 3.

In vitro luciferase gene silencing effect of different siRNA formulations (A) and the AAC (area above the curve) of different formulations in figure 3A (B). B16F10 cells were incubated with different siRNA formulations at 37°C for 24 h. At the end of incubation, formulations containing medium was removed and cells were washed. Luciferase activities of the cells (2×10^5) were analyzed at different time points after the treatment. Data = mean ± SD (n=3), * indicates $p<0.05$ compared to the free siRNA.

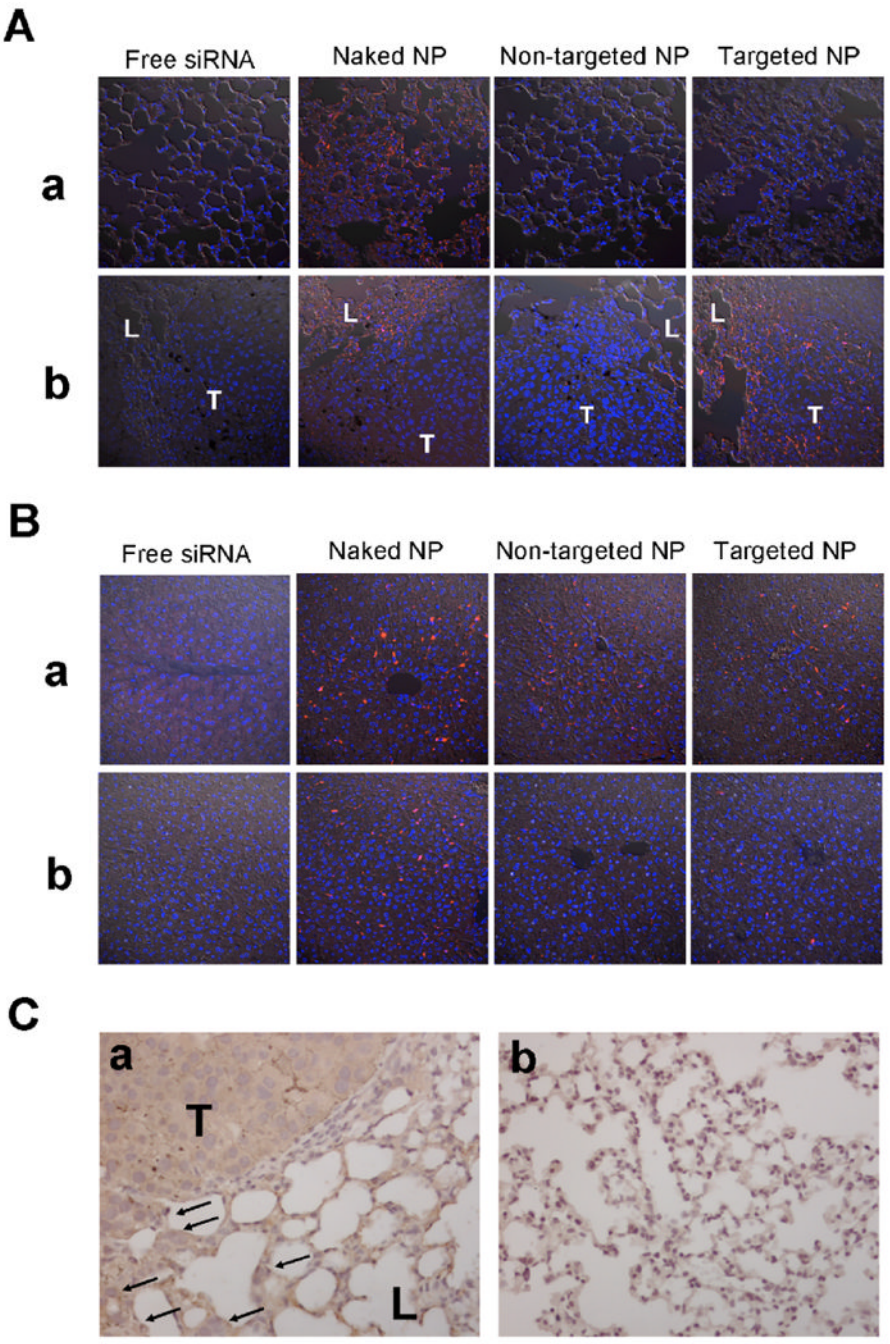

Figure 4.

Confocal microscopy photographs of the lungs (A) and livers (B) collected from the tumor free (a) and B16F10 tumor bearing mice (b). Mice were i.v. injected with cy3-siRNA in different formulations. After 4 h, mice were sacrificed and major tissues were collected and processed for paraffin embedded sections. Tissue sections were re-hydrated, mounted with the DAPI containing medium and imaged by confocal microscopy. Pictures are the overlay of phase contrast, DAPI fluorescence signal (blue, nuclei) and cy3 signal (red, siRNA). T and L indicate the presence of tumor and lung tissue, respectively. Magnification = 400×. C, immunohistochemistry of sigma receptor in tumor loaded lung (a) and tumor free lung (b). T

and L indicate the presence of tumor and lung tissue, respectively. Arrows indicate the presence of B16F10 cells. Magnification = 200 ×

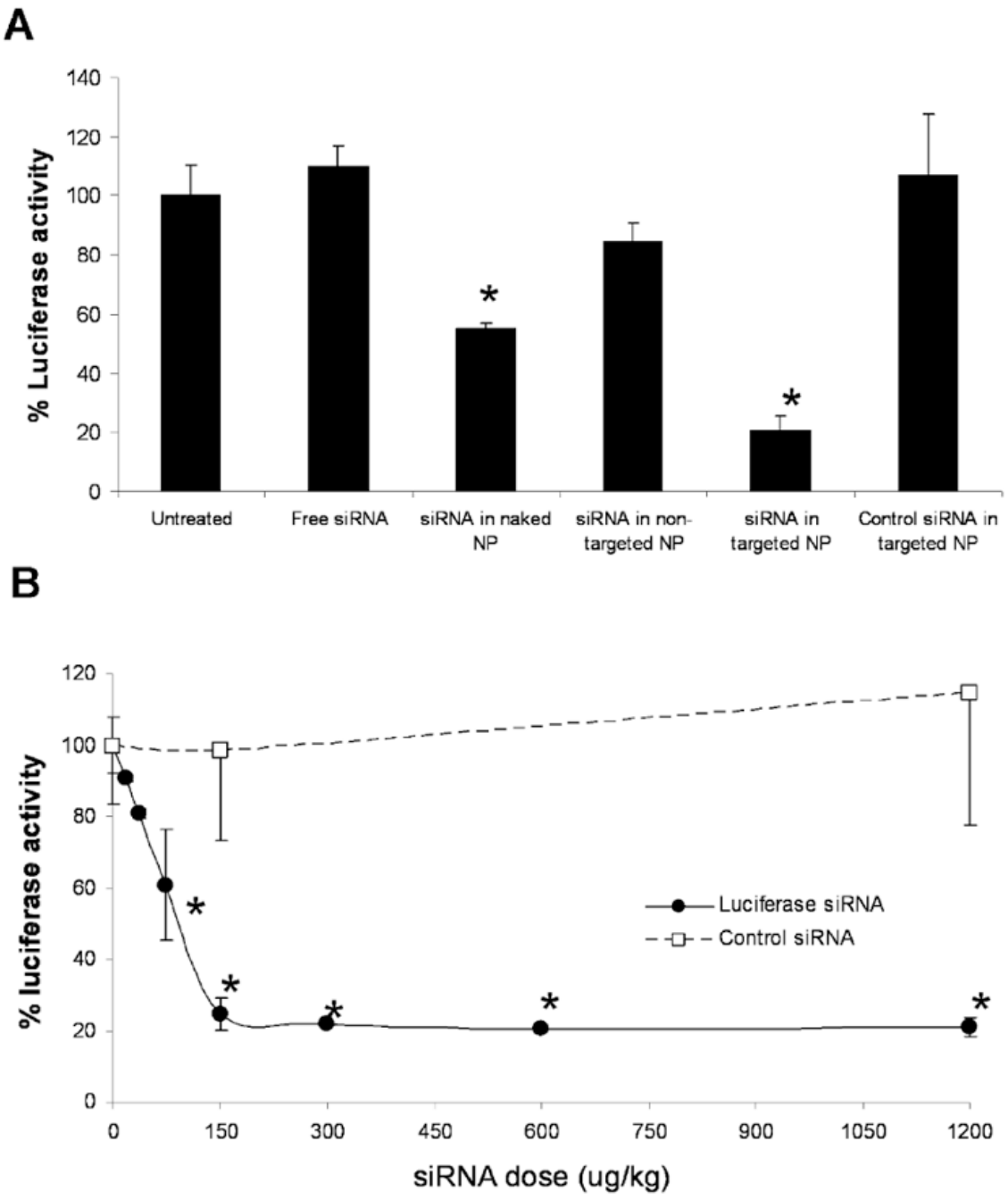

Figure 5.

In vivo luciferase gene silencing effect of different siRNA formulations at the dose of 150 μg/kg (A) and that of the targeted NP at various doses (B). B16F10 tumor bearing mice were i.v. injected with different siRNA formulations. After 48 h, mice were sacrifice and the tumor loaded lungs were excised for the luciferase activity assay. Luciferase activity was compared to the untreated control and expressed as % luciferase activity. Data = mean ± SD (n=3-8), * indicates $p<0.05$ compared to the untreated control.

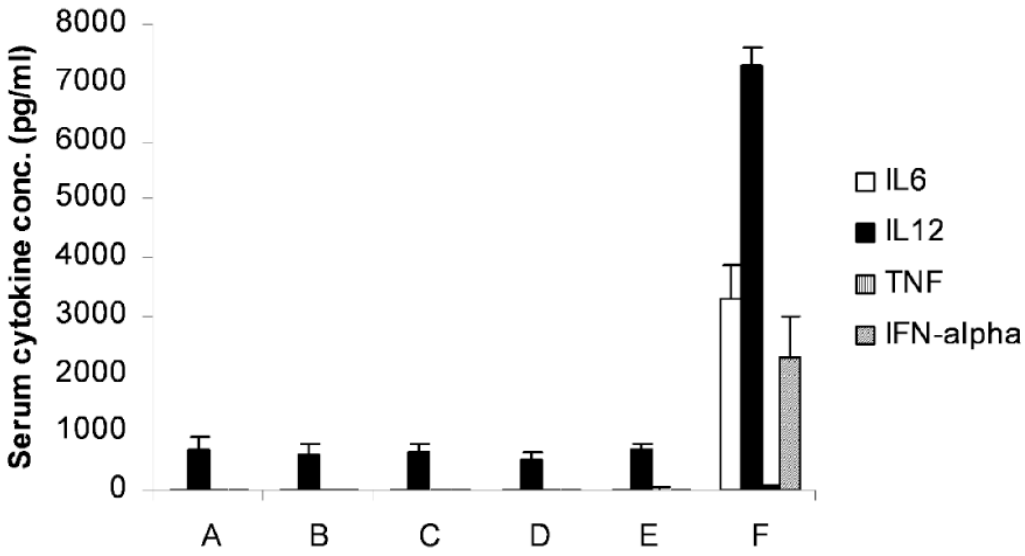

Figure 6.

The serum cytokine concentrations of the mice 2 h after the i.v. injections of siRNA in different formulations. C57BL/6 mice were i.v. injected with different siRNA formulations (A, untreated; B, siRNA in PBS; C, siRNA and calf thymus DNA in naked NP; D, siRNA and calf thymus DNA in non-targeted NP; E, siRNA and calf thymus DNA in targeted NP; F, siRNA and plasmid DNA in targeted NP. After 2 h, blood was collected from the tail artery and serum concentrations of the cytokines were analyzed by the ELISA method. Data = mean ± SD (n=4), * indicates $p<0.05$ compared to the untreated control.

Table 1

Characterization of NP formulations

	Naked NP	Non-targeted NP	Targeted NP
Particle size (nm)	117.6 ± 13.2	115.1 ± 14.8	110.6 ± 13.8
Zeta potential (mV)	43.2 ± 3.62	20.4 ± 2.08	25.51 ± 1.22

Data are representative data from repeated measures of 4-5 samples.