

# siRNA delivery by a transferrin-associated lipid-based vector: a non-viral strategy to mediate gene silencing

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## Abstract

**Background** RNA interference provides a powerful technology for specific gene silencing. Therapeutic applications of small interfering RNA (siRNA) however require efficient vehicles for stable complexation, protection, and extra- and intracellular delivery of these nucleic acids. Here, we evaluated the potential of transferrin (Tf)-associated liposomes for siRNA complexation and gene silencing.

**Methods** Cationic liposomes composed of DOTAP:Cholesterol associated with or without transferrin (Tf) were complexed with siRNA at different lipid/siRNA charge ratios. Complexation and protection of siRNA from enzymatic degradation was assessed with the PicoGreen intercalation assay and gel electrophoresis. Cellular internalization of these siRNA Tf-lipoplexes was detected by confocal microscopy. Luciferase assay, immunoblot and fluorescence-activated cell sorting (FACS) analysis were used to evaluate reporter gene silencing in Huh-7 hepatocarcinoma and U-373 glioma cells. c-Jun knockdown in HT-22 cells was evaluated by quantitative real-time polymerase chain reaction (RT-PCR). Cytotoxicity of the siRNA complexes was assessed by Alamar blue, lactate dehydrogenase and MTT assays.

**Results** Complexation of siRNA with the cationic liposomes in the presence of Tf results in the formation of stable particles and prevents serum-mediated degradation. Confocal microscopy showed fast cellular internalization of the Tf-lipoplexes via endocytosis. In the GFP glioma cells Tf-lipoplexes showed enhanced gene silencing at minimum toxicity in comparison to Tf-free lipoplexes. Targeting luciferase in the hepatocarcinoma cell line resulted in more than 70% reduction of luciferase activity, while in HT-22 cells 50% knockdown of endogenous c-Jun resulted in a significant protection from glutamate-mediated toxicity.

**Conclusions** Cationic liposomes associated with Tf form stable siRNA lipoplexes with reduced toxicity and enhanced specific gene knockdown activity compared to conventional lipoplexes. Thus, such formulations may constitute efficient delivery systems for therapeutic siRNA applications. Copyright © 2007 John Wiley & Sons, Ltd.

**Keywords** cationic liposomes; transferrin; siRNA delivery; serum protection; gene silencing; c-Jun; glutamate toxicity

## Introduction

RNA interference (RNAi) is an important biological process and a useful tool to modulate gene expression with implications in several fields, such as functional genomics, drug validation, and even transgenic design [1–4]. The discovery of RNAi has generated enthusiasm within the scientific community and its application for therapeutic purposes has been tested recently in several disease models that include viral infection [5–7], cancer [8–11], acute hepatitis [12,13], and dominantly inherited genetic disorders [14–16].

Sequence-specific post-transcriptional gene silencing is mediated by small interfering RNAs (siRNAs), about 21–23 nucleotides long, that originate from long double-stranded (ds)RNAs of endogenous or exogenous origin, after cleavage by a type III ribonuclease named DICER [17]. siRNA fragments are incorporated into a nuclease complex called RISC (RNA-induced silencing complex) where the RNA is unwound and one of the strands is released [18]. The active RISC complex is responsible for targeting and cleaving mRNA that is complementary to the siRNA [19].

RNAi holds a pronounced therapeutic potential, since application of siRNA may allow highly specific targeting of selected proteins without the common adverse effects of less specific drug-based therapies or the dangers of viral gene therapy. In addition, double-stranded siRNA is more stable and allows effective knockdown at lower concentrations and for longer time periods compared to single-stranded oligonucleotides used in antisense technology [20]. In addition, RNAi acts primarily in the cytoplasm, which is easier to access than the nucleus, thus avoiding one of the major obstacles of plasmid DNA delivery – the nuclear barrier. Nevertheless, the development of RNAi-based therapeutics faces some of the same challenges which have delayed the successful application of conventional gene therapy, including the design of suitable vectors able to cross the gap between cell culture and animal models, thus allowing an efficient and stable siRNA delivery *in vivo*. Currently, chemically synthesized siRNA is most effectively introduced into cells using electroporation [21,22] or commercially available lipid reagents, but these methods are poorly suited for *in vivo* delivery, due to their lack of efficiency or significant non-specific gene silencing [23]. So far, the vast majority of *in vivo* studies employing RNA interference technology have used high doses of non-modified naked siRNAs, usually delivered through hydrodynamic injection [24]. Although promising, this method has raised several safety concerns associated with the high siRNA dose necessary to achieve efficient silencing. Recent studies suggest that high shRNA concentrations can overwhelm the endogenous machinery responsible for siRNA and miRNA mediated silencing, and then induce considerable cytotoxic effects [25]. Although no such concerns have been reported regarding siRNAs, low concentrations of both siRNA and delivery agents should be preferred, in

order to avoid activation of the mammalian immune system [26–28]. In this context, cationic liposome carriers can help to significantly reduce necessary siRNA doses for efficient gene silencing by enhancing siRNA stability in the serum and improving cellular uptake. These lipid-based formulations also present a high degree of versatility that allows modifications which result in a significant increase of their targeting specificity and transfection capacity [29–32]. In this regard, DOTAP:Cholesterol/DNA complexes have been widely explored by us for both *in vitro* [30,33] and *in vivo* [29] gene therapy applications, mainly due to their capacity to mediate transfection in the presence of serum. The resistance to inhibition by serum exhibited by this formulation is related to the presence of cholesterol, a helper lipid, that can potentially reduce undesired interactions of lipoplexes with serum proteins, hence increasing their stability in biological systems [34–37].

Transfection activity can be enhanced by the association of targeting ligands such as antibodies, peptides or proteins to the complexes, which exploit the diversity of receptors existent at the surface of each cell type [31,32,38]. One useful cell-binding ligand is transferrin (Tf), an iron-transporting protein, which interacts with receptors ubiquitously expressed in various tissues [39]. Targeting Tf receptors has been successfully applied for DNA delivery by using antibodies against these receptors [40,41], by linking the ligand to pegylated liposomes [42], or by associating the protein to cationic liposomes through electrostatic interactions [32,38,43]. In this regard, we and others have recently demonstrated that association of Tf to lipoplexes significantly enhances transfection efficiency by promoting lipoplex internalization in a large variety of cells, including dividing and non-dividing cells, e.g. neurons [29,30,32,38,43–45]. In addition, we have reported that Tf can trigger cytoplasmic delivery of the carried nucleic acids through destabilization of the endosomal membrane under acidic conditions, thus further improving the transfection process [32,33].

However, despite the widespread use of protein-associated cationic liposomes, there are still very few reports concerning their application for siRNA delivery. The present study aims to show that Tf-lipoplexes, well known for their ability to deliver DNA *in vitro* and *in vivo*, can also be optimized for siRNA delivery, resulting both in efficient levels of intracellular siRNA and promising gene silencing effects. We applied the optimized Tf-lipoplexes to transfer siRNA targeting stably expressed reporter genes such as the green fluorescent protein (GFP) and firefly luciferase in U-373 glioblastoma and Huh-7 hepatocarcinoma cells, as well as siRNA targeting the endogenous transcription factor c-Jun in HT-22 immortalized mouse hippocampal neurons. Since c-Jun plays a key role in neuronal cell death in a wide variety of injury models relevant to neurodegenerative diseases [46], we finally addressed the therapeutic potential of c-Jun siRNA-Tf-lipoplexes against glutamate toxicity in the HT-22 neuronal cell line.

## Materials and methods

### Materials

The cationic lipid 1,2 dioleoyl-3(trimethylammonium) propane (DOTAP) and cholesterol (Chol) were purchased from Avanti Polar Lipids (Alabaster, AL, USA). Iron-saturated human transferrin (Tf) was obtained from Sigma (St. Louis, MO, USA). The anti-GFP siRNA (5'-GCAAGCUGACCCUGAAGUUCAU-3') and Cy3-labeled non-specific siRNA sequence were purchased from Ambion (Austin, TX, USA). The anti-luciferase siRNA (luciferase G12 duplex) and the non-silencing siRNA used as control were obtained from Dharmacon (Lafayette, CO, USA). The anti-c-Jun siRNA (5'-AGTCATGAACCACGTTAAC-3') was obtained from Thermo Byoproduts. All the other chemicals were obtained from Sigma unless stated otherwise.

### Liposome and complex preparation

Cationic liposomes composed of DOTAP : Chol (1 : 1 molar ratio) were prepared as previously described by Campbell [47]. Briefly, a mixture of the appropriate amounts of lipids, from stock solutions in chloroform, was dried under nitrogen in order to obtain a thin lipid film. The film was dissolved in 100  $\mu$ l of ultrapure ethanol and the resulting ethanol solution was injected in 900  $\mu$ l of HBS buffer (Hepes-buffered saline solution, 20 nM Hepes, 100 mM NaCl, pH 7.4) continuously maintained under vortex, employing a 250  $\mu$ l Hamilton syringe. The resulting MLV (multilamellar vesicles) were sonicated briefly to obtain SUV (small unilamellar vesicles), diluted with HBS to a final lipid concentration of 1 mg/ml, and stored at 4 °C until use.

Cationic liposome/siRNA complexes were prepared by sequentially mixing 100  $\mu$ l of HBS buffer with liposomes (whose volume was dependent on the desired (+/-) charge ratio) and with 10  $\mu$ l of a 5 pmol/ $\mu$ l siRNA solution. The mixture was further incubated for 15 min at room temperature.

Tf-lipoplexes were prepared by pre-incubating cationic liposomes with iron-saturated human Tf (32  $\mu$ g/ $\mu$ g of siRNA) for 15 min, before mixing 10  $\mu$ l of siRNA solution and further incubating the mixture for 15 min, at room temperature. All formulations were used immediately after preparation.

### PicoGreen intercalation assay

The accessibility of the fluorescent intercalating probe PicoGreen (Molecular Probes, Carlsbad, CA, USA) to the siRNA associated with the cationic liposomes was evaluated at 37 °C, for 2 min, in a SPEX Fluorolog spectrometer (SPEX Industries, Edison, NJ, USA). Briefly, PicoGreen fluorescence was detected at excitation and emission wavelengths of 480 and 520 nm, respectively.

The sample chamber was equipped with a stirring device and the temperature was controlled with a feedback-regulated circulating water bath. The fluorescence scale was calibrated such that the initial fluorescence of PicoGreen (1 ml of 1 : 200 solution added to the cuvette containing 1 ml of HBS) was set as residual fluorescence. The value of fluorescence obtained upon addition of 400 ng of siRNA (control) minus residual fluorescence was set as 100%. Plain complexes or Tf-lipoplexes, containing 400 ng of siRNA in 1 ml HBS buffer, were added to the cuvette containing 1 ml of PicoGreen solution. The amount of siRNA available to interact with the probe was calculated by subtracting the values of residual fluorescence (PicoGreen without siRNA) from those obtained for each measurement, and expressed as a percentage of the control that contained naked siRNA only, according to the following formula: % free siRNA = (PicoGreen fluorescence<sub>complexes</sub>/PicoGreen fluorescence<sub>naked siRNA</sub>)  $\times$  100. The proper controls were performed in order to exclude a possible non-specific decrease in PicoGreen fluorescence due to the presence of lipid or protein.

### Protection from RNase and serum-mediated degradation

Resistance of complexes to RNase and serum-mediated degradation were determined by electrophoresis and ethidium bromide staining. Complexes containing 500 ng of siRNA were submitted to 30 min incubation at 37 °C, in the presence of 0.1 U/ $\mu$ l RNaseV1 (Ambion), or to different incubation periods in 50% mouse serum (Jackson Immuno Research Laboratories Inc., West Grove, PA, USA) in HBS buffer. Parallel experiments were performed by incubating the samples under the same experimental conditions in the absence of serum or RNase V1 or in the presence of heat-inactivated mouse serum. After siRNA extraction using Tri-Reagent™ (Sigma) and precipitation overnight at -20 °C, the resuspended samples were analyzed in a 15% polyacrylamide gel for 2 h, applying a voltage of 110 V. The gel was stained for 5 min in ethidium bromide and the intensity of the siRNA bands analyzed using the Versa Doc imaging system (Bio-Rad, Hercules, CA, USA).

### Measurement of particle size and zeta-potential

Particle size of Tf-lipoplexes was measured by laser-light scattering using a Malvern Zetasizer 3000HS (Malvern Instruments, Worcestershire, UK). The Tf-lipoplexes containing 10  $\mu$ g siRNA were prepared at 3/2 or 2/1 lipid/siRNA (+/-) charge ratios and diluted to a final volume of 2 ml with HBS buffer containing 10% serum. For evaluation of the zeta-potential the Tf-lipoplexes were further diluted in 10 mM NaCl to give a final siRNA concentration of 2  $\mu$ g/ $\mu$ l. Zeta-potential and

particle size of the formulations were determined in at least two independent experiments, each with five or ten independent measurements, respectively.

### Generation of U-373/eGFP and Huh-7/eGFPLuc cell lines

For the generation of stably transfected cells, the vectors pEGFP or pEGFPLuc (Clontech, Palo Alto, USA) were linearized using the restriction enzyme Dra III, and U-373 or Huh-7 cells were transfected with the respective linearized vectors using linear polyethylenimine 22 kDa (PEI22) polyplexes generated in HEPES-buffered saline + HEPES-buffered glucose 1:1 (HBS1/2: 75 mM NaCl, 2.5% glucose, 20 mM HEPES, pH 7.2) with a PEI nitrogen to DNA phosphate (N/P) ratio of 6/1. Cells were incubated in fresh medium for 72 h and then selected with 60 to 400  $\mu\text{g}/\text{ml}$  G418 (geneticin; Invitrogen, Karlsruhe, Germany). After several days, surviving cells were seeded at low densities into six-well plates in order to generate separate colonies. Single cell clones were then isolated and expanded. The generated clones were analyzed for the percentage of GFP-positive (eGFPLuc stably transfected) cells. Clones with the highest amount of GFP-positive cells were then further selectively grown under the above described selective conditions and this procedure was repeated until all cells were positive for GFP. These stably transfected U-373/eGFP or Huh-7/eGFPLuc cells were then used for siRNA delivery experiments.

### Cell culture and siRNA delivery

U-373 cells (glioblastoma-derived cell line) stably expressing GFP, Huh-7 cells (hepatocarcinoma-derived cell line) stably expressing a GFP-Luc fusion protein, and HT-22 cells (immortalized mouse hippocampal neurons [48]) were incubated at 37 °C in an humidified atmosphere containing 5% CO<sub>2</sub>. Both U-373 and HT-22 cell lines were maintained in DMEM (Dulbecco's modified Eagles's medium) (Sigma), while Huh-7 cells were maintained in DMEM/Ham's F12 (GIBCO, Paisley, Scotland). Both culture media were supplemented with 10% heat-inactivated fetal bovine serum (FBS) (GIBCO), 100  $\mu\text{g}/\text{ml}$  streptomycin and 1 U/ml penicillin. In the case of the U-373 cell line, DMEM was also supplemented with 24  $\mu\text{l}/\text{ml}$  of the selection antibiotic Geneticin® (GIBCO) for a final concentration of 50  $\mu\text{g}/\text{ml}$ .

Twenty-four hours before any experiment, U-373, Huh-7 and HT-22 cells were plated at a density of 25 000 cells/cm<sup>2</sup>. Immediately before addition of the complexes, the cells were washed and the medium was replaced with fresh medium, and 100  $\mu\text{l}$  of plain or Tf-lipoplexes prepared at different lipid/siRNA (+/–) charge ratios were added to the cells to a final siRNA concentration of 1  $\mu\text{M}$ . As a positive control for siRNA delivery, Lipofectamine 2000 (Invitrogen, Carlsbad, CA,

USA) diluted in OPTIMEM (GIBCO) was used according to the manufacturer's instructions. In addition, a non-silencing siRNA sequence was used in parallel with the anti-GFP, anti-luciferase or anti-c-Jun sequences, in order to ensure that the decrease in protein levels was attributable to the selected siRNA and not to the vector *per se*. After 4 h of incubation the medium was replaced and the cells were further incubated for different periods of time, before evaluation of siRNA transfection.

### Epifluorescence and confocal microscopy studies

Following siRNA transfection, the number of GFP-positive cells was determined by epifluorescence microscopy using a Axiovert 200 microscope (Zeiss, Thornwood, NY, USA), equipped with the 20 $\times$  objective. Briefly, 48 h after complex delivery, U-373 cells were washed with phosphate-buffered saline (PBS), fixed with 4% paraformaldehyde for 15 min at room temperature, stained with the fluorescent DNA-binding dye Hoechst 33342 (1  $\mu\text{g}/\text{ml}$ ), rinsed again with PBS and mounted in Vecta Shield mounting medium (Vecta Laboratories Inc., Burlingame, CA, USA) for subsequent analysis. For each experimental condition, the number of blue nuclei and green cells was counted in nine separate coverslips (an average of 300 cells per coverslip) and results were expressed as the percentage of GFP-positive cells of total cell number per field. In addition, for analysis of cellular uptake of siRNA and Tf-lipoplexes, Huh-7 cells were incubated for 4 h with naked or complex-associated Cy3-labeled siRNAs. Following incubation, the cells were washed and mounted in PBS and immediately visualized by confocal microscopy. All observations were made using a Axiovert confocal scanning microscope (Zeiss, Jena, Germany), under the 60 $\times$  oil immersion objective.

### Fluorescence-activated cell sorting (FACS) analysis

Flow cytometric data were obtained in a Cyan™ MLE flow cytometer (DaKoCytomation, Copenhagen, Denmark). Seventy-two hours after siRNA delivery, U-373 cells were washed with PBS and treated with trypsin (10 min, 37 °C) to allow detachment from the wells. After trypsin inactivation with medium, cells were centrifuged at 1000 rpm and the supernatant was removed. The remaining pellet was resuspended in 600  $\mu\text{l}$  PBS with 4% paraformaldehyde and placed on ice until further analysis. To discriminate between viable and dead cells and to exclude doublets, cells were appropriately gated by forward/size scatter and pulse width and  $1 \times 10^4$  gated events were collected per sample. The FITC bandpass filter was used in emission detection. Tf-complex-mediated GFP silencing was expressed as percentage of GFP mean fluorescence in controls.

## Western blot analysis

Forty-eight hours after siRNA delivery, protein extracts were obtained from U-373 cells using a lysis buffer containing protease inhibitors (Sigma). Protein content was determined using the Bio-Rad protein quantification kit and 20 µg of total protein were resuspended in loading buffer (20% glycerol, 10% sodium dodecyl sulfate (SDS), 0.1% bromophenol blue), incubated for 2 min at 95 °C, and loaded onto a 10% polyacrylamide gel. After electrophoresis the proteins were blotted onto a PVDF membrane according to standard protocols. After blocking in 5% non-fat milk, the membrane was incubated with an anti-GFP antibody (1:1000) (Roche, Indianapolis, IN, USA) overnight at 4 °C, and with the appropriate secondary antibody (1:20000) (Amersham, Uppsala, Sweden) for 2 h at room temperature. Equal protein loading was shown by reprobing the membrane with an anti- $\alpha$ -tubulin antibody (1:10000) (Sigma) and with the same secondary antibody. After this incubation period, the blots were washed several times with saline buffer (TBS/T: 25 mM Tris-HCl, 150 mM NaCl, 0.1% Tween and 5 mg/ml non-fat powder milk) and incubated with ECF (alkaline phosphatase substrate; 20 µl of ECF/cm<sup>2</sup> of membrane) for 5 min at room temperature and then submitted to fluorescence detection at 570 nm using a Storm-860 (Molecular Dynamics, CA, USA). For each membrane, the analysis of band intensity was performed using the Quantity One software (Bio-Rad).

## Luciferase activity evaluation

Luciferase gene silencing, mediated by Tf-lipoplexes, was measured in protein extracts from Huh-7 cells using a luciferase detection kit, according to the protocol provided by the manufacturer (Promega, Madison, WI, USA). Briefly, 48 h after delivery of Tf-lipoplexes, cells were lysed with luciferase lysis buffer (Promega) for 15 min at room temperature. Then 20 µl of protein extract were removed from each well and added to 100 µl of luciferase assay buffer, containing luciferin. Luciferase activity, measured as relative light units (RLU), was immediately evaluated in a SpectraFluorPlus luminometer (Tecan, Männedorf, Switzerland) and the data were expressed as percentage of control (untreated cells).

## Cell viability assays

Following siRNA transfection of U-373 cells under the different experimental conditions, cell viability was quantified by a modified Alamar blue assay [49]. The assay measures the redox capacity of cells due to the production of metabolites as a result of cell growth, and allows the determination of viability over the culture period without detachment of adherent cells. Briefly, 1 ml of 10% (v/v) Alamar blue dye in complete DMEM medium was added to each well 24 or 48 h following

the initial incubation period with Tf-lipoplexes (4 h). After 2 h of incubation at 37 °C, 150 µl of supernatant were collected from each well and transferred to 96-well plates. The absorbance at 570 and 600 nm was measured in a microplate reader (SLT Lab instruments Spectra). Cell viability was calculated as percentage of control cells using the formula:  $(A_{570} - A_{600})$  of treated cells  $\times 100 / (A_{570} - A_{600})$  of control cells.

Alternatively, plasma membrane integrity after siRNA transfection was evaluated using the lactate dehydrogenase (LDH) assay. This assay monitors the conversion of NADH into NAD<sup>+</sup> by LDH at 340 nm. The LDH released into the extracellular medium by damaged cells was expressed as a percentage of the total LDH activity in the cells (% LDH released = extracellular LDH / (extracellular LDH + intracellular LDH)).

Viability of HT-22 cells, following delivery of therapeutic (anti-c-Jun) siRNAs and glutamate-induced toxicity, was determined in 96-well plates by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) reduction. Forty-eight hours after Tf-complex delivery, glutamate was added to a final concentration of 2 mM. After 18 h of glutamate exposure, the cells were washed with fresh growth medium and further incubated with MTT (0.25 mg/ml) for 2 h at 37 °C. The reaction was terminated by adding dimethyl sulfoxide solution and the absorbance was determined at 590 and 630 nm in an ELISA microplate reader (Spectra FluorPlus; Tecan, Durham, NC, USA). Cell viability was calculated as percentage of control cells using the formula:  $(A_{590} - A_{630})$  of treated cells  $\times 100 / (A_{590} - A_{630})$  of control cells.

## Extraction of RNA and cDNA synthesis

For each condition, total RNA was extracted from  $1 \times 10^6$  HT-22 cells using the RNeasy Mini kit (Qiagen, Hilden, Germany), according to the manufacturer's recommendations for cultured cells. Briefly, after cell lysis, the total RNA was adsorbed to a silica matrix, washed with the recommended buffers, and eluted with 40 µl of RNase-free water by centrifugation. After RNA quantification, cDNA conversion was performed using the Superscript III First Strand synthesis kit (Invitrogen, Karlsruhe, Germany), according to the manufacturer's instructions. For each sample, cDNA was produced from 0.5 µg of total RNA in an iQ5 thermocycler (Bio-Rad), by applying the following protocol: 10 min at 25 °C, 30 min at 55 °C and 5 min at 85 °C. After cDNA synthesis, a 30 min incubation period with RNase H at 37 °C was performed in order to remove any remaining RNA contamination. Finally, the cDNA was diluted 1:3 with RNase-free water prior to quantification by quantitative real-time polymerase chain reaction (QRT-PCR).

## QRT-PCR

QRT-PCR was performed in an iQ5 thermocycler (Bio-Rad) using 96-well microtiter plates and the iQ SYBR

Green Supermix kit (Bio-Rad). The primers for the target gene (c-Jun) and the two tested house-keeping genes (GADPH and HPRT) were pre-designed by Quia-gen (QuantiTect Primer, Quiagen). A master mix was prepared for each primer set, containing a fixed volume of SYBR Green Supermix and the appropriate amount of each primer, to yield a final concentration of 150 nM. For each reaction, 20  $\mu$ l of master mix were added to 5  $\mu$ l of template cDNA. All reactions were performed in duplicate (two cDNA reactions per RNA sample) at a final volume of 25  $\mu$ l per well, using the iQ5 Optical System software (Bio-Rad). The reaction conditions consisted of: enzyme activation and well-factor determination at 95 °C for 1 min and 30 s followed by 40 cycles at 95 °C for 10 s (denaturation), 30 s at 55 °C (annealing) and 30 s at 72 °C (elongation). The melting curve protocol started immediately after amplification and consisted of 1 min heating at 55 °C followed by 80 steps of 10 s, with a 0.5 °C increase in temperature at each step. Threshold values for threshold cycle determination ( $C_t$ ) were generated automatically by the iQ5 Optical System software. The percentage of c-Jun knockdown was determined by the  $\Delta\Delta C_t$  method, using HPRT as reference gene, according to the formulas:  $\Delta C_{t(c-Jun\ siRNA)} = C_{t(c-Jun\ gene)} - C_{t(HPRT\ gene)}$ ;  $\Delta C_{t(Mut\ siRNA)} = C_{t(c-Jun\ gene)} - C_{t(HPRT\ gene)}$ ;  $\Delta\Delta C_t = \Delta C_{t(c-Jun\ siRNA)} - \Delta C_{t(Mut\ siRNA)}$ ; % knockdown =  $100\% - (2^{-\Delta\Delta C_t} \times 100)$ . The  $\Delta\Delta C_t$  value indicates the changes in RNA transcription caused by treatment with anti-c-Jun siRNAs, normalized to RNA transcription changes in cells treated with Mut siRNAs. When positive, the  $\Delta\Delta C_t$  value indicates downregulation of the target mRNA.

## Statistical analysis

All data are presented as means  $\pm$  standard deviation (SD) of at least three independent experiments, each performed at least in triplicate. One way analysis of variance (ANOVA) combined with the Tukey posthoc test was used for multiple comparisons in cell culture experiments. Statistical differences are presented at probability levels of  $p < 0.05$ ,  $p < 0.01$  and  $p < 0.001$ .

## Results

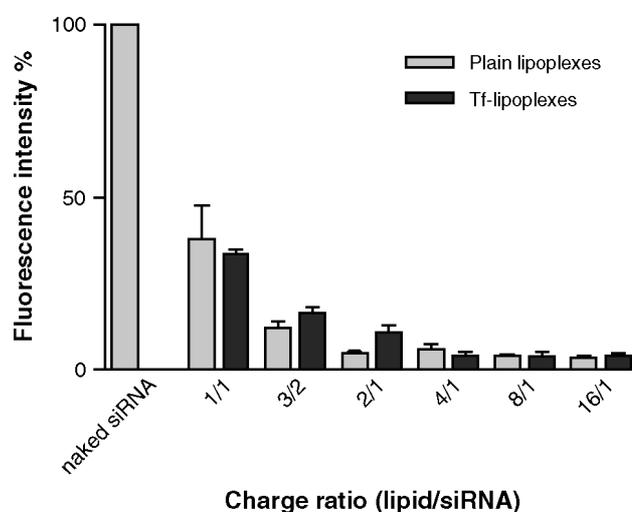
### Characterization of cationic liposome/siRNA lipoplexes

The PicoGreen intercalation assay was applied for monitoring complex formation of siRNA and cationic liposomes, composed of DOTAP and cholesterol at increasing lipid/siRNA charge ratios and in the presence or absence of transferrin (Tf).

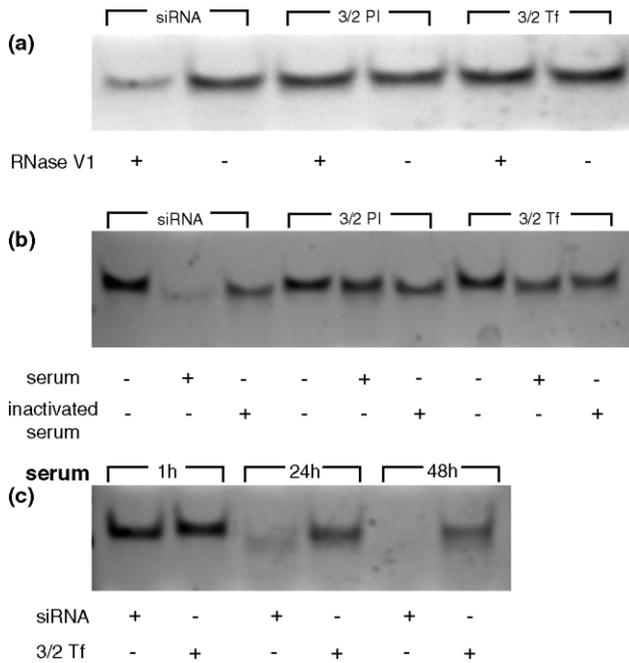
As shown in Figure 1, a significant quenching of the PicoGreen fluorescence was achieved at the lipid/siRNA charge ratio of 3/2 (+/-), this being almost complete for higher charge ratios, which indicates a very high

degree of siRNA complexation (approximately 90%) by cationic liposomes, in the absence or presence of Tf. These results demonstrate that complexes can be formed through electrostatic interactions between siRNA and cationic liposomes, although an excess of cationic lipid is required to ensure efficient nucleic acid protection. It is interesting to note that association of Tf to the cationic liposomes does not result in a significant increase of PicoGreen fluorescence over that observed for plain lipoplexes, which suggests that its presence does not lead to complex destabilization.

To examine stable binding and protection of siRNA in the lipoplexes under conditions that can mimic those *in vivo*, further measurements were performed after incubating both plain and Tf-lipoplexes in the presence of RNase V1 (0.1 U/ $\mu$ l) (Figure 2a) or mouse serum (Figures 2b and 2c). Following a 30 min or 4 h incubation period, respectively, the siRNA present in the mixtures was recovered and analyzed in a polyacrylamide gel stained with ethidium bromide. Both plain and Tf-lipoplexes significantly protected siRNA from RNase V1 and serum-mediated degradation, whereas naked siRNA was almost completely degraded, in both cases, after a short incubation period (Figures 2a and 2b). These results clearly indicate that the presence of cationic lipid is crucial to confer protection of siRNA molecules against serum nucleases. The observation that naked siRNA samples remained almost intact upon incubation with heat-inactivated serum suggests that the degradation observed in the presence of serum was of enzymatic origin. Moreover, results from parallel experiments carried out with longer incubation periods in the presence of serum (Figure 2c) indicate that the protection effect mediated



**Figure 1.** siRNA binding in plain or Tf-associated lipoplexes. DOTAP:Chol liposomes were complexed with 400 ng of siRNA in the presence or absence of transferrin (Tf) followed by incubation with the fluorescent probe PicoGreen. Relative fluorescence is presented as percentage of the control obtained with 400 ng naked siRNA. The percentage represents the fraction of siRNA in the sample available for PicoGreen interaction. Results are presented as mean values  $\pm$  SD from triplicates and are representative of three independent experiments



**Figure 2.** Stability of siRNA in plain or Tf-associated lipoplexes exposed to RNase V1 and mouse serum. Plain lipoplexes or Tf-lipoplexes containing 500 ng siRNA were incubated with RNase V1 or complete mouse serum. After the indicated incubation periods, siRNA was extracted and analyzed by electrophoresis in a 15% polyacrylamide gel and subsequent staining of the gel with ethidium bromide was performed. Naked siRNA, plain lipoplexes or Tf-lipoplexes prepared at a 3/2 lipid/siRNA charge ratio were incubated for 30 min in the presence or absence of (a) RNase V1 (0.1U/ $\mu$ l) or for 4 h (b) in normal or heat-inactivated mouse serum. (c) In parallel experiments, naked siRNAs and Tf-lipoplexes were incubated for 1 h, 24 h and up to 48 h in the presence or absence of mouse serum. The results are representative of three independent experiments

by Tf-lipoplexes is still present after 48 h of exposure to serum, which illustrates the high stability of these complexes over days.

In order to further characterize Tf-lipoplexes, zeta-potential and particle size of the two most promising formulations (3/2 and 2/1 lipid/siRNA (+/-) charge ratios) were evaluated in the presence of serum. Although

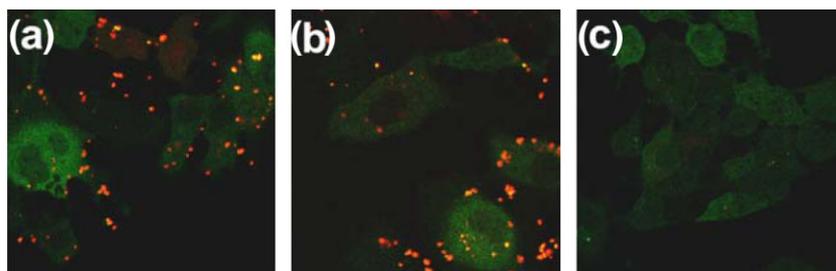
both formulations presented a negative surface charge, i.e.  $-27.5 \pm 3.1$  mV and  $-7.0 \pm 4.8$  mV at charge ratios of 3/2 and 2/1, respectively, Tf-lipoplexes prepared at the 2/1 charge ratio exhibited a zeta-potential value very close to neutrality, which may contribute to the significant difference observed in the size of both formulations. The negatively charged Tf-lipoplexes, prepared at the 3/2 charge ratio, exhibited a size of  $328 \pm 64$  nm, whereas the nearly neutral particles, prepared at the 2/1 charge ratio, were significantly larger, i.e.  $926 \pm 47$  nm. This may be attributed to reduced electrostatic repulsions at charge ratios of 2/1 with a consequent increase in particle aggregation compared to the negatively charged 3/2 lipoplexes, which may contribute to differences in siRNA delivery and biological activity.

### Cellular uptake of fluorescently labeled siRNAs

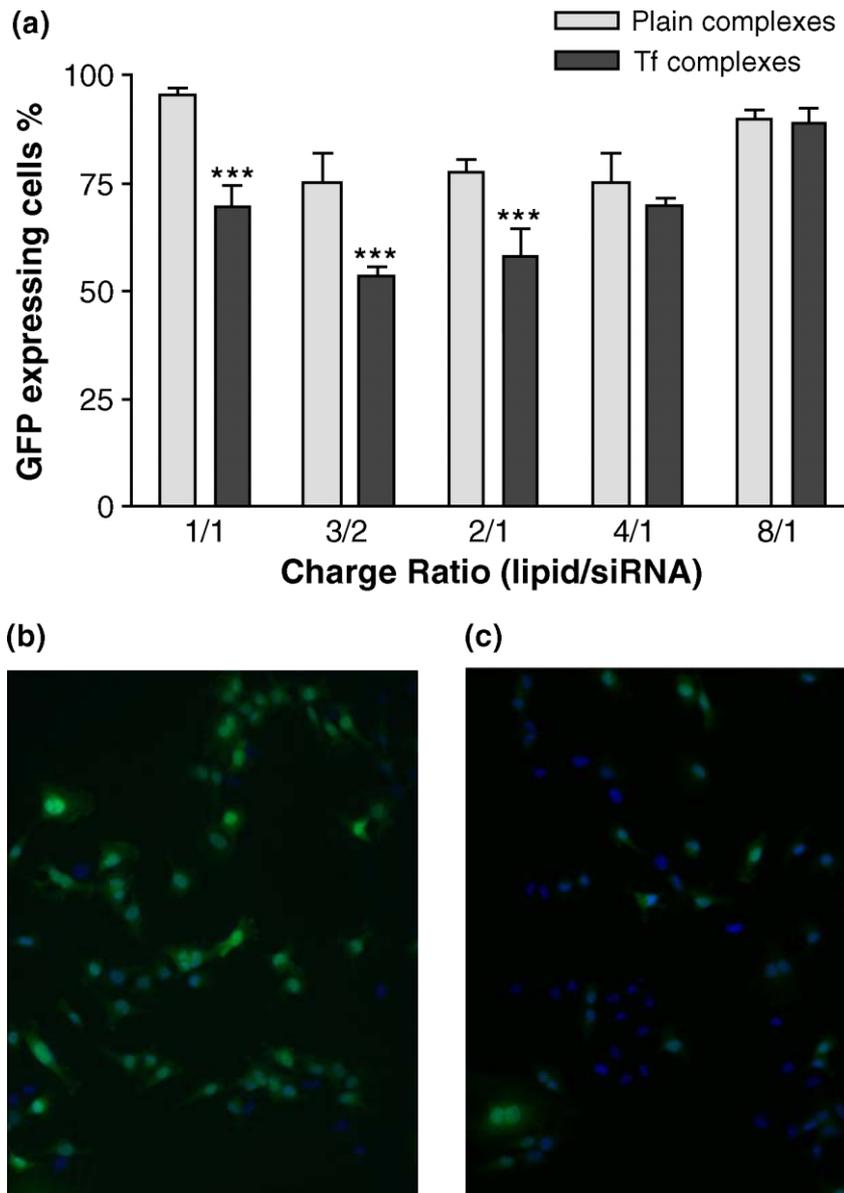
The internalization of Tf-lipoplexes was monitored in Huh-7 cells by confocal microscopy, using Cy3-labeled non-silencing siRNA. The results presented in Figure 3 reveal that 4 h after the delivery of Cy3-labeled Tf-complexes, prepared at 2/1 and 4/1 lipid/siRNA charge ratios (Figures 3a and 3b, respectively), fluorescent particles were found to be located within very large intracellular vesicles. In contrast, the delivery of a similar amount of naked Cy3-labeled siRNAs (Figure 3c) did not allow detection of red fluorescence within the cells or even in the extracellular medium, suggesting that siRNA degradation or removal upon washing occurs during the incubation period with the cells.

### Gene silencing efficiency

It has been demonstrated that Tf is highly effective in promoting lipoplex-mediated transfection in various cell lines and primary cell cultures [30,32,38,43]. Therefore, we evaluated whether this strategy would also result in an enhancement of siRNA transfer efficiency. Figure 4 illustrates the percentage of GFP-expressing U-373 cells,



**Figure 3.** Uptake of naked or Tf-lipoplex associated Cy3-siRNAs in Huh-7 cells. Huh-7 cells were plated 1 day before siRNA delivery and rinsed with DMEM supplemented with 10% FBS immediately before delivery of Tf-lipoplexes. DOTAP:Chol liposomes were pre-incubated in the presence of Tf (32  $\mu$ g/ $\mu$ g siRNA), complexed with 50 pmol of Cy3-labeled non-silencing siRNA at 2/1 or 4/1 lipid/siRNA (+/-) charge ratios, and added to the cells for 4 h. In parallel experiments, cells were incubated with the same amount of siRNAs in the absence of liposomes for the same period. After 4 h, the cells were rinsed twice with PBS and immediately observed by confocal microscopy. The panels show representative images at 600 $\times$  magnification of cells treated with (a) 2/1 lipid/siRNA Tf-lipoplexes, (b) 4/1 lipid/siRNA Tf-lipoplexes, and (c) naked Cy3-siRNA. Results are representative of two independent experiments

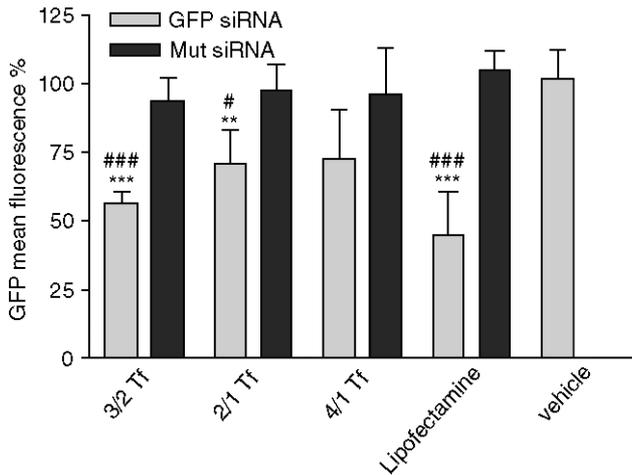


**Figure 4.** Effect of transferrin association to DOTAP:Chol liposomes on gene silencing in U-373 cells. U-373 cells stably expressing GFP were plated 1 day before siRNA delivery and rinsed with DMEM containing 10% FBS immediately before complex delivery. DOTAP:Chol liposomes pre-incubated in the presence or absence of Tf (32  $\mu\text{g}/\mu\text{g}$  siRNA) were complexed with 50 pmol of anti-GFP siRNA at the indicated lipid/siRNA charge ratios and added to the cells to a final siRNA concentration of 100 nM. After 4 h incubation, the medium was replaced with fresh medium and the cells were incubated for a further 48 h before evaluation of GFP knockdown by epifluorescence microscopy. (a) The number of GFP-expressing cells is presented as percentage of untreated controls. The data are given as mean values  $\pm$  SD of triplicates and are representative of three independent experiments. \*\*\* $p < 0.001$  compared to plain lipoplexes. Representative images of (b) control cells (200 $\times$ ) or (c) cells treated with Tf-lipoplexes prepared at 3/2 lipid/siRNA charge ratio (200 $\times$ )

48 h after delivery of plain or Tf-lipoplexes prepared at different lipid/siRNA charge ratios. Association of Tf to the lipoplexes resulted in a significant enhancement of GFP knockdown at low lipid/siRNA (+/–) charge ratios, the best results being observed with Tf-lipoplexes prepared at a charge ratio of 3/2.

In order to establish the average levels of protein knockdown mediated by Tf-lipoplexes at single cell level, GFP silencing in U-373 cells was also evaluated using FACS analysis (Figure 5). Tf-lipoplexes, prepared at different charge ratios and containing anti-GFP siRNA or a

non-silencing sequence (Mut siRNA), were incubated with the cells and, 72 h following delivery, the mean intensity of GFP fluorescence was analyzed for each condition. The values corresponding to the percentage of GFP mean fluorescence were compared to those obtained using the standard commercial lipid-based reagent, Lipofectamine 2000, which is frequently employed for *in vitro* DNA plasmid transfection and siRNA delivery. As illustrated in Figure 5, approximately 45% decrease in GFP levels was observed following siRNA delivery using Tf-lipoplexes prepared at a charge ratio of 3/2, a result very similar to

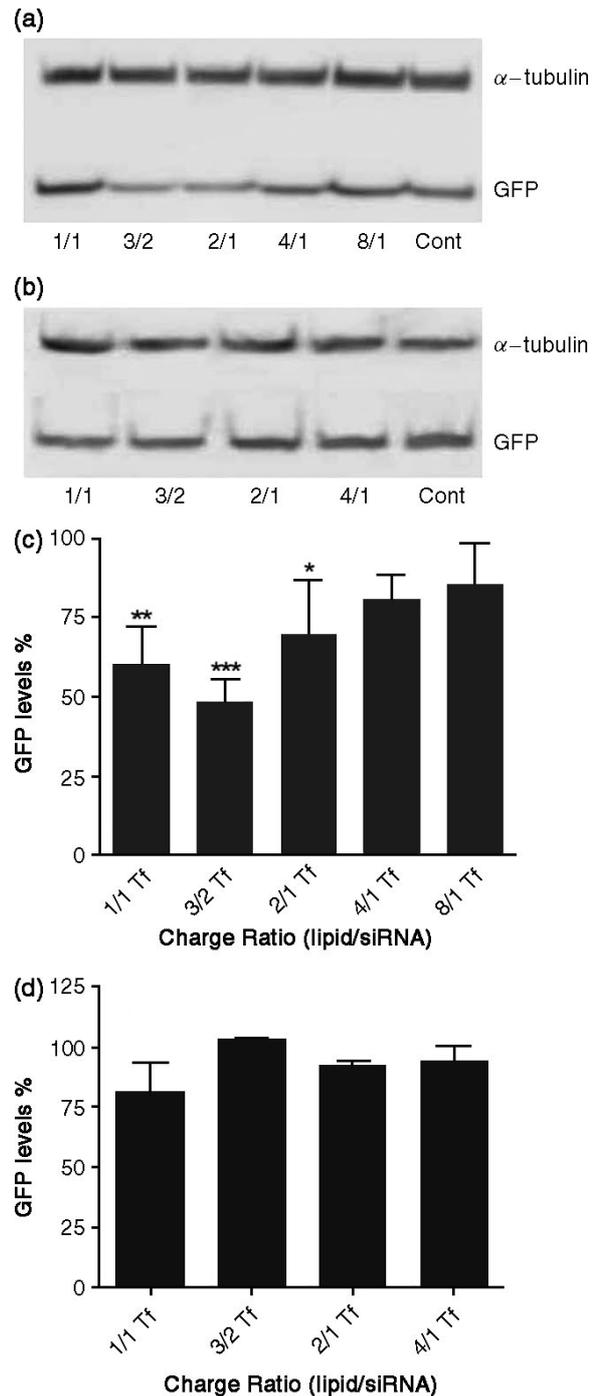


**Figure 5.** Effect of lipid/siRNA charge ratio of Tf-lipoplexes on gene silencing in U-373 cells. U-373 cells stably expressing GFP were plated 1 day before siRNA delivery and rinsed with DMEM containing 10% FBS immediately before delivery of the complexes. DOTAP:Chol liposomes were pre-incubated in the presence of Tf (32  $\mu\text{g}/\mu\text{g}$  siRNA) and then complexed with 50 pmol of anti-GFP or non-silencing (Mut) siRNA, at the indicated lipid/siRNA charge ratios, and added to the cells to a final siRNA concentration of 100 nM. After 4 h incubation, the medium was replaced and the cells were incubated for a further 72 h. In parallel experiments, cells were incubated with the same amount of siRNA complexed with Lipofectamine 2000 or with DOTAP:Chol liposomes associated to Tf in the absence of siRNA (vehicle). The results were obtained by flow cytometry 72 h after siRNA delivery. Mean GFP fluorescence levels in a population of 10 000 events/condition are expressed as percentage of untreated controls (mean  $\pm$  SD obtained from triplicates) and are representative of three independent experiments. \*\* $p < 0.01$ , \*\*\* $p < 0.001$  compared to controls, and # $p < 0.05$ , ### $p < 0.001$  compared to cells treated with Mut siRNA at the same lipid/siRNA charge ratio

the one obtained with Lipofectamine 2000. In contrast, DOTAP:Chol liposomes associated with Tf (vehicle) or Tf-lipoplexes containing non-functional siRNA (Mut siRNA) did not decrease GFP fluorescence levels, ruling out non-specific effects due to the vector *per se* or to the siRNA at the tested concentration.

The efficiency of siRNA-mediated gene silencing was confirmed by Western blot analysis employing both anti-GFP siRNA (Figure 6a) and Mut siRNA (Figure 6b). As shown in Figure 6c, among the different lipid/siRNA charge ratios tested, the most pronounced gene silencing (a mean decrease of 50% in GFP levels) was achieved after delivery of anti-GFP siRNA mediated by Tf-lipoplexes prepared at a 3/2 charge ratio, demonstrating that this is indeed the optimal charge ratio for DOTAP:Chol Tf-lipoplexes in U-373 cells. Gene silencing was found to be specific, since no GFP knockdown was observed with Mut siRNA (Figure 6d) and the levels of  $\alpha$ -tubulin were constant for all tested formulations.

In order to evaluate whether the gene silencing effect observed after siRNA delivery mediated by Tf-lipoplexes was not restricted to U-373 cells, parallel transfection experiments were performed in hepatocarcinoma cells (Huh-7), stably transformed to express a luciferase-GFP



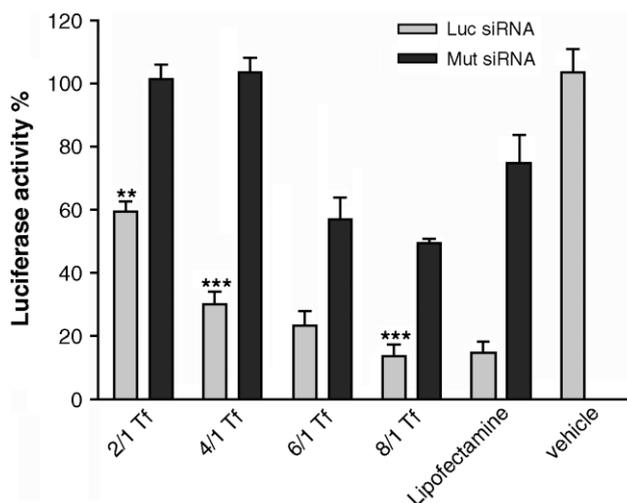
**Figure 6.** Western blot quantification of Tf-lipoplex-mediated GFP knockdown in U-373 cells. U-373 cells were plated 1 day before siRNA delivery and rinsed with DMEM supplemented with 10% FBS immediately before complex delivery. DOTAP:Chol liposomes were pre-incubated in the presence of Tf (32  $\mu\text{g}/\mu\text{g}$  siRNA), complexed with 50 pmol siRNA at the indicated lipid/siRNA charge ratios, and added to the cells to a final siRNA concentration of 100 nM. After 4 h incubation, the medium was replaced with fresh DMEM containing 10% FBS. After a further 48 h, GFP and  $\alpha$ -tubulin levels were quantified after immunoblotting of protein extracts obtained from U-373 cells treated with Tf-lipoplexes containing (a) anti-GFP or (b) non-silencing siRNAs. (c, d) Quantification of GFP silencing observed in (a) and (b), respectively, corrected for individual  $\alpha$ -tubulin signal intensity. Results are expressed as percentage of GFP expression levels in untreated controls. \* $p < 0.05$ , \*\* $p < 0.01$  and \*\*\* $p < 0.001$  compared to control

fusion protein. To assess the silencing efficiency mediated by Tf-lipoplexes, the levels of luciferase gene expression were measured 48 h after delivery of Mut siRNA or anti-luciferase siRNA (Luc siRNA), and transfection experiments with Lipofectamine 2000 were performed for comparison. As shown in Figure 7, the results are consistent with those obtained in U-373 cells, with the major difference that the levels of luciferase silencing in Huh-7 cells are even higher than those observed for GFP in U-373 cells (cf. Figures 5 and 7). Moreover, the most efficient gene silencing, taking into account the extent and specificity of action, was not observed at a 3/2 lipid/siRNA charge ratio (data not shown) but rather at a 4/1 lipid/siRNA charge ratio, which may be partially attributed to differences in the metabolic activity of U-373 and Huh-7 cells, and thus in their endocytotic capacity. It should be noted that some non-specific silencing of luciferase was observed for lipid/siRNA charge ratios higher than 4/1, as shown by the decrease in the percentage of luciferase activity obtained when employing Mut siRNA. Similar results were observed for gene silencing mediated by Lipofectamine 2000, although to a lower extent.

Overall, these results demonstrate that optimization of lipid/siRNA charge ratios resulted in efficient and specific gene silencing by Tf-associated siRNA/liposome complexes.

### Cytotoxicity induced by Tf-lipoplexes

To evaluate whether Tf-lipoplexes were toxic to cells, cell viability was assessed by the Alamar blue assay, 24 and 48 h after delivery of the complexes. As shown in Figure 8a, no significant toxicity was observed in U-373 cells, independently of the lipid/siRNA charge ratio. Similar results were observed in Huh-7 cells for the most efficient charge ratios (2/1 and 4/1 lipid/siRNA (+/−)) (data not shown), although some toxicity (25%) was observed at high charge ratios (6/1 and 8/1 lipid/siRNA (+/−)) which correlates with the non-specific effect observed for these formulations (Figure 7). Membrane integrity was evaluated in U-373 cells, using the LDH assay. The results illustrated in Figure 8b show that Tf-lipoplexes are overall well tolerated by the cells, even when prepared at high lipid/siRNA charge ratios.



**Figure 7.** Effect of lipid/siRNA charge ratio of Tf-lipoplexes on luciferase silencing in Huh-7 cells. Huh-7 cells were plated 1 day before siRNA delivery and rinsed with DMEM supplemented with 10% FBS immediately before delivery of the complexes. DOTAP: Chol liposomes were pre-incubated in the presence of Tf (32  $\mu\text{g}/\mu\text{g}$  siRNA), complexed with 50 pmol of anti-Luciferase or non-silencing (Mut) siRNA at the indicated lipid/siRNA charge ratios, and then added to the cells to a final siRNA concentration of 100 nM. After 4 h incubation, the medium was replaced and the cells were incubated for a further 48 h. Afterwards, relative light units (RLU) were measured in each sample and luciferase activity calculated as percentage of activity in untreated control cells. Mean RLU  $\pm$  SD values were obtained from triplicates and are representative of three independent experiments. \*\* $p < 0.01$  and \*\*\* $p < 0.001$  compared to cells treated with Mut siRNA

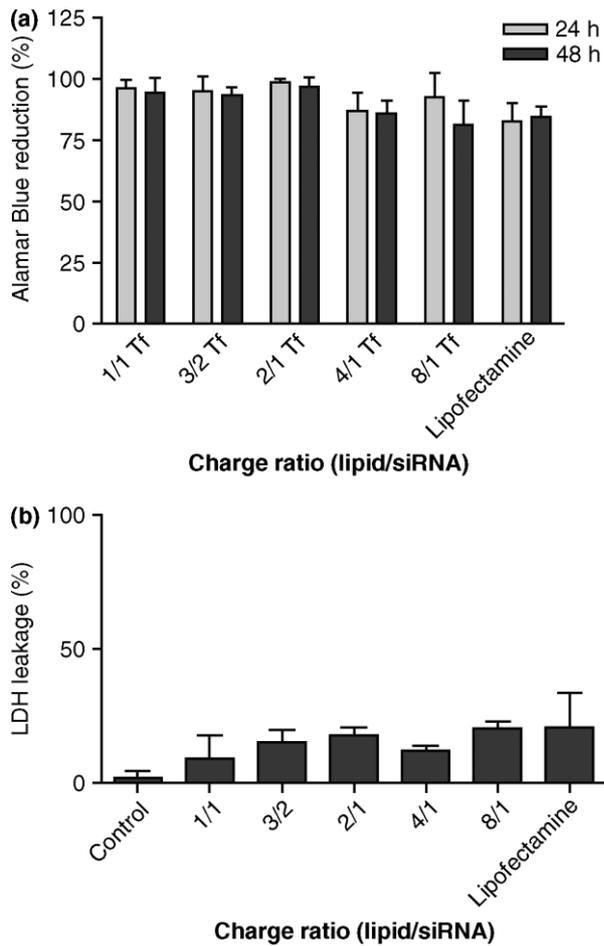
### c-Jun silencing and HT-22 protection mediated by Tf-lipoplexes

In order to demonstrate that Tf-lipoplexes are also able to promote efficient gene silencing of endogenous genes, an endogenous protein was chosen as target (c-Jun) and a complementary siRNA was synthesized based on the mouse c-Jun mRNA sequence. To establish the average decrease in c-Jun mRNA levels, HT-22 immortalized mouse hippocampal neurons were incubated with Tf-lipoplexes prepared at the 3/2 lipid/siRNA charge ratio and containing anti-c-Jun or non-functional Mut siRNAs. Forty-eight hours after Tf-lipoplex delivery, c-Jun silencing was evaluated using QRT-PCR (Table 1). The percentage of c-Jun knockdown in this cell line was calculated using the  $\Delta\Delta C_t$  method, as described in the

**Table 1.** QRT-PCR quantification of Tf-lipoplex-mediated C-Jun knockdown in HT-22 cells<sup>a</sup>

HT-22 cells	$C_t$ C-Jun	$C_t$ HPRT	$\Delta C_t$	$\Delta\Delta C_t$	C-Jun knockdown (%)	Average C-Jun knockdown (%)
Transfected with anti - C-Jun siRNA	29.6	23.5	6.1	1.0	50	51
	29.7	24.4	4.9	1.1	53	
	27.6	23.6	4.0	1.0	50	
Transfected with Mut siRNA	28.7	23.6	5.1			
	28.6	24.8	3.8			
	26.6	23.6	3.0			

<sup>a</sup>The levels of both C-Jun and HPRT genes were determined by quantitative RT-PCR 48 h after transfection. C-Jun knockdown was evaluated by the  $\Delta\Delta C_t$  method ( $n = 3$ ) using the HPRT gene as reference gene. Results are expressed as a percentage of C-Jun expression in cells treated with Mut siRNAs.



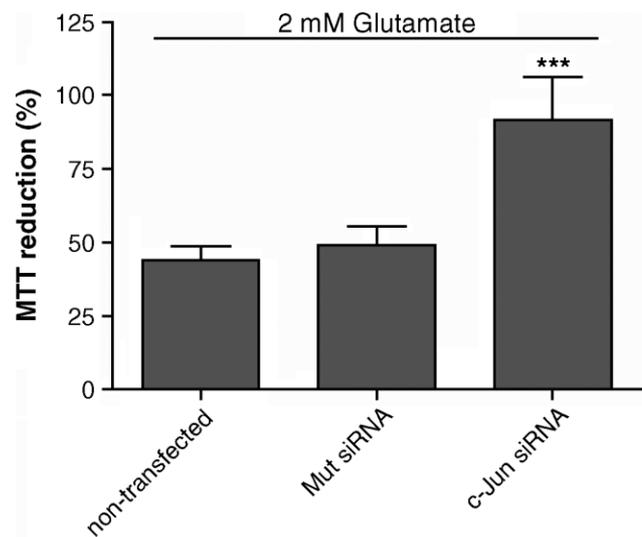
**Figure 8.** Effect of Tf-lipoplexes on the viability and membrane integrity of U-373 cells. U-373 cells were plated 1 day before siRNA delivery and rinsed with DMEM supplemented with 10% FBS immediately before delivery of Tf-lipoplexes. DOTAP: Chol liposomes were pre-incubated in the presence of Tf (32  $\mu\text{g}/\mu\text{g}$  siRNA), complexed with 50 pmol of non-silencing (Mut) siRNA at the indicated lipid/siRNA charge ratios, and added to the cells to a final siRNA concentration of 100 nM. After 4 h incubation, the medium was replaced with fresh DMEM with 10% FBS and the cells were incubated for a further 24 or 48 h. (a) Cell viability was determined by the Alamar blue assay. The data are expressed as percentage of untreated controls. (b) Membrane integrity was evaluated employing the LDH assay. Results are expressed as the percentage of total LDH (intracellular LDH + extracellular LDH). Mean values for LDH activity in the medium  $\pm$  SD were obtained from triplicates and are representative of three independent experiments. No significant statistical differences were observed

Materials and methods section. In this experiment, the mean  $\Delta\Delta C_t$  value found was 1.0, which translates into 51% of c-Jun knockdown. Moreover, two endogenous reference genes, whose expression should not differ between samples, were used in this study as normalization controls. The expression levels of both the glyceraldehyde-3-phosphate dehydrogenase (GAPDH) and Hypoxanthine-guanine phosphoribosyltransferase (HPRT) genes did not differ between control (non-transfected) cells and cells treated with Tf-complexes, which indicates that no non-specific mRNA downregulation occurs due to the vector *per se*.

C-Jun is a transcription factor that promotes cell death through transactivation of pro-apoptotic factors after glutamate-induced damage in neurons. Therefore, we also investigated whether the observed c-Jun knockdown mediated by Tf-lipoplexes was sufficient to attenuate cell death after glutamate exposure in HT-22 cells. The results presented in Figure 9 show that HT-22 neurons pre-treated with anti-c-Jun siRNA show 40% increase in viability after glutamate exposure compared to non-transfected cells or cells pre-treated with non-functional Mut siRNA. The protective effect by c-Jun siRNA was apparently specific, since Mut siRNA did not affect glutamate toxicity. Altogether, the results from QRT-PCR (Table 1) and the MTT assay (Figure 9) clearly show that the achieved c-Jun knockdown is capable of inducing a significant therapeutic effect in HT-22 cells, protecting them from glutamate-mediated cell death.

## Discussion

The recently discovered RNAi pathway is based on the cytoplasmic degradation of target mRNA by small double-stranded RNA molecules complementary to the mRNA sequence. siRNA molecules constitute already a



**Figure 9.** Effect of c-Jun silencing mediated by Tf-complexes on the viability of HT-22 cells after glutamate damage. HT-22 cells were plated 1 day before siRNA delivery and rinsed with DMEM supplemented with 10% FBS immediately before delivery of Tf-lipoplexes. DOTAP: Chol liposomes were complexed, in the presence of Tf (32  $\mu\text{g}/\mu\text{g}$  siRNA), with 25 pmol of anti-c-Jun siRNA (c-Jun siRNA) or non-silencing siRNA (Mut siRNA) at the 3/2 lipid/siRNA charge ratio, and added to the cells to a final siRNA concentration of 50 nM. After 4 h incubation, the medium was replaced with fresh DMEM with 10% FBS and the cells were incubated for a further 48 h. After this period transfected and non-transfected cells were treated with 2 mM glutamate and incubated for 18 h before cell viability evaluation by the MTT assay. The data are expressed as percentage of non-transfected and untreated controls. Mean RLU  $\pm$  SD were obtained from triplicates and are representative of three independent experiments. \*\*\* $p < 0.001$  compared to cells treated with Mut siRNA

popular tool in the fields of functional genomics [4,50,51] and target validation, and have recently started to be studied as a new strategy to control gene expression in disease models. As opposed to plasmid DNA delivery, this process does not present the delivery hurdle of nuclear entry which significantly hampers most of the conventional gene therapy approaches. Also, the process of RNA interference does not involve modifications of the cellular genome and employs highly specific and efficient endogenous enzymatic machinery which is ubiquitously distributed in the mammalian organism. Nevertheless, the successful application of the RNAi approach both *in vitro* and *in vivo*, especially in therapeutic approaches, depends on the development of delivery systems capable of reaching the target cells, in a stable, non-toxic and efficient way. Tf-associated cationic liposomes have been successfully employed for plasmid DNA delivery not only in cancer cells, but also in normal cells, such as neurons, both in cell culture and in animal models [29,30], where they mediated a significant enhancement in transfection efficiency as compared to conventional lipoplexes. Motivated by these results, we decided to examine the potential of Tf-associated lipoplexes for siRNA delivery.

While exhibiting some similarity, DNA and siRNA present several important differences that may prove crucial in terms of complex formation upon their interaction with cationic lipids. Due to their small size, synthetic siRNA molecules do not necessarily require the same process of condensation that leads to the collapse of plasmid DNA into nanostructures, in the presence of a cationic agent. Instead, siRNA molecules may retain their initial structure after interaction with the positively charged lipid headgroups and, therefore, it is not possible to predict siRNA/lipid interactions based on the previous knowledge of plasmid DNA behavior. The siRNA-specific properties may lead to the formation of particles that do not allow complete nucleic acid condensation, which in turn can result in degradation by macromolecules present in the extracellular environment and thereby in low gene silencing levels. For this reason, we initiated our study by assessing several parameters, such as the extent of lipid/siRNA association and the siRNA protection efficiency conferred by the cationic lipid, which helped predict the biological stability of the complexes. Results from these experiments revealed that even at low lipid/siRNA charge ratio (3/2), siRNA associated extensively with cationic liposomes both in the absence and presence of transferrin (Figure 1), resulting in stable complexes capable of conferring significant protection to the carried molecules (Figure 2), thereby inhibiting serum-mediated siRNA degradation even after long incubation periods (Figure 2c). The stability of lipid-based vectors in serum is especially important since the inhibitory effect of serum on transfection has been one of the major drawbacks when trying to transpose these systems from cell culture conditions to animal models, aiming at therapeutic applications. Serum increases the size of cationic liposomes by

inducing aggregation due to interaction of negatively charged proteins with the lipid headgroups. Although DOPE is usually the first choice as helper lipid for *in vitro* experiments, complexes containing cholesterol have been shown to be more active *in vivo*, which may be attributed to their high stability in biological fluids, as a consequence of a lower interaction with serum proteins.

Results from confocal microscopy on the intracellular uptake of Tf-lipoplexes demonstrated that for both tested charge ratios (3/2 and 2/1 lipid/siRNA), the lipoplexes were localized inside Huh-7 cells 4 h after delivery (Figure 3). In contrast, after incubation with naked Cy3-labeled siRNA, no fluorescence was observed inside or outside the cells, which may be due to siRNA degradation by serum enzymes present in the culture medium. These results reinforce the need to employ vectors capable of protecting the siRNA molecules following their administration.

In order to evaluate the biological activity of siRNA complexes with DOTAP:Chol liposomes we performed a series of studies focused on the major feature of the siRNA pathway, namely the ability to induce gene silencing and protein knockdown. Our results (Figures 4–7) clearly demonstrate that association of siRNAs to Tf-containing cationic liposomes resulted in a significant enhancement of gene silencing. This correlates to the observed decrease in the levels of reporter proteins, thus suggesting that an efficient knockdown of therapeutic-relevant targets may be achieved when employing this kind of vector. Consistent with results from our previous studies on plasmid DNA, coating of cationic liposomes with Tf enhances gene silencing by siRNA as compared to plain complexes (Figure 4), which is probably due to promotion of cellular uptake of the Tf-lipoplexes through the endocytotic pathway and cytoplasmic delivery of the carried siRNA molecules by destabilization of the endosomal membrane under acidic conditions [32].

Our observations that the optimal siRNA/lipid charge ratio albeit always positive varied considerably between the tested cell lines suggest that there is an ideal charge ratio for efficient gene silencing, depending on the cell type. As illustrated in Figures 5 and 7, a specific downregulation upon delivery of Tf-lipoplexes was demonstrated, which is of crucial importance for our work to be of value for future clinical applications [1,52].

Recently, several studies have suggested that activation of the mammalian immune system by siRNAs is dose-dependent [25], caused by nucleoside modifications [27,53], or stimulated by certain nucleotide sequences frequently found in viral RNA [54]. Careful analysis revealed that the siRNAs employed in our study do not contain any of the known sequences that may cause such immune responses. Moreover, we tested the lipid vehicle alone (liposomes associated to Tf), taking into consideration reports concerning a possible role of lipid-based vectors in non-specific silencing effects [28,55,56].

As shown in Figure 5, no GFP reduction was observed for this condition. However, at high lipid/siRNA charge ratios a significant luciferase silencing was observed for the Mut siRNA in Huh-7 cells (Figure 7) suggesting that the lipid, when present at high concentrations and associated with siRNAs, can indeed contribute to a non-specific protein decrease [56]. This may stimulate the activation of Toll-like receptors 7 and 8 [26,27,53,57] and the respective downstream pathways, which lead to expression of IFN and pro-inflammatory cytokines. This effect was also observed with Lipofectamine 2000, which reinforces the need to always employ the lowest effective dose of the nucleic acid delivery agent, as well as a non-silencing control, even when siRNA delivery is mediated by a commercial reagent [23]. Nevertheless, this was not verified in the U-373 cells, which indicates that these non-specific effects may be cell-type-specific, as has been recently suggested [58].

The toxicity assays revealed that Tf-lipoplexes were slightly better tolerated by the cells than Lipofectamine 2000 and, notably, the toxicity of lipoplexes prepared at low lipid/siRNA charge ratios was even negligible. This low toxicity level was observed not only for cell lines, but also for primary neuronal cultures (data not shown). Moreover, recent experiments involving siRNA delivery into neuronal cultures and after local injection into the mouse brain have clearly shown that Tf-lipoplexes are able to mediate high levels of gene silencing in this type of non-dividing cells *in vitro* and *in vivo* (Cardoso *et al.*, manuscript in preparation). These results are promising considering the potential applications of Tf-lipoplexes in experimental models of neurodegenerative disorders, where high toxic effects and low gene silencing efficiency are major drawbacks when employing available standard transfection reagents.

In order to clarify the potential of Tf-lipoplexes to mediate gene silencing of both reporter and endogenous genes, and as a first approach to a possible therapeutic application of these lipid-based vectors, we selected the c-Jun gene as target gene and investigated c-Jun knockdown in a glutamate-sensitive cell-line. The QRT-PCR results presented in Table 1 show a significant downregulation of c-Jun mRNA (51%) after delivery of Tf-complexes without detectable off-target effects.

Due to its important role as a transcription factor in several well-described pathways of apoptotic cell death in neurons, c-Jun is a promising target for therapeutic applications in neurodegenerative conditions, including glutamate-induced cell death [46]. Here, we demonstrate that c-Jun targeting siRNA mediates a pronounced decrease in c-Jun mRNA levels and significantly protects neuronal cells (Figure 9) from glutamate-induced damage, which confirms the potential of Tf-complexes to mediate an efficient therapeutic effect. The protective effect obtained with c-Jun siRNA in the present model system of glutamate-mediated

neurotoxicity *in vitro* is well in line with protective effects against excitotoxic neuronal death observed in mice expressing an inactive form of c-Jun [59]. It is important to note that, although c-Jun silencing in HT-22 cells was approximately 50%, the protective effect observed in this cell line was highly significant, indicating that for this target a complete knockdown is not necessary to obtain a relevant therapeutic effect.

Taken together, this study demonstrates that, similarly to what has been previously shown for plasmid DNA, DOTAP:Cholesterol liposomes associated to transferrin can mediate efficient intracellular siRNA delivery. Our findings clearly show that Tf-associated lipoplexes, when prepared at the adequate charge ratios, promote protein silencing *in vitro* which, together with previous results concerning their *in vivo* application [29], suggests that they may be a promising strategy to translate laboratory research to clinical application.

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