DISTRIBUTION OF INTRAVENOUSLY INJECTED SMALL INTERFERING RNAs IN ORGANS AND TISSUES

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There are a number of problems that need to be addressed when designing an effective RNA interference-based drug including distribution of intravenously injected exogenous small interfering RNAs (siRNAs) in the organs and tissues of the patient. Insufficient data on siRNA distribution obtained using isotopic/fluorescent labeling offers no insight into whether the polymer retains its original structure after the injection. Quantitative real-time polymerase chain reaction that we used in our work provides a better response to the challenge. In our experiment *LIVIN*–specific siRNAs injected intravenously were distributed unevenly between tissues and their accumulation was dose-dependent (the study was conducted in mice using 2.5 and 7.5 mg/kg doses). Maximal accumulation was observed in the liver and spleen where siRNA concentration continued to increase between 48 and 96 hours after its administration. This demonstrates that the studied cationic lisosome/miRNA complex has long circulation time. We believe that the obtained data will be instrumental in finding an effective therapeutic dose, designing adequate regimens and preparing for preclinical or clinical trials of siRNA-based drugs.

Keywords: small interfering RNA, cationic liposomes, mice, tissues, quantitative polymerase chain reaction

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РАСПРЕДЕЛЕНИЕ ТАРГЕТНЫХ МАЛЫХ ИНТЕРФЕРИРУЮЩИХ РНК ПОСЛЕ ВНУТРИВЕННОГО ВВЕДЕНИЯ

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Создание лекарственных средств на основе РНК-интерференции предполагает решение ряда задач, включая получение сведений о распределении по органам реципиента вводимых в его организм экзогенных малых интерферирующих РНК (миРНК). Имеющиеся данные по этому направлению исследований не являются полными и получены при помощи изотопных/флуоресцентных меток, которые не позволяют судить о сохранении первичной структуры введенного полимера. Использованный в данной работе метод анализа на основе количественной полимеразной цепной реакции в режиме «реального времени» позволяет решить эту проблему. Показано, что введенные внутривенно миРНК к гену *LIVIN* неравномерно распределяются между тканями и демонстрируют зависимое от дозы накопление в органах (исследование проводили на мышах, используя дозы 2,5 и 7,5 мг/кг). Максимальное накопление выявлено в печени и селезенке. Впервые обнаружено, что в этих органах концентрация анализируемой миРНК возрастает во временном интервале между 48 и 96 ч после введения. Это указывает на длительный период циркуляции миРНК в комплексе с катионными липосомами в организме. Полученные сведения актуальны для поиска эффективной терапевтической дозы, схем лечения, а также планирования доклинических и клинических исследований при разработке лекарственных препаратов на основе терапевтических миРНК.

Ключевые слова: малые интерферирующие РНК, катионные липосомы, мыши, ткани, количественная полимеразная цепная реакция

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There has been a steadily growing interest in the design of RNA interference-based therapeutics since the mechanism was discovered underlying the binding of exogenous small interfering RNAs (siRNAs) to the RNA-induced silencing complex (RISC) [1]. The therapeutic potential of siRNAs has been demonstrated in the animal model of autoimmune hepatitis [2]. The authors of the experiment used RNA interference to silence the expression of *Fas* (the gene that codes for an apoptosis-mediating receptor) in mouse hepatocytes *in vivo*. siRNA duplexes were injected in the tail vein of mice in doses of 2.0 to 2.5 mg/kg. To verify uptake of siRNAs by the cells, *Fas* siRNAs were labeled with Cy5 at their 3'-end.

The first therapeutically effective liposomal formulation of siRNA targeted apolipoprotein B (APOB) whose accumulation is implicated in the development of atherosclerotic vascular disease. It was shown that intravenous injections of APOB-specific siRNAs administered to monkeys in doses of 1.0 to 2.5 mg/kg were followed by a 90 % reduction in target gene expression [3]. In that experiment siRNAs were encapsulated into iLNPs (ionizable lipid nanoparticles) produced by Alnylam Pharmaceuticals that are considered to be effective siRNA carriers. These liposomes were also used to design revusiran (ALN-TTR01), a drug for treating transthyretin amyloidosis, successfully tested in the clinical setting [4]. Similar carriers were used in another siRNAs-based drug targeting *VEGF* and KSP in patients with liver metastases [5]. Such siRNA carriers are also reported to work against Ebola [6]. Unfortunately, only scarce data obtained using fluorescent tags are available on the distribution of siRNA-based complexes in organs and tissues [7].

A lot of effort is being put into discovery of other effective ways of delivering siRNAs into the cell *in vivo* [8]. Cationic liposomes seem to hold some promise as delivery vectors [9, 10]. A possibility to use them in *in vivo* studies was demonstrated recently when *Bid* (the BH3-interacting domain death agonist) was silenced using a siRNA/Invivofectamine complex (Invivofectamine is a commercial reagent by Thermo Fisher Scientific, USA). The drug was administered to mice intravenously in doses of 0.5 to 4.0 mg/kg; efficiency of RNA interference was evaluated using liver tissues [11]. However, distribution of the drug in organs and tissues was not studied. Such information is essential for understanding the patterns of drug accumulation in the target organ and gives an idea of what possible adverse effects might be expected

The aim of this work was to evaluate distribution of cationic liposomes/siRNA complexes targeting gene *LIVIN* using a new, recently proposed real-time PCR-based method for siRNA quantification. The composition of cationic liposomes was as follows. Cationic lipid: 1,26-bis(cholest-5-en-3β-yloxycarbonylamino)-7,11,16,20-tetraaza hexacosane tetrahydrochloride; neutral lipid: 1,2-dioleoyl-sn-glycero-3-phosphoethanolamine). *LIVIN* is an apoptosis inhibitor. It is often overexpressed in malignant tumors and is a promising therapeutic target [12].

METHODS

Animals

All experiments on animals were carried out in strict compliance with the *Principles of Good Laboratory Practice* (GOST 53434-2009, 2010) and Order No. 708n of the Ministry of Health and Social Development of the Russian Federation *On the Approval of Rules of Laboratory Practice* dated October 13, 2010. The study was approved by the local Ethics Committee of the Research Center of Medical Genetics (Protocol No. 8 dated December 23, 2016). The experiment was carried out on 18 healthy male Balb/c mice weighting 21–22 g bred in Blokhin Russian Cancer Research Center. The mice were housed under standard conditions with free access to food and water. Prior to the actual experiment the mice were examined and found healthy.

The animals were randomly distributed into 3 groups. The first group (the controls) received intravenous injections of 0.5 ml normal saline. Group 2 received intravenous injections of 2.5 mg/kg siRNA against *LIVIN*; group 3 received 7.5 mg/kg siRNA against *LIVIN*. Before the experiment all mice were tagged, weighted and placed into cages 6 animals per cage. Tissue samples for biochemical analysis were collected 48 and 96 hours after the injection. The animals were sacrificed in strict compliance with the existing guidelines. Lung, liver, spleen, kidney and brain samples were collected from each mouse, immediately frozen and stored at –70 °C.

Quantification of exogenous siRNA in tissues

Exogenous siRNAs in tissue samples were quantified by polymerase chain reaction (PCR) as proposed by Liu et al. [13]. Probes specific to *LIVIN* siRNA were selected in strict accordance with the Custom TaqMan Small RNA Assays Design and Ordering Guide (Applied Biosystems, USA). Nucleic acids were isolated from tissue samples homogenized in liquid nitrogen, by a standard proteinase-K-based technique and phenol-chloroform extraction. Reverse transcription and quantitative PCR were performed using Custom TaqMan Small RNA Assays by Applied Biosystems according to the vendor's protocol. Three replicates were run for each probe. Duplex concentrations were inferred based on the Ct (threshold cycle) value. This value represents a point during the reaction at which the fluorescent signal from the analyzed samples crosses the preset threshold. Data were processed using the Applied Biosystems StepOne and StepOnePlus Real-TimePCR Systems. In all studied samples gene expression was normalized to snoRNA202 as proposed by Wong et al. [14]. The relative amount of duplexes in the samples was calculated using DataAssist v3.0 Software (Applied Biosystems, USA) [15]. Time zero calibrator samples were obtained from the corresponding tissues of the controls.

Preparation of the injectable formulation

The injectable formulation was prepared in the lab in full compliance with the technical guidelines. Cationic liposomes used in our experiment are described in the work by Maslov et al. [10]. Interfering RNAs against *LIVIN* were obtained by annealing two complementary oligos as described in [12]. The complexes were assembled in sterile water. At the final step, 0.9 % sodium chloride was added to the loaded liposomes, and the composition was nanopore-filtered (pore size of 10 μm). The final concentration of the duplex in the injectable formulation was 0.125 μkg/ml. The minimal dose for *in vivo* injections was determined based on the guidelines for the use of Invivofectamine, which is a similar formulation for siRNA delivery.

RESULTS

A series of preliminary *in vivo* experiments showed that siRNAs delivered into the cells by cationic liposomes are eliminated at a slow rate and therefore can be detected in the isolated nucleic

acid fractions within at least 3 days following the transfection (data are unpublished). Based on these observations, the time of sampling was determined. Samples were collected 48 and 96 hours after the siRNA injections.

No signs of toxicity of the liposomal siRNA formulation were observed in the animals who received 2.5 and 7.5 mg/kg doses. The body weights of mice included in the experimental groups and the weights of their removed organs were physiologically normal and did not differ significantly from those in the control group.

Before proceeding to the biochemical analysis, we carried out a series of preparatory experiments. Specifically, we determined a lower threshold at which siRNA concentrations in the sample could be detected by reverse-transcription real-time PCR. Using the calibrator, we constructed a 5-point calibration curve encompassing a range of siRNA duplex concentrations from 23.04 pg/ml to 230 ng/ml. The lower detection threshold was 3–5 pg per reaction. After the threshold had been identified, we proceeded to the analysis of exogenous siRNA distribution in tissues and organs.

The results are shown in the figure below. The obtained data demonstrate that distribution of intravenously injected siRNA in organs and tissues of mice had a pattern. Its maximal accumulation was observed in the liver, whereas minimal — in the brain.

It was found that within 48 h after the injection siRNA accumulation in the liver, kidneys and brain was dose-dependent. This pattern was not observed for the lungs and spleen.

Gradually siRNA concentrations in the lungs, kidneys and brain went down. In contrast, the liver and spleen demonstrated a different pattern: siRNAs accumulated in these organs within the first 4 days after the injection. To find out whether siRNAs were eliminated beyond this time point, we carried out another experiment in which tissue samples were collected 12 days after the injection (the administered dose was the same). No trace amounts of the formulation were detected in the liver, kidneys, lungs and brain, suggesting that siRNA accumulation was time-limited.

DISCUSSION

Information about the distribution of therapeutic siRNAs (including those attempted to treat cancer) in organs and tissues is very important. Tumors found in the organ that readily accumulates siRNA will be more responsive to treatment. Predominant accumulation of miRNA in the liver makes malignant hepatic tumors an attractive target for RNAi-based drugs. Our data suggest that accumulation of intravenously injected miRNA in the liver is directly proportional to the administered dose and lasts for at least 4 days.

The pattern of siRNA distribution observed in our study is consistent with the data obtained by other researchers who used fluorescent labeling [7]. However, our analysis of dose- and time-dependencies yielded some new and critically important data. In particular, an increase in dosage stimulated a manifold increase in miRNA concentrations in the kidneys. This effect was observed for the first 2 days after the injection; then siRNA concentrations started to go down. This information can be helpful in designing an siRNA-based treatment for patients with kidney cancer. Similar, though less conspicuous associations between time, dosage and siRNA concentrations were observed in the brain tissue. Interestingly, increase in dosage did not have any effect on the siRNA concentrations in the lungs and spleen.

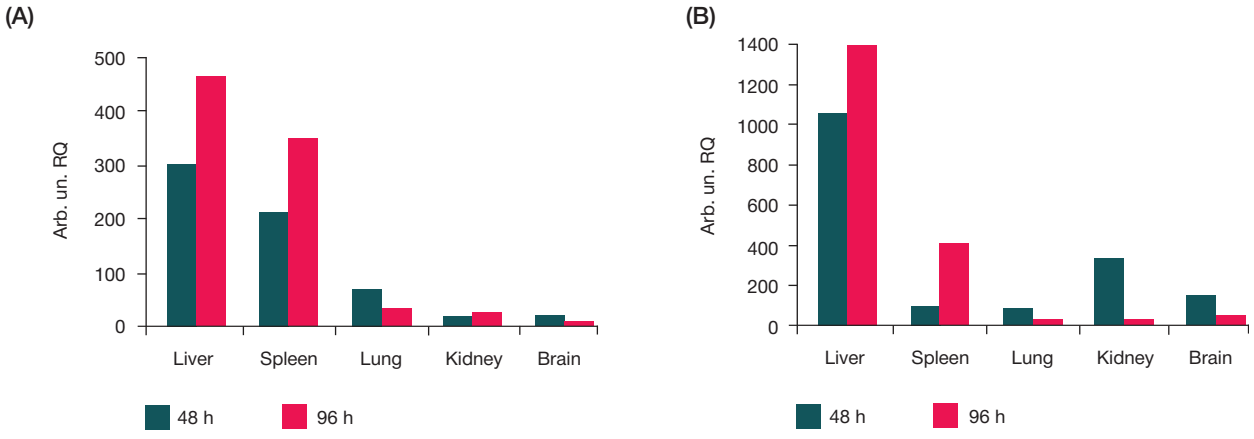
The obtained data about time- and dose-dependent accumulation of siRNA could be instrumental in devising a treatment regimen. Earlier treatment strategies based on RNA interference did not take into consideration the previously unnoticed time-related pattern of siRNA accumulation in the liver and spleen.

Dynamics of dose-dependent siRNA accumulation shows that kidney tissue is particularly sensitive to growing concentrations of the cationic liposome/siRNA complexes in the blood. It means that liposomal siRNA formulations targeting malignant cells, such as kidney carcinoma cells, should be administered at a higher dose for the enhanced effect.

In our study we used a recently proposed method for siRNA quantification by quantitative real-time PCR. Unlike fluorescence labeling [2, 7], this method can detect only those siRNAs that have retained their primary structure responsible for their interfering function [13], meaning that only functional siRNAs are quantified, which is important for preclinical and clinical studies.

Once dose and time dependencies have been identified, siRNA delivery into the target organ can be optimized to achieve the highest specificity possible and to prevent drug accumulation in "non-target" organs.

For this work we chose an siRNA against *LIVIN* that had proved to work well in *in vitro* experiments and has a good therapeutic potential [12]. We did not observe any visible toxic effects, which is consistent with the data provided by other authors [2, 3, 7, 11]. The doses of 2.5 and 7.5 mg/kg administered to the animals in our experiment are typical for



Accumulation of siRNA in the organs of mice after the intravenous injection. Differences between the experimental and control groups were significant for liver, spleen, kidney and lung tissues ($p < 0.05$). **(A)** Administered dose of 2.5 mg/kg. **(B)** Administered dose of 7.5 mg/kg

the majority of siRNA-based drugs. Single injections did not cause any damage to the internal organs or affect the behavior of the mice.

CONCLUSIONS

The discovered dose and time dependencies of siRNA distribution in the organs and tissues following the intravenous administration of the liposomal siRNA formulation prompt us to conclude that the liver is highly sensitive to RNAi-based treatment. Accumulation of siRNAs in the liver depends on the dose and time elapsed after the injection. The analysis of siRNA accumulation by other organs revealed that an increase in dosage leads to a proportional increase of siRNA concentrations in the kidney and brain within 48 hours after the injection, followed by a gradual reduction of siRNA levels by hour 96. In general, distribution of siRNA in the organs is uneven and must be taken into consideration when designing target RNAi-based drugs.

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