Delivery systems for enhanced siRNA delivery

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ABSTRACT

The development of reagent to deliver small amount of siRNA is necessary. We have explored the potential of *in vivo*-jetPEI™ (optimized linear polyethylenimine) for the delivery of siRNA in animal models. Our results show an efficient delivery of siRNA in different organs. Moreover, we have designed sticky siRNA (ssiRNA) in order to increase delivery of siRNA and silencing efficiency with *in vivo*-jetPEI™. We show that ssiRNAs are more effective than siRNAs when using *in vivo*-jetPEI™.
We have also developed a cationic lipid system for delivery of siRNA *in vitro* at picomolar concentration and we are currently testing an adapted formulation for *in vivo* delivery. Our data highlight the potency of these molecules as non-viral reagents of choice for siRNA based therapy.

Keywords: polyethylenimine, sticky siRNA, cationic lipid, delivery, animals.

1. RNA INTERFERENCE

RNA interference (RNAi) is a potent technology for gene silencing at the level of early gene function, *i.e.* at the mRNA level [1]. The principle is an extremely selective interaction of short RNA duplexes (siRNA; small interfering RNA) with a single target in the mRNA, providing sequence-specific mRNA degradation and inhibition of protein production [2, 3]. When introduced by transfection, siRNA shows effective silencing of exogenous and endogenous genes in a variety of mammalian cells [4].

RNAi is emerging as a powerful tool for human therapy, which could lead to entirely novel therapeutic developments for severe diseases such as cancer or viral infections. However, to fully exploit the vast potential of RNAi, the generation of robust and safe vectors and strategies of delivery in animals is necessary to improve the bioavailability of siRNA by the target and to achieve effective RNA interference. Two approaches are proposed for introducing siRNA into cells: the delivery by transfection of synthetic siRNA duplexes into the cytoplasm and the *in situ* delivery of siRNAs expressed from a plasmid (or DNA cassettes) preliminary introduced by gene transfer into the nucleus. In this report, we present many delivery systems enhancing siRNA delivery.

2. siRNA DELIVERY WITH NON-VIRAL VECTORS

Non-viral vectors offer appealing alternatives to viruses for nucleic acid delivery in animals. In particular, the cationic linear polyethylenimine (PEI) is the most widely used vector for *in vivo* gene delivery [5]. Currently, *in vivo*-jetPEI™, the optimized form of linear PEI available from Polyplus-transfection, is being tested in humans for cancer gene therapy [6]. Many non-viral systems are used or under development for the delivery of synthetic siRNA, including cationic polymers, such as PEI-based reagents, or cationic lipid-based formulations. As major requirements, gene silencing has to be obtained with minimal amount of siRNA as well as with the absence of toxicity and side or off-target effects. Non-viral systems have also to be designed for overcoming their limitations of diffusion, targeting and instability following systemic applications.

2.1 Delivery with cationic polymer

To explore the promising potential of *in vivo*-jetPEI™ to deliver active siRNAs in animals, we administrated retro-orbitally the GL2 luciferase gene (GL2-Luc) as a model of targeted gene in the lungs where the luciferase expression is maximal and significant compared to others organs. After optimization of gene delivery conditions (dose of 40 µg was used, N/P ratio of 8), siRNA matching the GL2-Luc sequence (40 µg per mouse) was mixed with the plasmid and retro-orbitally injected. As control, a non-specific GL3-siRNA, matching the GL3-Luc sequence, was injected in the same conditions. Mice were sacrificed one day later and luciferase expression determined in lung homogenates using a standard assay. Systemic administration of GL2-siRNA showed a mean silencing superior to 90% (n=9) of the luciferase expression compared to the luciferase expression obtained with the non-specific GL3-siRNA (Figure 1). This silencing effect was reproduced with many doses of plasmid injected intraveneously (Figure 1 and 2).

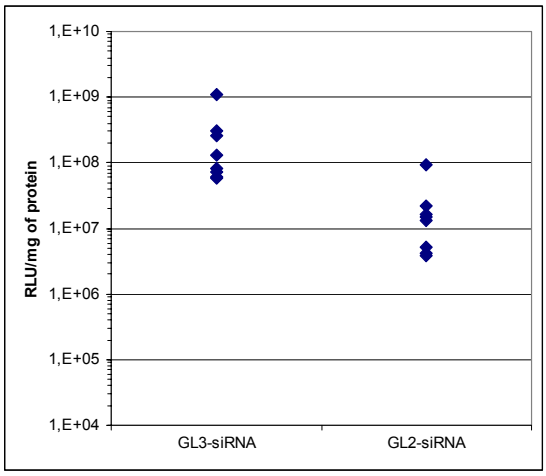
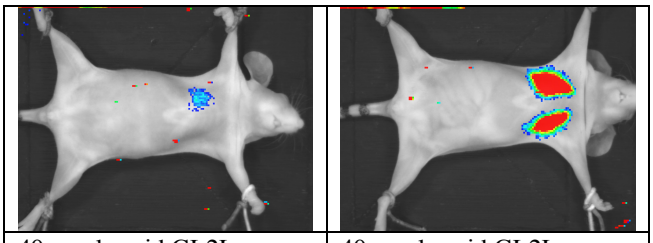


Figure 1: Inhibition of luciferase gene expression in the lungs. Forty micrograms of Luciferase specific siRNA (GL2) were injected retro-orbitally with 40 µg of pCMVLuc (GL2Luc) complexed with *in vivo*-jetPEI™ at N/P 8 in 200 µl of 5% glucose solution. As control, mice were injected with 40 µg of unspecific siRNA (GL3) co-transfected with the same amount of pCMVLuc. The lungs were harvested 24 h post-injection. Luciferase expression was measured and expressed relative to the total amount of protein present in the extract. Each point represents one mouse, n=9.

A second route of systemic injection was investigated where siRNA and plasmid complexed with *in vivo*-jetPEI™ were delivered *via* the tail vein. The luciferase expression was monitored by bioluminescent imaging of living mice using a cooled CCD camera 24 h after injection. A high luciferase expression is observed in the lungs when the plasmid was injected without siRNA. Highly reduced luciferase expression was observed when GL2-siRNA was co-delivered with the plasmid whereas a GL3-siRNA in the same conditions showed no effect on the transgene expression (Figure 2). We show here that *in vivo*-jetPEI™ can deliver active siRNAs silencing a target gene expressed in lung after intravenous injection in mice.



40 µg plasmid GL2Luc + 10 µg GL2-siRNA	40 µg plasmid GL2Luc + 10 µg GL3-siRNA

Figure 2: Inhibition of luciferase gene expression in the lungs. Forty micrograms of pCMVLuc (GL2Luc) with 10 µg of siRNA (Gl2- or GL3-siRNA) and complexed with *in vivo*-JetPEI™ at N/P 8 in 200 µl of 5% glucose solution were injected in the tail vein of nude mice. As control, mice were injected with 10 µg of unspecific siRNA (Gl3) co-delivered with the same amount of pCMVLuc. Twenty four hours later, the luciferase gene expression was monitored by bioluminescent imaging of live mice using a cooled CCD camera. This work was done in collaboration with Dr. Jean-Luc Coll and Olivier Freund, INSERM U578, Institut Albert Bonniot, Grenoble, France.

Many reports confirmed the potency of *in vivo*-jetPEI™ for siRNA delivery in animals. Ge and collaborators developed a therapy of *influenza* virus infections in mice [7]. They showed antiviral activity of siRNA in virus-infected mice with a selective inhibition of virus production in the lungs when siRNA were introduced by *i.v.* administration with *in vivo*-jetPEI™. Both prophylaxis (administration of complexed siRNA before virus infection) and therapeutic strategies (administration of *in vivo*-jetPEI™/siRNA post-infection) provided efficient protection against viral infection.

Song and collaborators [8] have retro-orbitally injected PTEN-specific siRNA complexed with *in vivo*-jetPEI™ showing a suppression of diabetes-enhanced apoptosis and Aki inhibition in streptozotocin-induced diabetic mice.

jetPEI™ has shown also promising results for anticancer RNA interference in murine models. Urban-Klein and collaborators [9] have shown first in a subcutaneous mouse tumor model that PEI-mediated delivery of low amounts of siRNA directed against a proto-oncogene results in a significant decrease of tumor growth while naked siRNA has no effect. The potency of siRNA administrated through systemic routes was confirmed in a second report [10] showing inhibition of tumor growth in orthotopic mouse glioblastoma models.

These publications and our own work highlight the possible use of jetPEI™/siRNA complexes for RNAi applications in animals. The low amount of siRNAs injected (10 to 60 µg per injection) in the reports cited are in favour of a more efficient gene silencing effect by RNA interference than the conventional antisense strategy. The use of a delivery vector such as jetPEI™ allows one to reduce both the siRNA dose injected and the side effects as compared to chemically modified siRNAs. Indeed, when using siRNA-cholesterol conjugates 1 mg siRNA per mouse (50 mg/kg, 3 injections) is needed to show a silencing effect [11]. Exploiting the protective effect of jetPEI™ against siRNA degradation means that one does not need chemical modifications of siRNA to improve siRNA stability in the physiological fluids, modification that are required if delivering naked siRNA by systemic injection. However, once cellular uptake has occurred, chemical modifications may be requested to increase the half time of intracellular siRNA. Further investigations are needed to fully address this question.

2.2 Sticky siRNAs

Cationic polymers can be used for siRNA delivery. However, siRNA/cationic polymer complex is less stable than complex

formed with DNA plasmid because of the lack of cooperativity and electrostatic binding forces offers by siRNA (a rigid two-turn double helix). The major consequence of these stability differences is unwanted exchange with large polyanions found outside cells. We proposed to concatemerize siRNA «mimicking a gene, a long double stranded nucleic acid», *via* short complementary A_n/T_n 3'-overhangs to increase the stability of complex (Figure 3). Concatemerization is therefore reversible and restricted to within the complex and siRNAs are released as non-oligomerized in the cytoplasm [12].

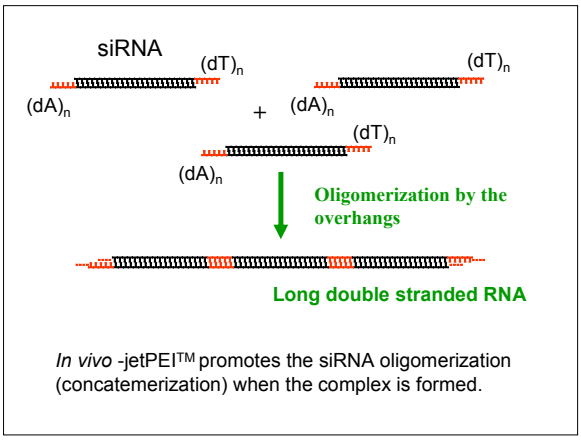


Figure 3: Concatemers formation with sticky siRNA is promoted during the siRNA complexation with *in vivo*-jetPEI™.

The effect of concatemer formation on gene silencing efficiency was compared between sticky $(dA)_5$-siRNA-$(dT)_5$ and standard $(dT)_2$-siRNA-$(dT)_2$, using the GL3 luciferase gene stably expressed by A549 epithelial cells (Figure 4).
Sticky small interfering RNAs (ssiRNAs) enhance gene silencing *in vitro* up to tenfold when complexed with *in vivo*-jetPEI™ compared to standard siRNA [12].

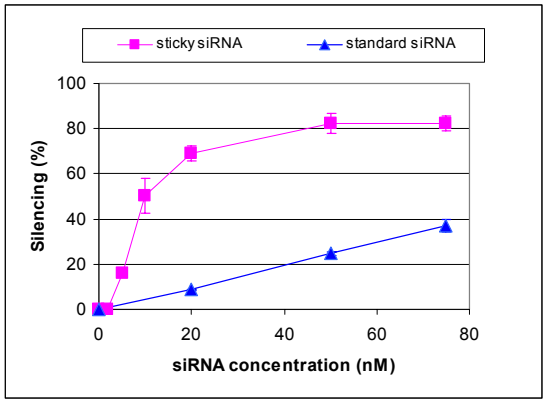


Figure 4: Endogeneous luciferase silencing with sticky siRNAs complexed with *in vivo*-jetPEI™. A549-GL3Luc cells were incubated with $(dA)_5$-siRNA-$(dT)_5$ and standard $(dT)_2$-siRNA-$(dT)_2$ complexed with *in vivo*-jetPEI™ (N/P=5). Luciferase silencing efficiency was determined after 48 h.

Sticky siRNAs would be especially interesting for use with linear PEI *in vivo* by restoring the original delivery power of PEI well-known for gene transfer. Preliminary experiments showed increased silencing efficiencies of sticky siRNAs versus standard siRNAs in the mouse lungs when administrated complexed with *in vivo*-jetPEI™ [12].

2.3 Cationic lipid-based formulations

Cationic lipid-based formulations are probably the most potent class of siRNA delivery vector *in vitro* because they are able to release siRNA directly in the cytoplasm while the lipid molecules remain associated to the membrane of endosomes, according to their mechanism of action [13]. However, generation of cationic lipid system fully adapted to the RNAi requirements was needed in order to limit toxicity, side effects, off-target effects and decrease the level of active siRNA into cells at picomolar concentration.
We have developed a transfection reagent, INTERFERin™, which fullfils these requirements for *in vitro* use. As shown in the Figure 5, transfection with INTERFERin™ results in high gene knockdown using picomolar siRNA concentrations.

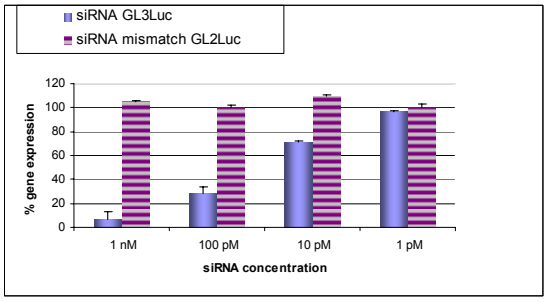


Figure 5: Gene silencing using picomolar concentration of siRNA.
A549-GL3Luc cells were transfected with decreasing concentrations of GL3Luc siRNA or mismatch GL2Luc siRNA duplexes using INTERFERin™. Luciferase gene expression was measured 48 h post-transfection.

Formulations based on INTERFERin™ are currently developed for *in vivo* use, by incorporating compounds needed to avoid aggregation of cationic liposomes, increase stability and extend its circulation time into the blood.

Another formulation of cationic lipids was found efficient for gene silencing in newborn mouse brain [14]. Combining jetSI™ (a mixture of cationic lipids) with a 2 molar excess of the fusogenic lipid DOPE produced a silencing effect of over 80% of co-transfected *Photinus pyralis* luciferase. A variety of stringent controls showed that the silencing was target gene specific and the control mutated siRNA to be without effect. Most importantly the effect was dose-dependent, with 80% silencing being obtained with picomolar amounts of siRNA .

3. CONCLUSIONS AND FUTURE DIRECTIONS

The futures directions are now to exploit RNAi *in vivo* for functional genomic studies and therapeutic applications. This challenge will require addressing many pharmacological aspects including pharmacokinetics, pharmacodynamics, immune response and target-tissue bio-availability. Most of theses aspects can be resolved by effective and targeted delivery systems based on non-viral vectors. In gene delivery, some of them have shown *in vivo* potentialities when administrated via intracerebral injection, via portal vein injection for the liver, by nasal instillation for the lung, or after intratumoral injection for some tumor models.

The possibility of delivering siRNA in animals by many routes with jetPEI™ for example is promising both for targeting different organs or tumours and developing different therapeutic approaches. Based on our accumulated experience in polynucleotide delivery and the different delivery systems that we propose, it is reasonable to predict a promising future for at least one of our systems as carrier of therapeutic siRNAs.

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