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Studies on Efficiency and Toxicity of in vivo Delivery Systems for siRNA and plasmid DNA

vorgelegt von

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Erklärung

Diese Dissertation wurde im Sinne von § 13 Abs. 3 bzw. 4 der

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Ehrenwörtliche Versicherung

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Für meine Eltern

Brigitte und Joachim Tietze

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1 Introduction

1.1 Gene Therapy

Gene therapy offers tremendous potential for treatment of inheritated as well as acquired diseases. Traditional gene therapy utilizes DNA to correct genetic by inserting functional genes into an organism to replace defective ones. If the abnormal production of proteins is the cause for disease, gene therapy follows a different approach which is posttranscriptional regulation of gene expression. It works by silencing specific messenger RNA, for example by using short interfering RNA (siRNA), thereby preventing disease-causing proteins from being made. For cancer therapy siRNAs offer the potential to eliminate neoplastic cells by silencing of genes that are crucial for cell growth and viability.

Gene therapy is likely to become more and more important for disease treatment. The number of clinical trials using gene therapy is steadily increasing. Most of the current studies are in phase I stage but some have already reached phase II or even III (http://www.wiley.co.uk/genmed/clinical/). However, for effective treatment, transfer of genetic material needs to reach specific cells of a patient. The easiest way to do so is the *ex vivo* approach. Here the relevant target cells are isolated from the patient, exposed to the gene of interest *in vitro* and then reinjected into the patient. In most cases the *ex vivo* approach is not feasible due to difficult culturing of the cells, reimplantation difficulties or the needed cells cannot be solely isolated. Therefore the far more challenging way of *in vivo* gene therapy has to be chosen. Here the cells are directly transfected within the patient´s body, which gives rise to several problems which have to be overcome. First, one has to consider that “naked” DNA or RNA is unstable under in vivo conditions due to rapid degradation by serum nucleases (Houk et al. 2001, Kawabata et al. 1995). Only in few approaches, like intramuscular injection or electroporation, injection of free plasmid leads to effective gene transfection (Isaka and Imai 2007, Nishitani et al. 2000). The usage of free siRNA is also effective as shown in the treatment of age related macula degeneration in mice (Shen et al. 2006) and has already reached phase III clinical trials in humans (http://clinicaltrials.gov). However, for this application a stabilized modification of

free siRNA has to be used. Therefore, if the nucleic acid (NA) cannot be protected through chemical modifications within the molecule, carriers or vectors are needed to protect nucleic acids from degradation and also to specifically target the DNA / nucleotide complex to the cells of interest. These vectors can be divided into two major classes: viral and synthetic vectors, wherein synthetic vectors include cationic polymers and lipids.

1.2 Synthetic vectors for gene therapy

Currently more than two thirds of clinical gene therapy trials use viral vectors (http://www.wiley.co.uk/genetherapy/clinical/). Nevertheless, domestication might pose inherent problems including immune and toxic reactions as well as the potential for viral recombination (for review see: Nair 2008, Yi 2005) Chemical modifications for targeting reasons are also difficult.

As a consequence, a broad range of synthetic vectors have been developed of which the use of liposomal formulations and cationic polymers have achieved some prominence (Duzgunes et al. 2003, Liu et al. 2003). One main advantage of synthetic vectors is that they can be tailored to specific needs, including subsequent functionalizations, like targeting or shielding moieties. Nevertheless, their transfection efficiency is rather low compared to viral vectors (Brown et al. 2001). Great efforts have been made during the last years to improve efficiency, but still a lot of work has to be done to reach satisfactory levels.

The group of cationic lipids have become one of the most studied synthetic vectors. They are amphiphilic molecules mainly composed of three parts: (i) a hydrophobic lipid anchor group; (ii) a linker group, such as an ester, amide or carbamate; and (iii) a positively charged head-group, which condenses with negatively charged nucleic acids, leading to so called lipoplexes.

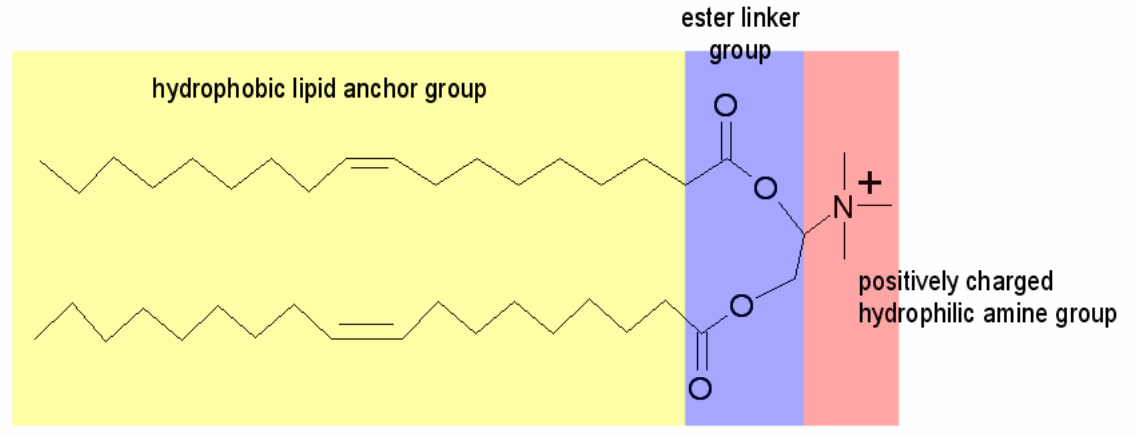


Fig. 1-1: *Schematic representation of the cationic lipid DOTAP (1,2-dioleoyl-3-trimethylammonium-propane)*

These characteristics cause the cationic lipids to spontaneously assemble into nanospheric liposomes when put into aqueous solutions (Mahato 2005). By this, nucleic acids (NAs) are compacted and protected against nucleases. Cationic lipids are often used in combination with so called helper lipids such as dioleoylphosphatidylethanolamine (DOPE) or cholesterol. Both lipids potentially promote conversion of the lamellar lipoplex phase into a non-lamellar structure, which presumably rationalizes their ability to often improve cationic lipid mediated transfection efficiency (Igarashi et al. 2006). But even though liposomes are successfully used in clinical gene therapy trials (for review see: Fenske and Cullis 2008), concerns were raised regarding immunotoxicity (De Smedt et al. 2000, Li et al. 1999).

In the group of polycations polyethyleneimine (PEI) has achieved some prominence and is successfully used in *in vitro* and *in vivo* (Godbey et al. 1999, Lungwitz et al. 2005) gene therapy.

Like cationic lipids cationic polymers show extraordinarily good potential to condense NAs into nanosized complexes (polyplexes). It is worth mentioning that even viruses use “polycations” for NA assembly: NA condensing agents in viruses consist of basic and therefore, under physiological conditions, cationically charged amino acids. In summary, NA condensation is one of the main features needed for efficient gene transfer and can occur with polycations.

Even though the immunogenicity of PEI complexes is less distinctive than that of liposomal complexes (Kawakami et al. 2006), it is still a major concern. Furthermore a correlation between efficiency and toxicity has been stated *in vitro* and *in vivo*. To summarize, the ideal vector should combine efficient gene delivering properties with a decreased toxicity potential.

1.3 Decreasing immidiate vector toxicity

One reason for immidiate toxicity (within one hour) observed *in vivo* is the cationic nature of polyplexes (for review see: Lv et al. 2006). The positive surface charge on the one hand tends to improve uptake into cells *in vitro* due to higher affinity to negatively charged membrane constituents. On the other hand, *in vivo* applicability of these complexes becomes problematic, since the positive surface charge leads to increased non-specific interactions. The charge leads to uptake of the complexes by macrophages and rapid removal from the blood stream. Furthermore the highly negative zetapotential directs aggregate formation with erythrocytes that can cause fatal lung embolism (Ogris et al. 1999, Plank et al. 1996, Kircheis and Wagner 2000, Chollet 2002). Shielding the particle surface with agents such as polyethyleneglycol (PEG) reduces toxicity and improves circulation time (Ogris et al. 1999).

1.4 Decreasing intermediate vector toxicity

In addition to the immidiate toxic effects, intermediate toxicity of the polycation can be observed due to extremely slow, if any, degradation. This will not be distinctive until about 24 hours or more after administration. PEI as an example tends to accumulate primarily in the liver and causes side effects like microvesicular fatty liver (Ogris et al. 2007). The synthesis of biodegradable polycations is one possible opportunity to overcome this problem, while maintaining high transfection efficiency.

One proposal is the crosslinking of low molecular weight polycations with biodegradable linkers. Ideally these biodegradable polycations will securely guide the nucleid acid into the target cell and thereafter get degraded into smaller

molecules which can be excreted. One example for this approach that was used in the present work are the polycations OEI-HD, lt-OEI-HD1.2 and OEI-HA10, wherein OEI stands for oligoethyleneimine, a 600-800 Da low molecular weight polyethyleinimine (PEI) fraction used.. The suffixe HD abbreviates the linker 1, 6-hexanedioldiacrylate that was used to polymerize OEI fractions. In case of OEI-HD and lt-OEI-HD1.2 the HD linker reacts with OEI to an ester-based polymer (figure 1-2B) and in case of OEI-HD, due to reaction temperature, the free amino groups of OEI lead to aminolysis of the esters, generating the branched structure displayed in figure 1-2A. The advantage of these polymers over the classical PEI lies in their potential of degradability, thus avoiding toxic side effects due to accumulation *in vivo*.

For OEI-HA10 (figure 1-2C) approximately ten (of approximately 18) nitrogens in OEI are reacted with hexylacrylate (HA), producing a hydrophobic polymer, which stabilizes formed polyplexes by hydrophobic interactions and potentially increases the membrane interaction potential.

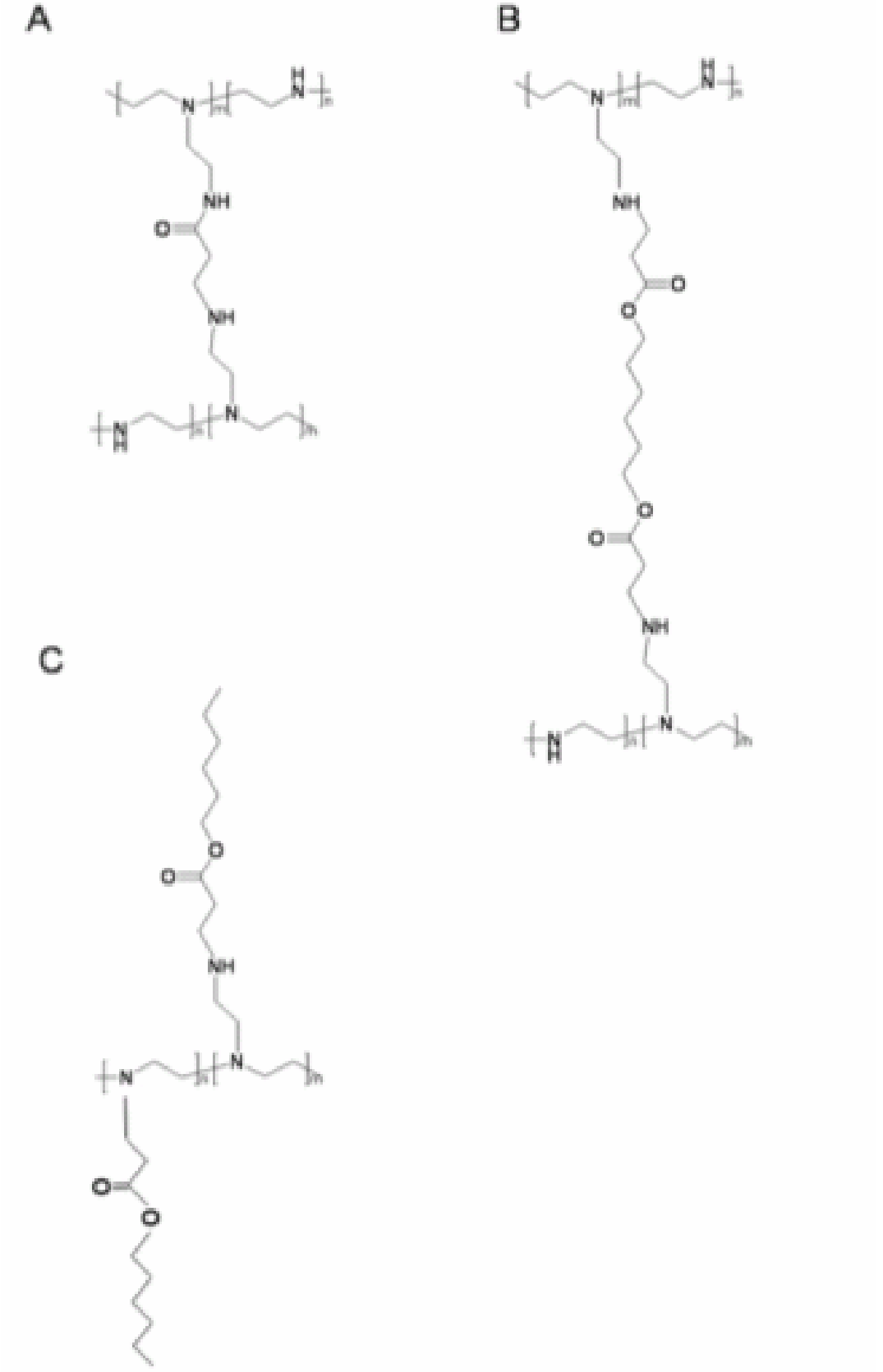


Fig. 1-2: *Structure of OEI-HD (A), lt-OEI-HD1.2 (B) and OEI-HA10 (C)*

However, even with polycations showing good complexation efficiency and improved *in vivo* tolerability, intra-cellular delivery of NAs remains a major hurdle.

1.5 Targeted gene therapy

Especially for *in vivo* therapy it is important to target complexes to the designated cell of interest to avoid unwanted side effects in normal cells. Targeting can be achieved with different strategies:

(i) By incorporation of targeting ligands that actively facilitate the uptake into specific cells or tissues.

(ii) By passive uptake due to special physiological conditions, such as irregular fenestration in tumors in connection with tailored complex properties.

(iii) By the use of cell type specific promoters or enhancers to facilitate gene expression in specific cell types.

Notwithstanding the large amount of research devoted to this subject, active targeting is still the largest field of interest. Several ligand-receptor systems have been investigated to date. Ligands include antibodies (Jeong et al. 205), peptides (Kunath et al. 2003), transferrin (Kircheis et al. 1997), sugars (Zanta et al. 1997), mannuronic acid (Weiss et al. 2006), folate (Chul et al. 2005) and growth factors such as EGF (Blessing et al. 2001). In this work, transferrin (Tf) was chosen as targeting ligand to provide for efficient intracellular uptake, as transferrin receptors are known to be overexpressed by proliferating cells such as tumor cells used as *in vivo* model (Wagner et al. 1991, Wagner et al. 1994). Just recently a siRNA nanoparticle delivery system, also containing a transferrin receptor targeting agent on the surface, entered Phase I in clinical trials (Press release Calando Pharmaceuticals, June 02, 2008, www.insertt.com).

1.6 Intracellular pathway of complexes

Spontaneously formed complexes of polycation and NA (where applicable modified with shielding moieties and or targeting ligands), can be injected into the

patients vein and will be delivered to the target tissue by systemic circulation. After binding of the vector to the cell surface they are internalized. Once inside the cell, release from the endosome is crucial for efficient gene transfer. In the case of PEI, a unique endosome-disruptive mechanism, also designated as proton-sponge-effect (Boussif et al. 1995) is described, making it one of the most efficient polycationic gene transfer agents. During processing of endosomes, the pH decreases to ~6 in early, ~5 in late endosome and even to pH 4.5 in lysosomes. PEI possesses high buffering capacity leading to increased influx of protons, followed by water and chloride ions, thereby inducing osmotic swelling and finally by postive charge/membrane interaction to rupture of the endosomal membrane. The high transfection efficiency is likely to be linked to this proton sponge effect.

After release into the cytoplasm, siRNA can directly interact with the mRNA, representing a clear benefit over DNA which needs to be translocated into the nucleus to exert its function. The hypothetical process of polycation/NA complex delivery is shown in figure 1-3. Nevertheless, it should be emphasized that many steps of this gene delivery process are still under investigation.

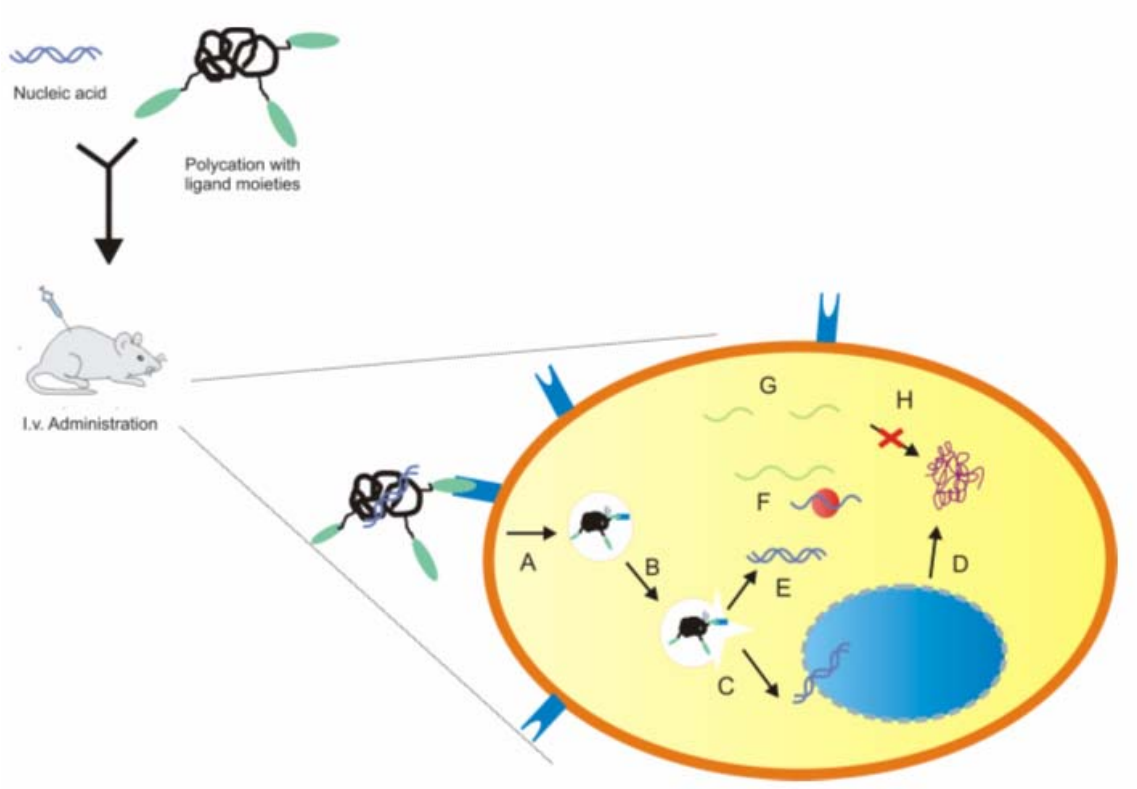


Fig. 1-3: *Hypothetical process of polyplex delivery in vivo. Polycation is modified with targeting ligands and shielding moieties. Polycation and NA upon mixing spontaneously form polyplexes, which deliver the cargo NA through systemic circulation to the target tissue (e.g. tumor). The targeted polyplexes bind to the cell surface via ligand-receptor interaction and are internalized (A). Later, complexes are released from the endosome (B) and in case of DNA polyplexes DNA is translocated into the nucleus (C), resulting in protein expression (D). In case of siRNA its mechanism of action can take place already in the cytoplasm (E). After incorporation into RISC the antisense strand of the siRAN guides the RISC to the homologous mRNA (F), resulting in cleavage of target mRNA (G), and gene silencing (H).*

1.7 RNA Interference

RNA interference (RNAi) is the process by which specific genes can be silenced. It is proposed to be an ancient method of inactivating viral gene expression (Lindbo et al. 1993). In fact, plants can recover from infection by RNA viruses via RNA silencing (Ratcliff et al. 1999, Baulcombe 2003). RNAi was first reported in plants (Napoli et al. 1990). Studies with the germ line of *C.elegans* (Fire et al. 1998) lead to the recognition that double-stranded (ds) RNA plays a pivotal role in this mechanism. However, in the beginning this approach seemed unworkable in mammals, because here dsRNA provokes the interferon response and causes apoptosis. For normal situations of dsRNA encountering a cell (viral infection), this is useful, because it prevents replication and spreading of the virus to neighbouring cells. However, for gene therapy this obstacle needed to be overcome. By using small synthetic dsRNAs, so called small interfering (si) RNAs (Zamore et al. 2000, Elbashir et al. 2001) with a length of only 21-25 base pairs this could be achieved. As shown in figure 1-4 siRNAs are a natural occuring intermediate of RNA interference. But by using siRNA instead of dsRNA Tuschl and colleagues overcame the problem of Toll-like receptor-detection and immune response, however still obtaining gene silencing. The multi-step process of RNA interference is presented in the following figure.

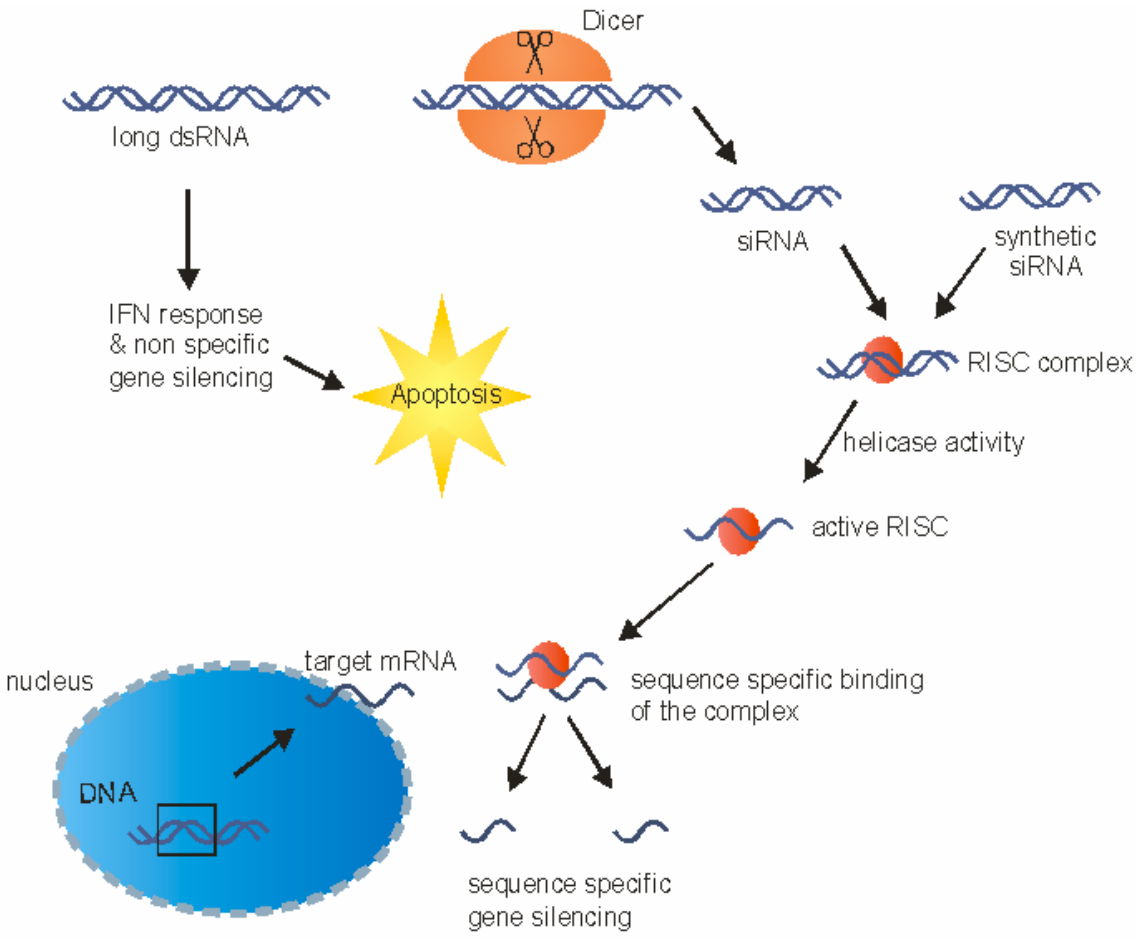


Fig. 1- 4: *Mechanism of RNAi: After entering the cell, the double-stranded RNA is cleaved into shorter fragments of 21 to 25 nucleotides, known as short interfering RNA (siRNA), in the cell by an enzyme called Dicer, and the 'sense' strand is degraded. The remaining 'antisense' strand is then incorporated into a protein complex called RNA-induced silencing complex (RISC). The antisense strand of the duplex siRNA guides the RISC to the homologous mRNA, where the RISC-associated endoribonuclease cleaves the target mRNA resulting in silencing of the target gene.*

1.8 Ran protein

In this work the potential therapeutic effect of mRNA knockdown in tumor cells was studied. As the Ran protein was recently identified from an RNAi based screen as possible target in cancer therapy (Morgan-Lappe et al. 2007), we have worked with an siRNA directed against the Ran mRNA.The Ran protein is a small GTPase and has been implicated in a large number of nuclear proc-

esses including regulation of nuclear transport and formation and organisation of the microtubule network (Sazer and Dasso 2000, Gruss and Vernos 2004).

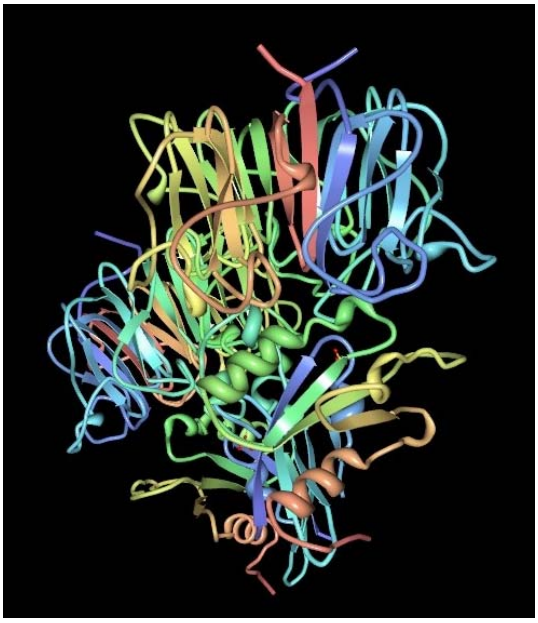


Fig. 1-5: *Ran (ras-related nuclear protein)*

High expression of Ran GTPase is associated with cancer (Abe et al. 2008). Because of its pivotal role in nuclear transport (see figure 1-6), our hypothesis is that downregulation of the Ran protein results in apoptosis.

In the cell Ran exists in two nucleotide-bound forms: GDP-bound and GTP-bound. Transport through the nuclear pore complex is driven by a gradient of Ran/GTP, with a high concentration of Ran/GDP in the cytoplasm and a high concentration of Ran/GTP in the nucleus. Proteins that should be transported into the nucleus contain a nuclear localization signal (NLS) that forms complexes with importin α and importin β in the cytoplasm where Ran is in the GDP-bound form. Following transport through the nuclear pore complex, Ran/GTP binds to importin β, releasing importin α and the protein within the nucleus. GTPase-activation then leads to transportation of this complex to the cytoplasm. Here hydrolysis of the bound GTP occurs, forming a Ran/GDP complex and releasing importin β, thus closing the Ran cycle.

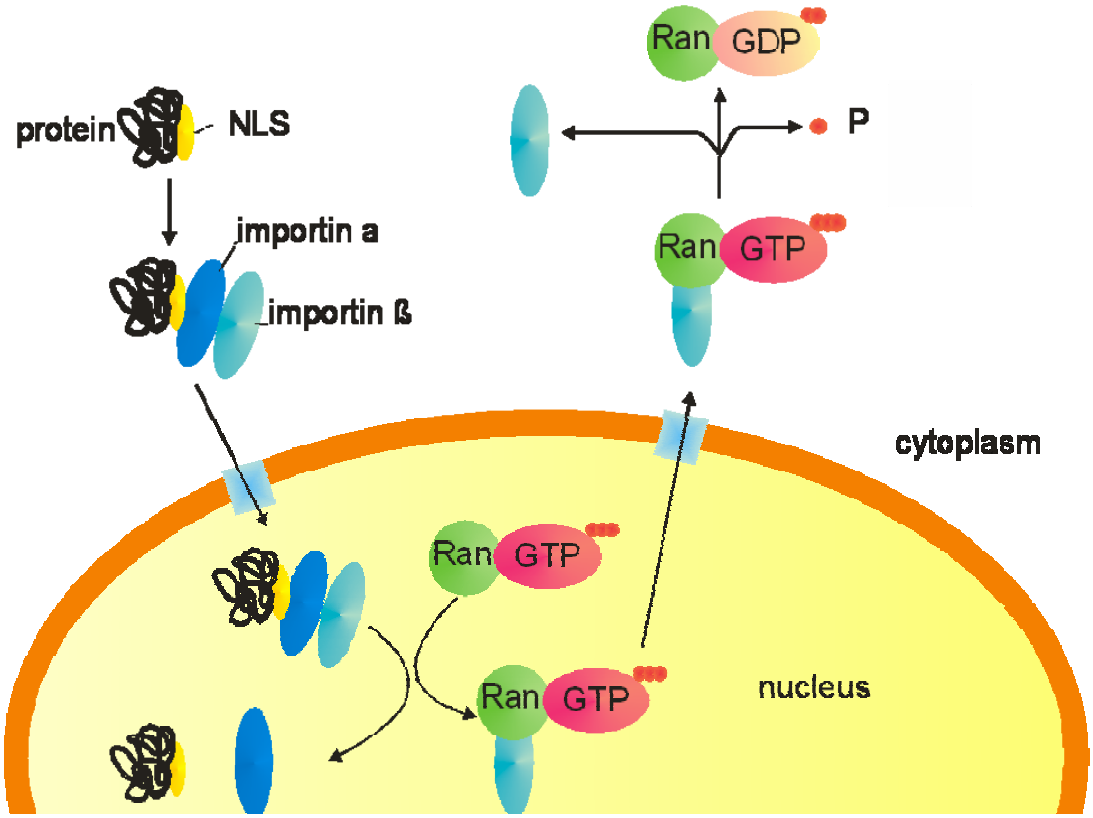


Fig. 1-6: *Role of Ran protein in nuclear transport*

1.9 Plasmid DNA - design and its impact on gene expression

The delivered vector in gene therapy can also be bacterial plasmid DNA. However expression following transfection with systemically delivered plasmid DNA (pDNA) complexes is commonly short-lived, due to induction of immune response.

In addition to its therapeutic cargo, such bacterially derived plasmids necessarily contain a backbone with immunstimulatory bacterial CpG motifs. The mammalian immune system has been tuned by evolution to recognize the CpG motifs by activation of Toll-like receptor 9 (TLR-9) as foreign antigens and to mount an inflammatory response (Krieg et al. 1995, Hemmi et al. 2000). In case of DNA vaccination this is a favoured process, and has made it very attractive for immunotherapy against cancer. Nevertheless, for other cases approaches to overcome this problem have to be evaluated. To date, there are two different ways of minimizing unmethylated CpG sequences. One is the enzymatic methy-

lation of pDNA and the other is the removal of bacterial sequences, thereby generating a minimized DNA vector.

For comparison studies on the influence of CpG sequences within a plasmid we have used two different plasmids (see figure 1-6): pEGFPLuc as a classical vector for luciferase expression and pCPGLuc which was cloned by Plasmid-Factory based on pCpG-mcs and pMOD-LucSh and which shows reduced CpG sequences due to removal of bacterial sequences.

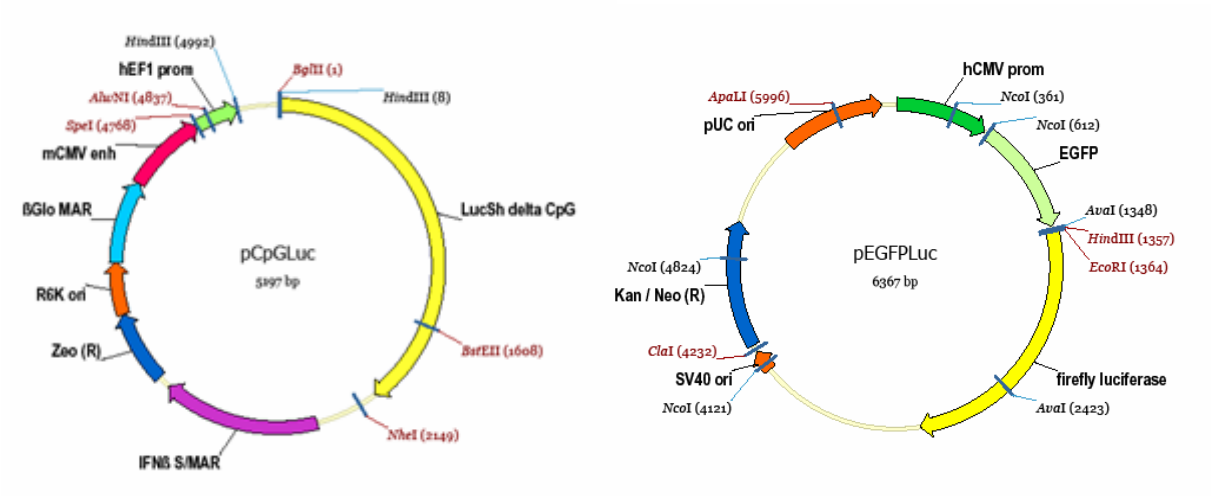


Fig. 1-6: *Structures of pEGFPLuc and pCPGLuc plasmids that were used for analysis of CpG content influence.*

Another problem might appear in the fact that plasmid solutions still contain a significant degree of undesired process-related impurities such as bacterial genomic DNA. Through purification by high performance liquid chromatography (HPLC) this amount can be reduced from ~ 10 to 15 % down to ~ 1 %.

In this work we have analyzed the influence of CpG depletion and purification of plasmids concurrently to further enhance transgene expression both *in vitro* and *in vivo.*

1.10 Specific aims of this work

In this dissertation, systemic delivery of NAs, alone and complexed with polycationic delivery systems, was analysed. The analysis was focused on tolerability and efficiency of the treatment.

The in vivo administration is so far hampered by the lack of stable delivery systems that are able to protect their NA cargo in the blood stream and savely lead it to the desired target cells. However, two novel polycationic vectors that had been established in our lab, led to promising results for siRNA delivery in vitro. In the current work we have therefore tried to optimize siRNA / polycationic complexes for in vivo administration. Furthermore, we have used the therapeutically relevant RAN siRNA to influence the growth of subcutaneous Neuro2A tumors in an in vivo model.

Another aim was to evaluate different biodegradable polymers, that had been established in our lab, for their efficiency and safety in in vivo application. Polymers were compared with L-PEI as a gold standard regarding efficiency. However, L-PEI can not be degraded within the body and can thereby lead to a long-term toxicity. In contrast, we wanted to show that our biodegradable polymers can reach efficiency levels as high as L-PEI, yet compared with a much better tolerability and the possibility of repeated application.

Furthermore we were interested in clarifying the impact of the NA on efficiency and toxicity of the treatment. In this case we have compared plasmid DNA containing bacterially derived CpG motifs, with plasmid DNA that was exempt from CpG sequences.

But because plasmids are generated in bacteria and the plasmid purification step leaves behind a certain amount of bacterial genomic DNA, we also wanted to clarify the impact of this (CpG containing) impurities. One approach was to compare the effect of the plasmids alone. For this analysis we had chosen the possibility of hydrodynamic tail vein injection of plasmid solutions in mice. Another approach was to compare the plasmids when they were complexed to L-PEI as a gold standard for polyplex delivery.

2 Materials and Methods

2.1 Reagents and chemicals

The plasmid pEGFPLuc (Clontech Laboratories, Heidelberg, Germany) containing a CMV promoter driven fusion of the genes encoding for enhanced green fluorescent protein and luciferase was used for generation of stably transfected cells. Lipofectamine 2000 (LF2000) was obtained from Invitrogen (Karlsruhe, Germany). Linear PEI with an average molecular weight of 22 kDa (PEI22) was synthesized by acid-catalysed deprotection of poly(2-ethyl-2-oxazoline) (50 kDa, Aldrich) in analogous form as described (Brissault et al. 2003) and is also commercially available from Polyplus (Strasbourg, France). Branched PEI with an average molecular weight of 25 kDa (PEI25) was obtained from Sigma-Aldrich (Munich, Germany). PEIs were used as a 10 mg/ml stock solution neutralized with HCl. Transferrin was obtained from Biotest (Dreieich, Germany). Transferrin conjugated OEI-HD (Tf-PEG-OEI) was synthesized and purified in analogous manner as Tf-PEG-PEI conjugate described in (Kursa et al 2003). Oligoethylenimine derivative OEI-HD (Kloeckner et al. 2006-1, Kloeckner et al. 2006-2, Kloeckner et al. 2006-3) is based on OEI 800 Da crosslinked by ß-aminoproprionamide linkages and was synthesized by Abbott GmbH (Chicago, IL, USA). The optimized procedure as described in (Tarcha et al. 2007) generates OEI-HD polymers with a typical molecular weight of 25-30 kDa. OEI-HA10 was synthesised in our work group (Arkadi Zintchenko, unpublished data), lt-OEI-HD1.2 was synthesized as described in (Kloeckner et al. 2006-3),using a polymer to linker ratio of 1:1.2.

All other chemicals were purchased from Sigma-Aldrich (Munich, Germany). Cell culture media, antibiotics and fetal calf serum (FCS) were purchased from Invitrogen (Karlsruhe, Germany). siRNA duplexes were purchased from Dharmacon (Lafayette, CO, USA); LucsiRNA: GL3 luciferase specific duplex: 5'-CUUACGCUGAGUACUUCGAUU-3', non-specific control siRNA siCONTROL: 5'-UAGCGACUAAACACAUCAAUU-3' und RAN specific therapeutic siRNA RANsiRNA: 5’-AGAAGAAUCUUCAGUACUAUU-3’ were used. RNA stabilization solution RNAlater was purchased from Qiagen (Hilden, Germany). RT-PCR primers were purchased from Eurofins MWG Operon (Ebersberg, Germany) and Roche Diagnostics (Mannheim, Germany) and are further described in chapter 2.21. All other re-

agents for RT-PCR were also obtained from Roche Diagnostics. D-Luciferin, sodium salt is available from Promega (Mannheim, Germany).

2.2 Plasmid design

Three different types of DNA were used in this work. pCMVLuc carrying the firefly luciferase coding region under the promoter control of cytomegalovirus (CMV) was provided by Plasmid Factory (Bielefeld, Germany).

pCpGLuc and pEGFPLuc were amplified by Plasmid Factory and provided in two different purification grades. (i) Research grade (RG, containing up to 15 % genomic DNA) (ii) Covalently closed circular grade (ccc, containing max. 1 % genomic DNA). The amount of genomic DNA in the ccc versions is ~ 1 % compared to ~10-15 % in RG plasmids.

EGFPLuc plasmid was in addition amplified in our group with a competent E. coli strain JM 109 (Promega) and purified with the EndoFree Plasmid Kit according to a protocol from Qiagen (Hilden, Germany).

2.3 Formation of transfection complexes

Formulations for siRNA and pDNA delivery, respectively were prepared as follows: First, different concentrations of NA and polymer were diluted at various polymer/NA ratios (w/w: weight/weight) in separate tubes in equivalent HBG amounts (HEPES buffered glucose solution; 20 mM HEPES, 5% glucose, pH 7.4). Then, the HBG solution of the polymer was added to the NA, mixed and incubated for 30 to 40 min at room temperature to form polymer/NA complexes. Transferrin-conjugated polymer/NA-formulations were prepared as described above with the exception that the polymer was partially replaced with corresponding weight percentages (5, 10, 25, 35, 50 %) of Tf-conjugated polymer, where the weight percentages refer to the polymer component of the Tf conjugate. Replacing for example 5, 10, or 50 % of the OEI-HD with Tf-PEG-conjugated OEI results in transferrin: OEI weight/weight ratios of 0.21/1, 0.41/1 or 2.1/1, respectively, with 17 %, 29 %, or 68 % of the weight of the blended carrier being composed of transferrin protein.

2.4 Measurement of particle size and zeta-potential

Particle size of various polymer/NA formulations were measured by dynamic laser-light scattering using a Malvern Zetasizer Nano ZS (Malvern Instruments, Worcestershire, UK) equipped with a 4 mW He–Ne laser at a wavelength of 633 nm at 25 °C. Scattered light was detected at a 173° backward scattering angle. The viscosity and refractive index of water at 25 °C was used for data analysis. For particle sizing complexes were prepared as for gene transfer and diluted in HBG to give a final NA concentration as used for transfection. For estimation of the zeta-potential, transfection complexes were first prepared in HBG, then 1 mM NaCl was added to achieve appropriate volume for measurement and the zeta potential was determined. Dispersion Technology Software 5.00 (Malvern Instruments, Herrenberg, Germany) was used for data analysis. All data represent the mean of three independent measurements.

2.5 Cell culture experiments

For pDNA delivery experiments, mouse neuroblastoma cells Neuro2A (ATCC; CCL-131) were used. The cells were grown in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10 % serum and 4 mM stable glutamine. All cells were seeded 24 hours prior to pDNA delivery using 5,000 cells per well in 96-well plates for transfection and cytotoxicity experiments.

2.6 Transfection in vitro

For standard in vitro transfection cells were seeded in 96-well plates using 5,000 cells per well 24 hours prior to transfection. Experiments were performed in 100 µL growth medium containing 10 % fetal bovine serum (FCS). Transfection complexes with indicated amounts of pDNA were then added to each well. Complex containing medium was removed after 4 hours, 100 µl of fresh medium was added and cells were analyzed for cytotoxicity of complexes or luciferase reporter gene expression, respectively after 24 hours.

2.7 Cytotoxicity assay

The cells were grown in 96-well plates and treated with different amounts of pDNA complexes as described in chapter 2.6. Cell viability was determined 1 day following

transfection by addition 5 µg of MTT [2,3-bis(2-methoxy-4-nitro-5-sulfophenyl)-2H-tetrazolium-5-carboxyanilide] (Sigma) to each well. Cells were incubated at 37°C/5% CO_2 for up to two hours. Medium was removed and samples were frozen at – 80 °C for at least 2 hours. 100 µl of dimethyl sulfoxide (DMSO) was added and samples were further incubated at 37 °C/5% CO_2 for 30 min under constant shaking. Absorbance was measured spectrophotometrically at 450 nm.

The relative cell viability (%) was related to control wells containing cell culture medium without polyplex and was calculated by: absorption test x 100 % / absorption control.

2.8 Luciferase reporter gene expression

Twenty four hours following pDNA delivery (see chapter 2.6), medium was removed, cells were washed with phosphate-buffered saline (PBS; pH 7.4) and lysed with 50 µL of lysis buffer (25 mM Tris, pH 7.8, 2 mM EDTA, 2 mM DTT, 10 % glycerol, 1 % Triton X-100). Luciferase activity was measured using a Lumat LB9507 instrument (Berthold, Bad Wildbad, Germany). Luciferase light units were recorded from an aliquot of the cell lysate with 10 s integration time after automatic injection of freshly prepared luciferin (Luciferase Assay System, Promega, Mannheim, Germany). Luciferase activity was measured in triplicate and the relative light units (RLU) were determined.

2.9 Hemolysis of erythrocytes

A/J mouse erythrocytes were isolated from fresh citrate treated blood and washed in phosphate-buffered saline (PBS) by four centrifugation cycles at 800 *g* each for 10 min. at 4° C. The erythrocyte pellet was diluted with PBS to give 6000 erythrocytes per ml . Polymer was diluted in HBG, containing 10 % FCS to reach final concentrations of 0.01 to 0.1 mg/ml. Erythrocyte suspension was added to each well and plates were incubated at 37 °C for 30 min under constant shaking. After centrifugation at 300 *g* for 10 min, 80 µl supernatant was analyzed for hemoglobin release at 405 nm using a microplate plate reader (Spectrafluor Plus, Tecan Austria GmbH, Grödig, Austria). Erythrocyte suspensions treated with HBG / FCS buffer (10 % v/v FCS) only and 1 % Triton-X-100 served as negative and positive controls, respectively.

2.10 Polymer induced erythrocyte aggregation

To study polymer induced aggregation of erythrocytes, erythrocyte pellets were used (see chapter 2.9). Pellets were resuspended in 1 ml HBS and transferred to a 24 well plate. Pictures were taken using an Axiovert 200 microscope (Carl Zeiss, Jena, Germany) equipped with an Infinity 2 CCD camera (Lumenera Corporation, Ottawa, Ontario, Canada). Aggregation was visualized at a 51-fold magnification.

2.11 Ethidium bromide exclusion assay

DNA condensation of OEI-HD and lt-OEI-HD1.2 was measured by quenching of ethidium bromide fluorescence. Ethidium bromide was diluted either in HEPES-buffered saline (HBS, 20 mM HEPES, 150 mM NaCl, pH 7.4) or HEPES-buffered glucose (HBG, 5 % (w/w) glucose, 20 mM HEPES, pH 7.4) to give a final concentration of 0.4 µg/ml. Two ml of ethidium bromide solution were mixed with 20 µg of pDNA. After 3 minutes incubation time, polycation was added and fluorescence intensity was measured over time. The fluorescence was measured on a Cary Eclipse fluorescence spectrophotometer (Varian Deutschland GmbH, Darmstadt, Germany) at λex = 510 nm and λem = 590 nm. Results are given as relative fluorescence and the value of 100% is attributed to the fluorescence of free DNA with ethidium bromide (rel. F = Fsample/FDNA solution).

2.12 Polyplex stability in heparin

Increasing amounts of heparin in 10 µL pure water were added to 100 µL polyplex solution, yielding heparin concentrations of 0.05 to 2.5 international units (IU) per µg plasmid, and incubated for 30 minutes at room temperature. 10 µL of this mixture was applied to a 1% agarose gel containing 0.5 ng/ml of ethidium bromide. Gels were run for 70 minutes at 70 V, prior to the scanning with a gel documentation system (Raytest Isotopenmeßgeräte GmbH, Straubenhardt, Germany). Images were processed with Image J (National Institute of Health, Bethesda, MD, USA).

2.13 Animals

A/J mice (8-12 weeks, 20-23 g) were purchased from Harlan Winkelmann (Bicester, UK) and BALB/c mice (8-12 weeks, 20-23g) were purchased from Janvier (Le Gen-

est-St-Isle, France). Mice were kept under specific pathogen free conditions with a 12 h day/night cycle. Standard breeding chow and water were provided *ad libitum*. All animal procedures were approved and controlled by the local ethics committee and carried out according to the guidelines of the German law of protection of animal life.

2.13.1 In vivo applications for RNAi experiments

A/J mice were injected subcutaneously with $1x10^6$ Neuro2A cells. After 1 week, when tumors had reached approximately 3 mm in size, polymer/siRNA formulations were applied using intravenous injection of complexes via the tail vein (2.5 mg siRNA / kg). OEI-HD/Tf-PEG-OEI/siRNA polyplexes were formed at the OEI-HD/siRNA w/w ratio 0.6/1 containing 10 % targeting Tf conjugate, and were applied at concentrations of 175 µg/ml siRNA, 94.5 µg/ml OEI-HD and 10.5 µg/ml OEI conjugated to Tf-PEG in sterile HBG. The blended carrier in this case contained 43 µg/ml transferrin, i.e. 29 % transferrin by weight of total polymer. Mice were separated into three groups (n = 5 per group): (i) animals treated with therapeutic RAN siRNA, (ii) animals treated with unspecific mismatched Mut siRNA and (iii) non-treated animals. Injections were applied 3 times, every three days. Tumor size was measured over time every day during the whole experiment using a digital caliper. The size is given in length x width x hight (mm^3). Two days following the last administration, animals were sacrificed and blood was collected. For histological analysis one half of each organ explanted (liver, lung, spleen, tumor) was fixed for 24 h in formalin solution (4 % paraformaldehyde in PBS). Afterwards organs were rinsed with water before tissue was dehydrated under vacuum in 70 %, 80 % and 100 % (v/v) ethanol and three times in 100 % xylol. Next, organs were placed in paraffin three times for three hours under vacuum follwed by embedded in paraffin. The remaining organ parts were frozen in liquid nitrogen and stored at -80 °C until further analyzed by Western Blot and qPCR.

2.13.2 In vivo applications of plasmid DNA

2.13.2.1 Low pressure tail vein injections

BALB/c mice were treated intravenously with 200 µl of complexes containing 50 µg plasmid per 20 g mouse. Complexes were formed with L-PEI, as described above, in the L-PEI nitrogen / DNA phosphate (N/P) ratios 5/1, 6/1 and 9/1. Polyplexes with the

following plamids were analyzed (n = 3-4): (i) pCpGLuc RG, (ii) pCpGLuc ccc, (iii) pEGFPLuc RG and (iv) pEGFPLuc ccc.

After intraperitoneal luciferin injection, transfection efficiency was measured over time by a CCD camera (see chapter 2.12) 6 h, 24 h and 72 h after transfection. One supplementary group of animals was sacrificed 24 h post transfection, blood was collected for cytokine expression detection and organs were explanted for additional luciferase expression analysis by tube luminometer.

2.13.2.2 High pressure pDNA tail vein injections

Eight week old female BALB/c mice were placed into a restraining device and anesthetized with isoflurane, which was administered by inhalation (2.0% (v/v) isoflurane in oxygen at a flow of 1 liter/min). Intravenous injections were performed by injecting a plasmid solution (12.5 µg /ml in isotonic sodiumchloride solution (B Braun Meslungen AG, Melsungen, Germany)) in a volume corresponding to 12 % of the total body weight within approx. 5 seconds (this corresponds to 2.4 ml in case of 20 g body weight) using a 30 gauge needle (0.3 x 12 mm) (Sterican, B Braun Melsungen AG).Transfection efficiency was measured over time 10 minutes after intraperitoneal luciferin injection by a CCD camera (see chapter 2.12) for a period of several weeks.

2.13.3 In vivo applications for polymer comparison

For in vivo experiments A/J mice received 1 x 10^6 Neuro2a cells in 100 µl PBS subcutaneously. After tumors reached sizes of about 7 mm in diameter, pDNA complexes containing 50 µg of EGFPLuc plasmid, diluted in sterile HBG, were injected into the tail vein. Injection volume was 10ml/kg bodyweight. The following polymers were applied as complexes: (i) L-PEI, (ii) OEI-HD1 and (iii) lt-OEI-HD1.2.

Forty-eight hours after application animals were sacrificed and blood was collected for liver enzyme detection. Organs were cut into half and luciferase activity was quantified in tumor and organs as described in chapter 2.11.3 with one half. The remaining organ parts were kept for histopathological analysis.

2.14 In vivo Imaging

For luciferase expression experiments mice were anesthetized before imaging at various time points using isoflurane (2.0% (v/v) isoflurane in 1 liter/min oxygen flow). D-Luciferin (30 mg/ml, pH 7) was injected intraperitoneally into the mice at a dose of 150 mg/kg. A greyscale reference image of the mice position was taken prior to bioluminescent image. At 10 min post-luciferin injection bioluminescent signals were assessed at an integration time of 1 to 5 min using an in vivo imaging system that utilizes a cooled-charge-coupled device (CCD) camera (Ivis 100, Caliper Life Sciences, Hopkinton, MA, USA). All images were analysed using the Living Image 2.50 software.

2.15 Luciferase gene expression detection in situ

For additional luciferase expression anaylsis, explanted organs were homogenized in cell lysis buffer (Promega, Mannheim, Germany) with an Ultra-Turrax T8, dispersing tool S8N-5 G (IKA Werke, Staufen, Germany), and centrifuged for 10 min at 4000 g and 4 °C. The supernatant was analyzed for relative light units of luciferase with 30 s integration time after automatic injection of freshly prepared luciferin using the Lumat LB9507 instrument (Berthold, Bad Wildbad, Germany).

2.16 Blood analysis

Blood was collected by heart puncture immediately after sacrification using heparinized syringes. Counts of white blood cells, red blood cells and platelets were determined immediately following sample collection using a Sysmex XE-2100 whole blood analyser (Sysmex, Norderstedt, Germany). For the determination of various blood enzymes samples were allowed to clot at 37 °C for 4 h, overnight at 4 °C, then centrifuged at 3000 g for 20 min at 4 °C and the supernatants were collected for serum analysis. Alkaline and aspartate aminotransaminases (ALT, AST) as well as alkaline phosphatase (AP) were determined using a kinetic UV test from Olympus (Olympus Life and Material Science, Hamburg, Germany). Glutamate Dehydrogenase in plasma was analyzed using a kinetic UV test from Hitado (Hitado Diagnostic Systems, Möhnesee Delecke, Germany).

2.17 Cytokine antibody array

Mouse Cytokine antibody array 1.1 (Ray Biotech Inc., Norcross, GA, USA) consisted of 40 different cytokine and chemokine antibodies spotted in duplicate onto a membrane. Cytokine membranes were processed according to the manufacturer's instructions. In brief, membranes were blocked with 10 % bovine serum albumin in Tris-buffered saline for 30 minutes at room temperature. Membranes were then incubated with mouse plasma samples (10-fold dilution with blocking buffer) for 2 hours at room temperature. After extensive wash with Tris buffered saline (TBS; 0.5M Tris Base, 9 % NaCl, pH 7.6) / 0.1 % Tween 20 and TBS alone to remove unbound cytokines, membranes were incubated with biotin-conjugated anti-cytokine antibodies. Membranes were again washed and then incubated with horseradish-peroxidase-conjugated streptavidin (2.5 pg/ml) for 1 hour at room temperature. Unbound material was washed out with TBS / 0.1 % Tween 20 and TBS. Spots were visualized using a peroxidase substrate detection buffer. Membranes were exposed to Kodak X-Omat radiographic film for 1 minute for image. Membranes were scanned and spots were quantified using NIH image analysis software (Image J). Membranes were compared by normalizing the positive control readings.

2.18 Histopathological examination

For histopathological analysis organs were dehydrated and embedded in paraffin using a Shandon Excelsior (Thermo Fisher, Schwerte, Germany). After paraffin effusion organs were cut into 5 µm thick sections and stained with hematoxylin and eosin for histopathological examination. Pictures were taken using an Axiovert 200 microscope (Carl Zeiss, Jena, Germany), equipped with an Infinity 2 CCD camera (Lumenera Corporations, Ottawa, Canada). Histology was visualized at a 51-fold magnification.

2.19 TUNEL staining of tissue sections

For determination of apoptosis the ApopTag® Fluorescein kit from Qbiogene (Heidelberg, Germany) was used according to the manufacturers' protocol. Briefly, organs were embedded in paraffin as described in 2.15 and cut into 5 µm thick sections. After that sections were deparaffinized and treated with 5 mg/mL proteinase K for 15 min at room temperature and inactivated endogenous peroxidase with 3 % H_2O_2.

Sections were then incubated with TdT enzyme and biotin-labeled and -unlabeled deoxynucleotides at room temperature for 30 min in the dark. Nuclei were counter-stained with 4',6-Diamidino-2-phenylindol (DAPI). Cell imaging was performed using a confocal laser scanning microscope (LSM 510 Meta, Carl Zeiss, Jena, Germany) equipped with an argon and two helium / neon lasers delivering light at 488, 543 and 633 nm, respectively. Light was collected through a 63 x oil immersion objective (Zeiss). Simultaneous scans for fluorescein and DAPI were carried out with an excitation wavelength of 488 nm (emission 505 nm long-pass) and 351 nm (385-470 nm and-pass) respectively. Digital image recording and image analysis were performed with the LSM 5 software (Zeiss).

2.20 Western Blot Analysis

For *in vivo* experiments 50 µg protein per lane was separated by SDS-PAGE under reducing conditions. Proteins were then transferred on a PVDF membrane and blocked with 5 % skimmed milk for one hour at room temperature. Immunostaining was performed using primary Ran antibody (Cell Signaling/Sigma RBI, Taufkirchen, Germany; 1:500) over night at 4°C according to manufacturer's protocol and peroxidase labelled anti-rabbit-IgG (Cell Signaling; 1:2000) as the secondary antibody for two hours. Ran protein was visualized using ECL western blotting detection system (Upstate, Temecula, CA, USA). In addition, for the internal correlation of protein expression of each sample, detection of the housekeeping protein for α-tubulin was performed using mouse primary antibody (Cell Signaling, 1:5000) for one hour following incubation with anti-mouse HRP-secondary antibody (Cell Signaling, 1:5000) for another hour. For quantification, first Ran protein expression of each sample was normalized for the expression of the housekeeping protein for α-tubulin and quantification was performed using ImageJ (National Institute of Health, Bethesda, MD, USA).

2.21 RNA isolation

For evaluation of siRNA experiments RNA from mouse tumors was isolated after stabilising tissue over night at 4°C with RNAlater (Qiagen). Tumors were then snap frozen with liquid nitrogen and crushed in a plastic bag with a hammer. The tumor powder was further processed for RNA isolation by using the NucleoSpin RNA II Kit (Macherey Nagel) as described by the manufacturer. Briefly, tissue was denaturated by adding of ß-mercaptoethanol. Viscosity was reduced by filtration of the lysate

through filter units. 70 % ethanol (v/v) was used for precipitation of nucleic acids and precipitate was loaded on silica filters. The matrix was desalted and then DNA was removed by treatment with DNAse. Silica matrix was then washed and RNA diluted with RNAse free water.

2.22 Reverse transcription of RNA to cDNA

RNA was reverse transcribed by High Capacity RNA-to-cDNA Master Mix (Applied Biosystem, Darmstadt, Germany) as described by the manufacturer. Briefly, 100 ng of extracted RNA was added to 4 µl of a cDNA synthesis reaction mixture containing random hexamers (total volume of 20 µl). Samples were then incubated at 37°C for 60 min, followed by 5 min at 95°C.

2.23 Real-time polymerase chain reaction (RT-PCR)

For determination of the most stable housekeeping gene, preliminary RT-PCR was performed using the four commonly used housekeepers ATP synthase, ß-subunit (ATP5B), Cytochrom C1 (CYC1), ß-Actin (ACTB), glyceraldehydes-3-phosphate dehydrogenase (GAPDH). All primers were designed using the ProbeFinder qPCR assay design software, which is freely accessible at www.universalprobelibrary.com, and constructed as follows:

Ran:

forward 5´ACCCGCTCGTCTTCCATAC

reverse 5´ATAATGGCACACTGGGCTTG, 121 bp

ATP5B:

forward 5´GGCACAATGCAGGAAAGG

reverse 5´TCAGCAGGCACATAGATAGCC, 77 bp

CYC1:

forward 5´ACCTGGTGGGAGTGTGCTAC

reverse 5´CATCATCATTAGGGCCATCC, 84 bp

ACTB:

forward 5´CTAAGGCCAACCGTGAAAAG

reverse 5´ACCAGAGGCATACAGGGACA, 104 bp

GAPDH:

forward 5´AGCTTGTCATCAACGGGAAG

reverse 5´TTTGATGTTAGTGGGGTCTCG, 62 bp

RT-PCR amplification mixture (20 µl) contained template cDNA (1:500 dilution), 200 nM forward and reverse primer, 100nM Universal Probe Library probe and the Light Cycler 480 Probes Master. Reactions were run on a Light Cycler 480 (Roche Diagnostics GmbH, Mannheim, Germany). RT-PCRs were performed with a predenaturing step of 95°C for 10 minutes and 45 cycles of 95°C (10 seconds), 60°C (30 seconds), and 72°C (10 seconds) followed by a cooling step at 40°C for 1 minute. The crossing-point (Cp) value from each signal was calculated based on the second derivative maximum method performed by the LightCycler 480 quantification software (version 3; Roche Diagnostics GmbH) followed by geNorm analysis (geNorm VBA applet: http://medgen.ugent.be/genorm/) was used for detection of the most stable gene. A set of serially diluted cDNAs was used to construct a 4 data-point standard curve for every PCR system.

After detection of the most stable housekeeping gene, a multiplex assay for simultaneous detection of target and reference gene was used. For ACTB new primers were designed using the ProbeFinder qPCR assay design software:

forward: 5´GCCAACCGTGAAAAGATGAC

reverse 5´ GAGGCATACAGGGACAGCAC, 95 bp.

All RT-PCR procedures were performed as described above.

2.24 Statistical analysis

All experiments were performed at least in triplicate. Values are expressed as means ± standard deviation (SD). Statistical significance of differences was evaluated using Graph Pad Prism 4 software (Graph Pad Software, San Diego, CA, USA). Experimental groups were compared by Mann-Whitney-U test. The level of significance is indicated with a single asterisk if $p \leq 0.05$, two asterisks if $p \leq 0.01$, and three asterisks if $p \leq 0.001$.

3 Results

3.1 Therapy with siRNA

3.1.1 OEI-HD as a delivery vector

3.1.1.1 Biophysical properties of OEI-HD/siRNA polyplexes

Particle size and zeta potential of complexes formed at various polymer (OEI-HD, OEI-HD/Tf-PEG-OEI) to siRNA ratios prepared in HBG were determined using dynamic laser-light scattering (Table 3-I). Non-targeted OEI-HD/siRNA complexes tend to aggregate at a polymer/siRNA ratio of 0.5/1. In contrast, the targeted (10 weight % Tf conjugate) OEI-HD/siRNA formulations, showed no aggregation and sizes of about 160 nm were achieved. For polymer/siRNA ratios of 1/1 as well as 2/1 (w/w) both, targeted and non-targeted OEI-HD/siRNA formulations appeared smaller in size and showed multimodal distribution (1 - 1000 nm). The low scattering intensity implies that predominantly smaller particles are present which is in accordance with findings seen by (Meyer at al. 2008).

All standard OEI-HD/siRNA formulations had positive zetapotentials in the whole range tested (Table 3-I). In correlation with the increasing polymer/siRNA ratio, the charge of the particles increased continuously from +2.1±0.6 mV at the ratio 0.5/1 to +19.4±4.0 mV at the ratio 2/1. In contrast, Tf containing OEI-HD/siRNA formulations showed lower zeta-potential due to transferrin which also serves as a shielding agent (Kircheis et al. 2001). Using 10 weight % Tf and a polymer/siRNA (w/w) ratio of 0.5/1, particles were even slightly negatively charged. At higher ratios (up to 2/1) the Tf-containing formulations showed increased zetapotentials of up to 11 mV.

w/w ratio:	*0.5/1*	*1/1*	*2/1*
		Particle size (nm)	
OEI-HD	*1193 ± 217*	**	**
OEI-HD/ Tf*	*155 ± 3*	**	**
		Zeta Potential (mV)	
OEI-HD	*+ 2.1±0.6*	*+ 11.5±2.5*	*+ 19.4±4.0*
OEI-HD/ Tf*	*- 6.0±3.0*	*+ 7.8±2.3*	*+ 11.3±1.6*

Table 3- I: *Particle size and zeta potential of OEI/siRNA formulations prepared in HBG. Formulations were prepared in HBG using the same concentration of siRNA as for in vivo applications; 10 µg siRNA in 40 µL HBG were used for measurement of particle size (Z-Average). For zeta potential, 1 mL 1 mM NaCl was added to the formulations prepared in HBG prior to measurement.*

** 10 weight % of OEI-HD are present as Tf-PEG-OEI conjugate.*

*** no data presentation: particles appeared smaller and showed multimodal distribution (1 - 1000 nm) - no in depth evaluation was performed.*

3.1.1.2 Preliminary in vivo studies

Preliminary studies on dose finding revealed that acute toxicity of the polymer was significantly reduced if applied as polyplex in a polymer/siRNA ratio of 0.5/1. Seventy µg of free polymer killed mice within minutes after application. In contrast, animals receiving 70 µg OEI-HD complexed to siRNA even survived two applications at an 24 h interval. We also carried out preliminary experiments using Tf-free complexes. OEI-HD siRNA formulation was tested in a subcutaneous Neuro2A tumor mouse model using OEI-HD/siRNA formulations with polymer/siRNA ratio 0.5/1. Treatment by intravenous tail vein injection (2.5 mg formulated siRNA / kg applied 3 times at 3 days intervals) was started after subcutaneous tumors had reached a size of about 3 mm in diameter. Although transferrin free OEI-HD/siRNA complexes aggregate and therefore are less suitable for intravenous injection (see also Table 3-I), mice survived applications, but nevertheless showed only 40 % of Ran protein knockdown in tumors. The same conditions were chosen for analysis of *in vivo* potential of transferrin-

shielded OEI-HD siRNA formulation, using 10 % Tf conjugate. Complexes with si-CONTROL and untreated animals served as a control here.

3.1.1.3 Tumor growth reduction in vivo

Data representing the tumor growth following the systemic treatments described in 2.13.1 are shown in figure 3-1.

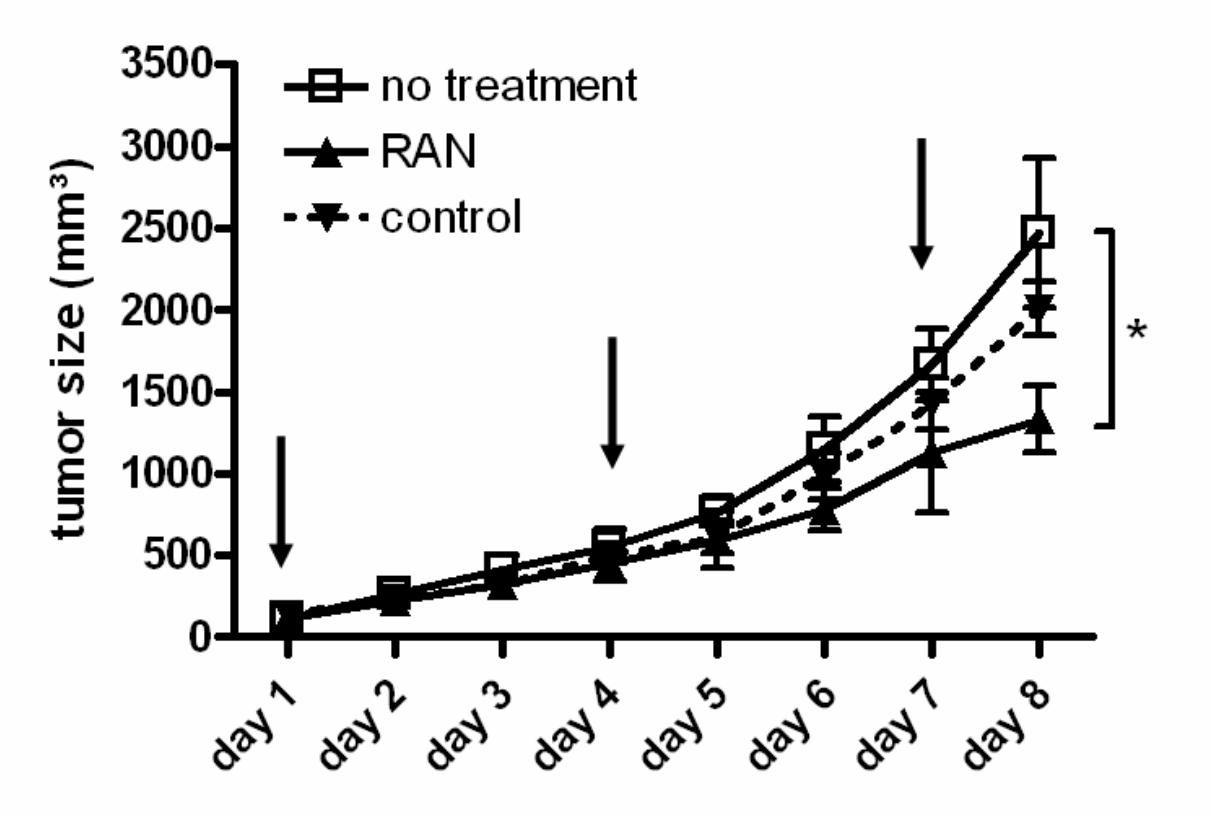


Fig. 3-1: *Tumor growth reduction after systemic treatment of experimental animals by using Tf-targeted OEI-HD polyplexes of RAN siRNA compared with siCONTROL or non-treated A/J mice. Therapeutic application started after tumors had reached a size of about 125 mm³ and was repeated 3 times every 3 days. Tumor volume was measured using a digital caliper. Mean tumor volume and SD of five animals are shown for each day of tumor growth. Arrows indicate days of application. Data marked with asterisk are statistically significant relative to the non treated group as calculated by ANOVA (*, $p < 0.05$).*

Treatment with the RAN siRNA but not siCONTROL formulation significantly reduced the tumor growth. In addition, no obvious toxicity was observed in animals during the whole treatment period. Behavior of the animals and weights of mice were not significantly different among the three groups of animals.

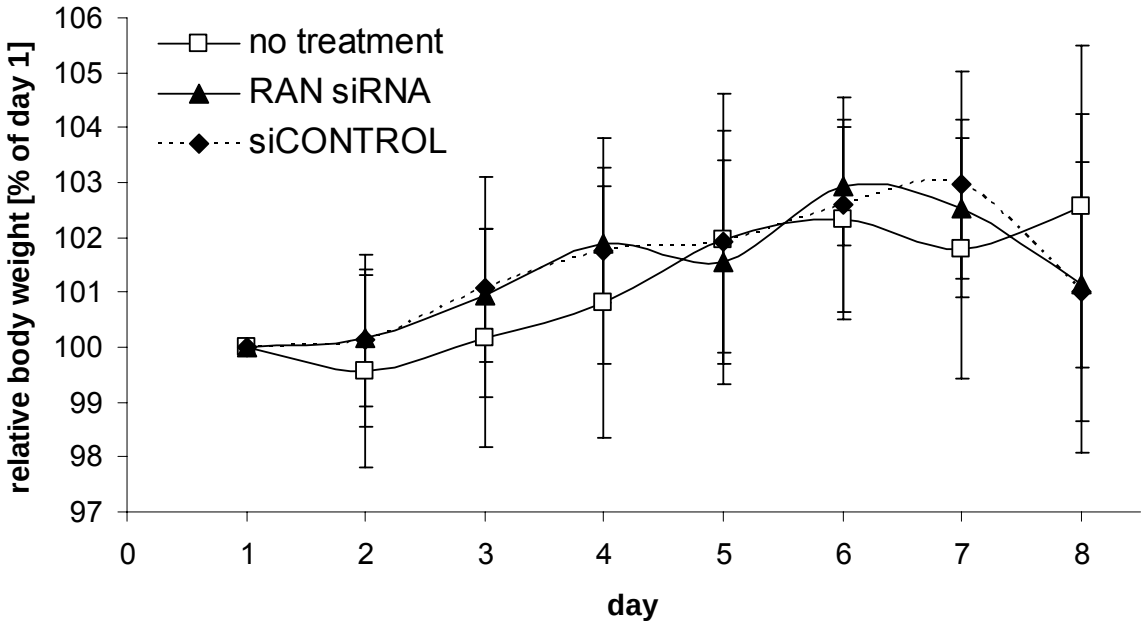
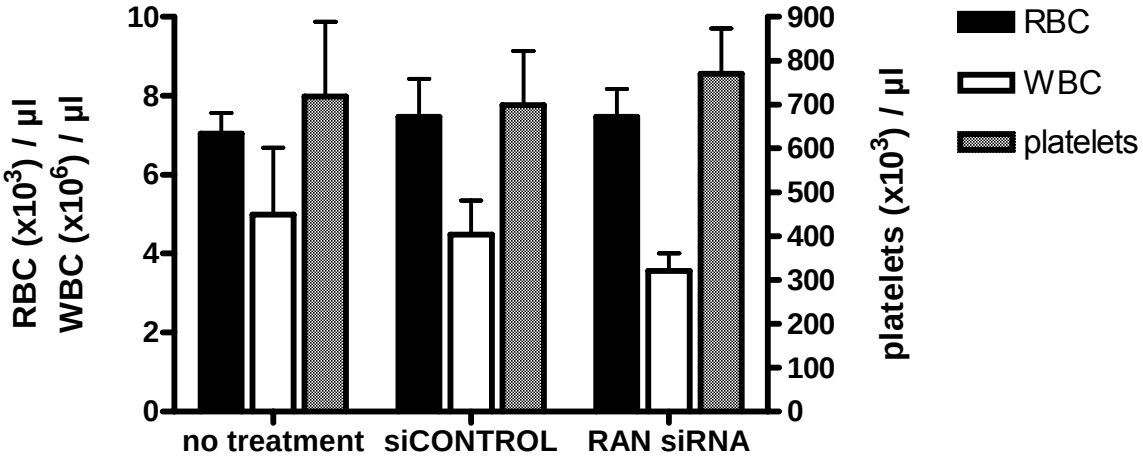


Fig. 3-2: Relative body weight of mice during systemic treatment. Mean values and SD of five animals are shown for each day.

3.1.1.4 Blood cell and liver enzyme levels in treated mice

Counting of blood cells and determination of various liver enzymes (figure 3-3) did not reveal any significant changes in hematology parameters nor in the activities of AST, ALT, AP, or GLDH.

A



B

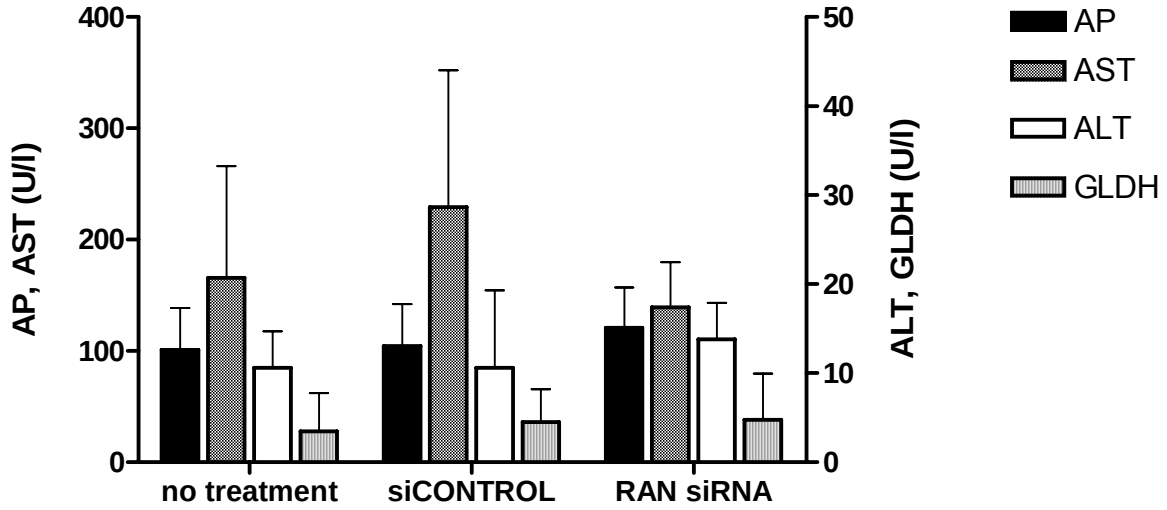


Fig. 3-3: *Biocompatibility (hematology and liver enzyme parameters) of Tf-targeted OEI-HD/ RAN siRNA or siCONTROL formulation, in comparison to non-treated animals. Whole blood samples were obtained from A/J mice 24 hours following the last of three injections and measured for blood counts (A) and liver enzymes (B). Data shown are mean levels ± SD out of 5-6 animals.*

3.1.1.5 Reduction of RAN protein expression in Neuro2A tumors

The effects of systemically applied RAN siRNA or siCONTROL formulations were determined by western blot analysis of Ran protein in Neuro2A tumors and in livers of the same animals at 24 hours following the last application in comparison to non-treated animals.

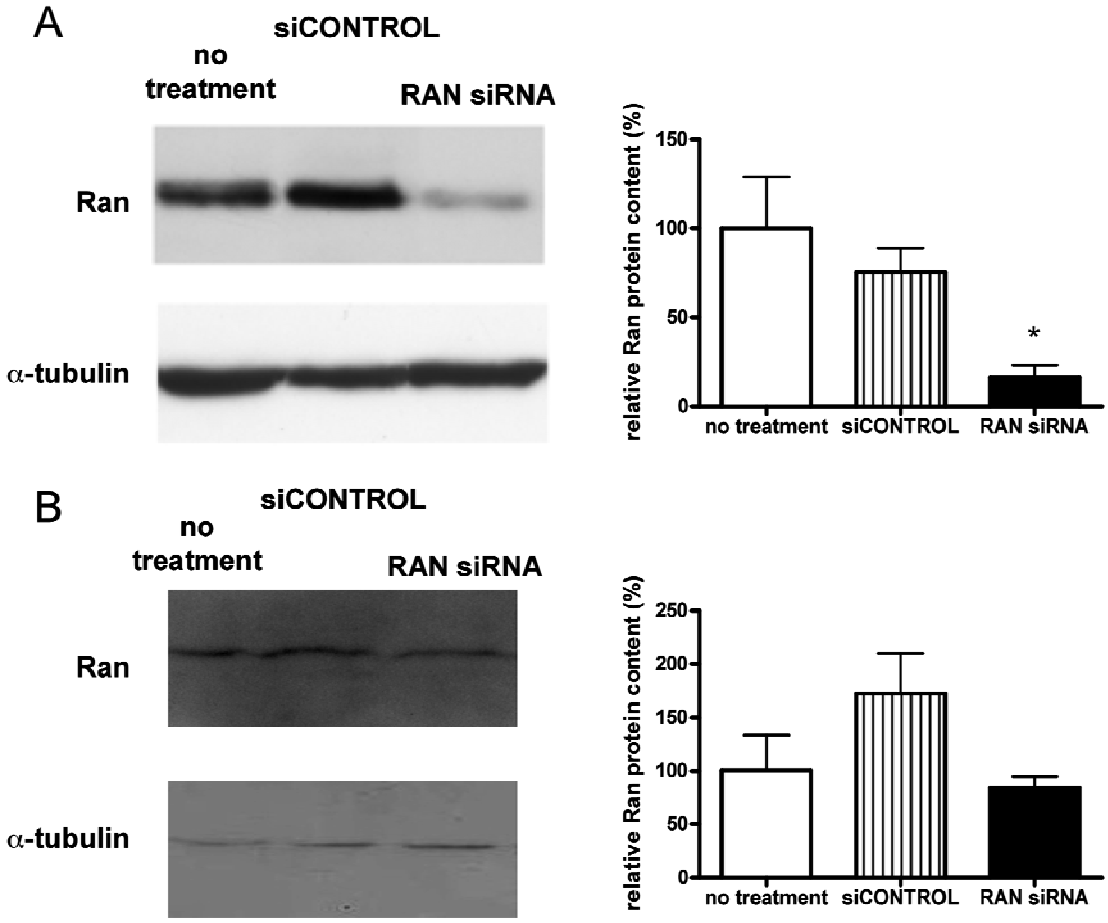


Fig. 3-4: *Analysis of Ran protein expression in A/J-mice by western blot, following application of Tf-shielded RAN siRNA polyplexes, in comparison to siCONTROL polyplex-treated and non-treated animals. Protein levels (A) in Neuro2A tumors, and (B) in livers of the same animals. Protein expression levels of each sample were normalized for the expression of α-tubulin set as 100 %. Samples were assessed one day after the last of three systemic applications. Data shown are mean ± SD. All data sets collected have a group size of five animals. Asterisks indicate statistical significance compared with the non treated group (*, $p < 0.05$; ANOVA).*

The transferrin shielded RAN siRNA formulations reduced Ran protein expression by more than 80 % in tumors of treated mice as compared to non-treated animals. Animals treated with the analogous siCONTROL formulation did not show a significant Ran protein reduction in tumors (figure 3-4A). Ran protein expression in livers was not affected in any group (figure 3-4B). Transferrin free RAN siRNA formulations as additional control only led to knockdown of Ran protein of 40 % in comparison to 20 % with transferrin-shielded Ran siRNA polyplexes (figure 3-5).

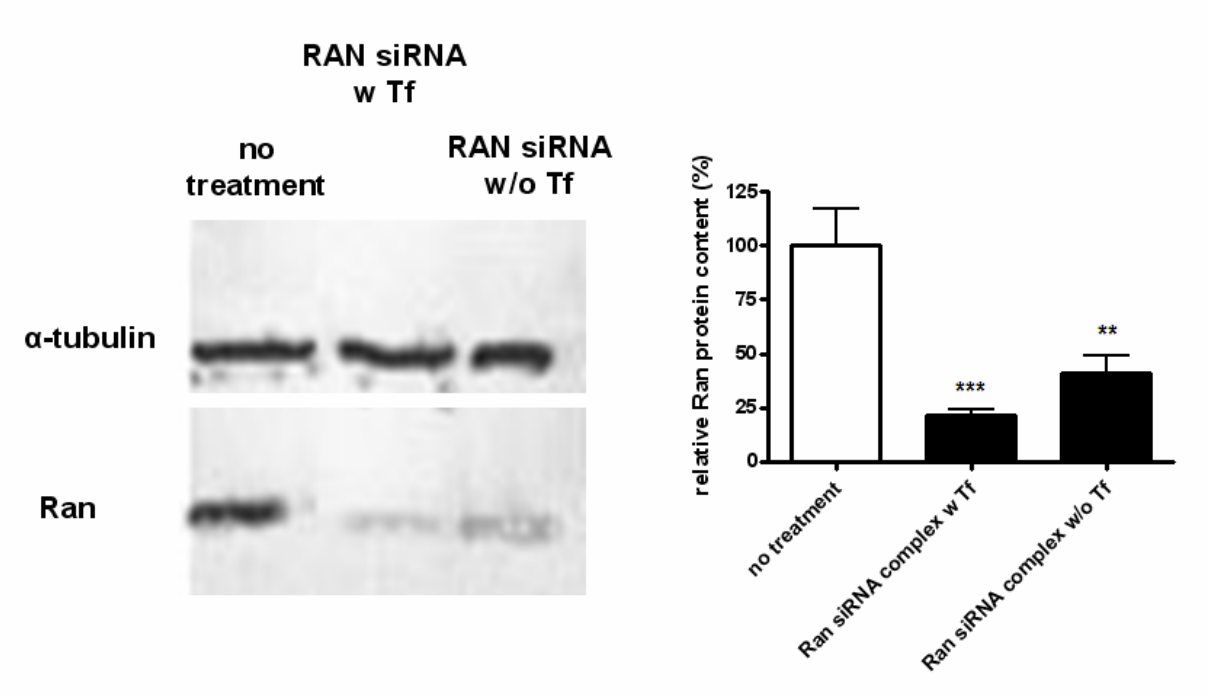


Fig. 3-5: *Analysis of Ran protein expression in tumors of A/J mice following application of Tf-shielded and unshielded Ran siRNA polyplexes in comparison to untreated animals (n = 3).*

3.1.1.6 Reduction of RAN mRNA expression in Neuro2A tumors

In addition to Western blot results of Ran protein knockdown in tumors, qPCR anaylsis was performed to analyze the reduction of Ran mRNA. For normalization at first the four possible reference mRNAs glyceraldehydes-3-phosphate dehydrogenase (GAPDH), ß-actin (ACTB), cytochrome c-1 (CYC1) and ATP synthase, beta subunit (ATP5ß) were analyzed for minimal variations across siRNA treatment using the geNorm software (Vandesompele et al. 2002). The most stably expressed gene was ACTB, resulting in M = 0.277, M describing the average expression stability (lowest for the most stably expressed genes). The expression of ATP5B (M = 0.344), CYC1 (M = 0.312) and GAPDH (M = 0.389) were less stable. Hence ACTB was the best candidate for normalization of Ran mRNA.

Due to this results, for the following qPCR experiment a dual color approach was chosen to determine Ran mRNA and ACTB mRNA levels concurrently. Analysis showed a significant ($p < 0.001$) knockdown of Ran mRNA in Neuro2A tumors treated with Ran siRNA polyplexes, supporting the thesis of RNA interference (figure 3-6).

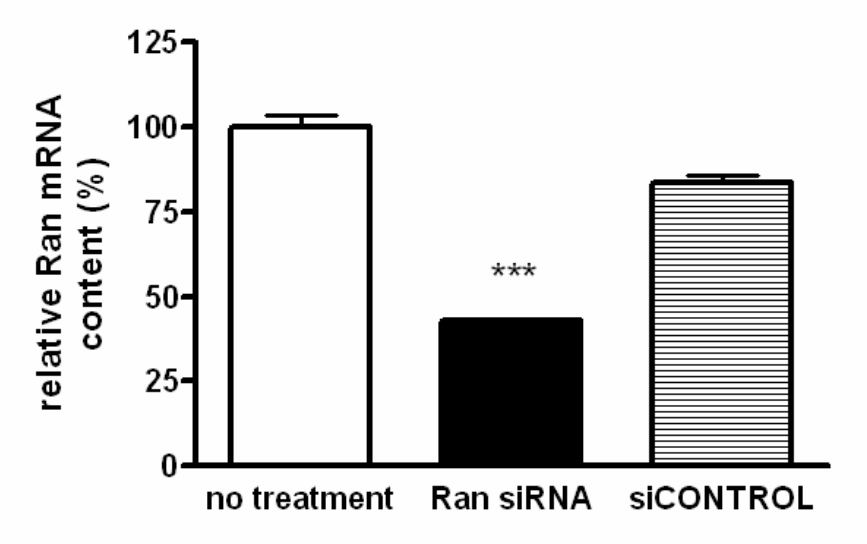


Fig. 3-6: *Content of Ran mRNA in Neuro2A tumors measured by qPCR. All values are normalized to ACTB content within the same samples. Values are mean ±SD out of 6 animals. Significance was determined using 1way ANOVA with Bonferroni´s Multiple Comparison; *** p < 0.001.*

3.1.1.7 Apoptosis in tumors of RAN siRNA treated mice

In order to analyze the cellular consequences of siRNA-mediated silencing of the RAN gene expression, TdT-mediated dUTP biotin nick-end labeling (TUNEL) analysis of treated tumor tissues was performed, which allows for the detection of apoptotic cells. In order to corroborate the notion that this effect was specific to the tumor cells, liver tissue samples were TUNEL stained and likewise analyzed.

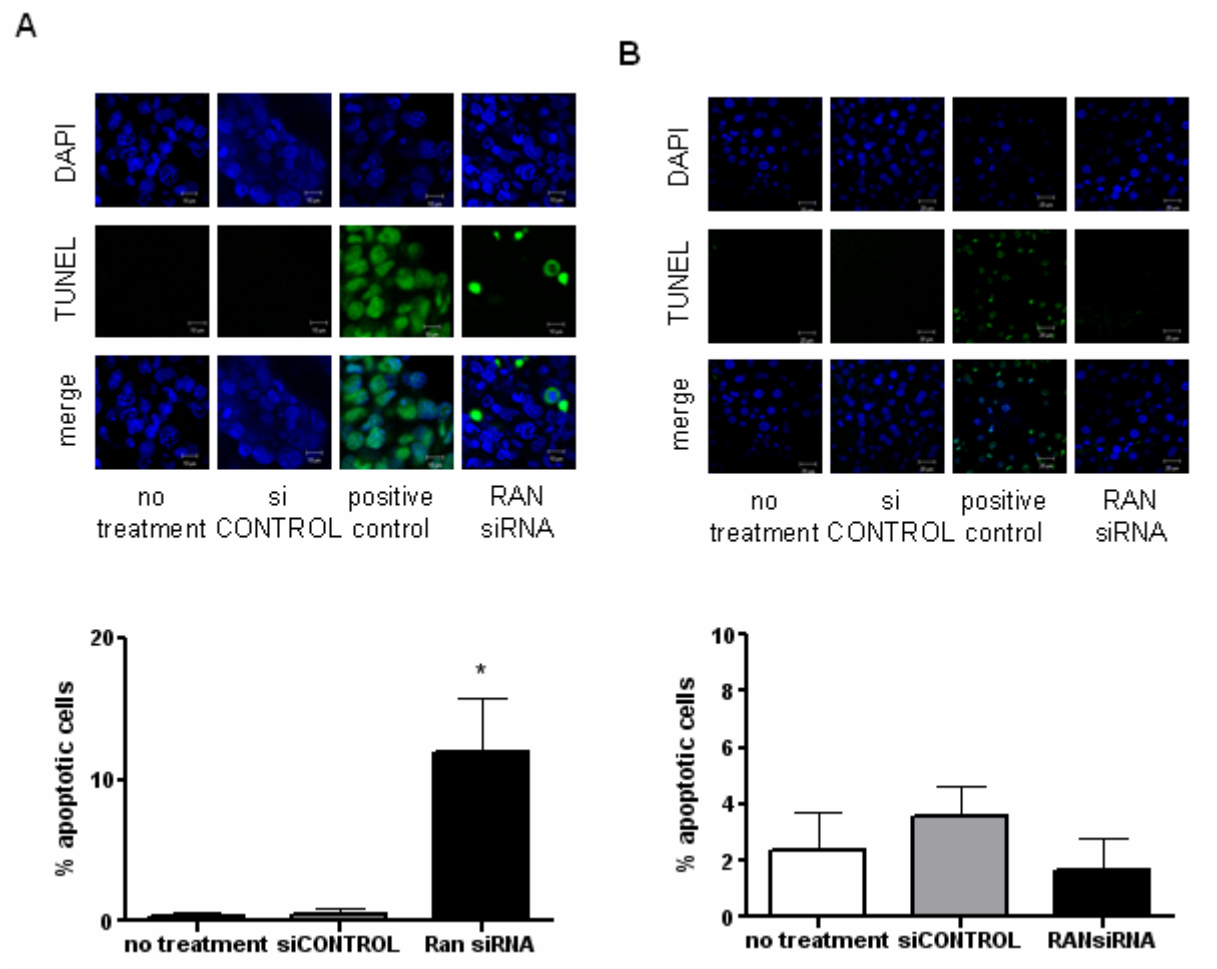


Fig. 3-7: *Analysis of apoptosis in Neuro2A tumors (A) and liver (B) following application of Tf-shielded RAN siRNA polyplexes compared to siCONTROL polyplexes or non-treated animals by TUNEL staining of 5 µm tissue sections. The apoptotic fraction was derived by counting the TUNEL-positive fractions (green) of 100 cells from up to 20 random fields in each tumor section. DAPI was used for nuclear counterstaining (blue). Upper part of the figure shows selected examples of staining demonstrating the average findings. Lower part shows the statistical evaluation of all fractions analyzed. Values are means from five animals per group ± SD. Asterisk marks statistical significance compared with the non treated group (*, $p < 0.05$).*

The number of apoptotic cells in the tumors treated with RAN-specific siRNA was increased to 12 % compared with non-treated animals (0.2 %) (figure 3-7A). Furthermore, non-treated animals and animals treated with the siCONTROL formulation showed no significant increase in apoptotic cells within the tumors. In contrast to apoptosis in tumors, therapeutic RAN siRNA did not significantly affect the liver cells of the same animal (figure 3-7B). Neither the specific RAN siRNA nor the control

siRNA formulation changed the number of apoptotic cells within the liver as compared to non-treated animals.

3.1.2 OEI-HA10 as a delivery vector

Due to preliminary in vitro data with siRNA on Neuro2A cells that led to promising knockdown and cytotoxicity data (unpublished data, Alexander Philipp and Arkadi Zinchenko), this vector was tested for its in vivo delivery potential similarly to OEI-HD1 (see chapter 3.1.1).

3.1.2.1 Acute toxicity of free OEI-HA10 polymer

Polymer concentrations of 50 µg, 75 µg and 100 µg (per 20 g body weight, in 200 µl HBG) were intravenously injected up to three times every 24 h in BALB/c mice. Of these concentrations the latter (100 µg/20g) led to immediate sealing of the vein and was therefore not possible to apply. The two lower concentrations were possible to be applied even three times. Over the first two days of application animals appeared healthy and did not show any signs of acute toxicity, like loss in body weight. Not until the third application could necrotic tails be observed in animals treated with 50 and 75 µg free OEI-HA10. Our hypothesis is that low concentrations of OEI-HA10 did not lead to immediate damage of the vein. However after repeated applications at the same injection site these injuries multiply, leading to necrosis.

3.1.2.2 Lytic activity of free OEI-HA10 polymer

In order to measure membrane destabilization activity of OEI-HA10, lytic activity was measured on erythrocytes. Freshly isolated murine erythrocytes (6000 erythrocytes / ml) were incubated with free polymer of final concentrations 0.01, 0.025, 0.05 and 0.1 mg/ml in HBG (pH 7.4), containing 10% (v/v) FCS for 45 minutes at 37°C. For comparison, other polymers like L-PEI and OEI-HD1 that were tolerated well *in vivo* served as a control. While L-PEI and OEI-HD1 did not lead to more than 20 % erythrocyte lysis even if applied in concentrations of up to 0.1 mg/ml, OEI-HA10 showed a strongly enhanced lytic effect. Already at concentrations of 0.05 mg/ml erythrocyte lysis of about 75 % was detected.

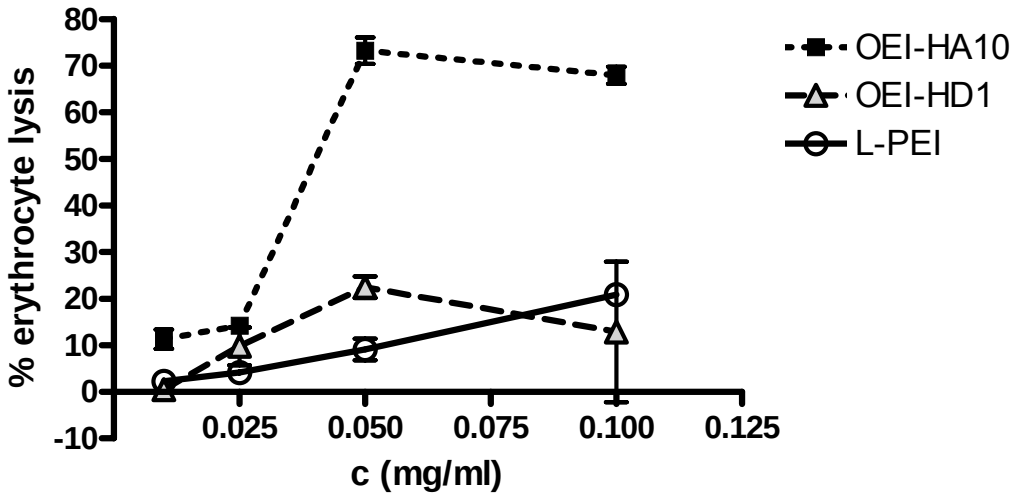


Fig. 3-8: *Quantification of hemoglobin release at 450 nm, after incubation of OEI-HA10 polymer with murine erythrocytes for 45 minutes at 37°C. 100% lysis refers to TritonX treatment of erythrocytes.*

In summary, addition of the hexyl chain strongly increased the membrane destabilising activity of OEI. The toxicity of OEI-HA10 and the corresponding complexes seems to be related to the high membrane destabilizing activity of OEI-HA10. This leads to membrane blebbing and cell lysis.

3.1.2.3 Erythrocyte aggregation induced by free OEI-HA10 polymer

The influence of free OEI-HA10 polymer on erythrocyte morphology was analyzed by incubation of freshly isolated murine erythrocytes as described in 3.1.2.1. Aggregation was visualized by microscopy. No aggregation was detected with the analyzed polymer concentrations, however with higher concentrations of 0.05 and 0.1 mg/ml due to lysis almost no erythrocytes were detected.

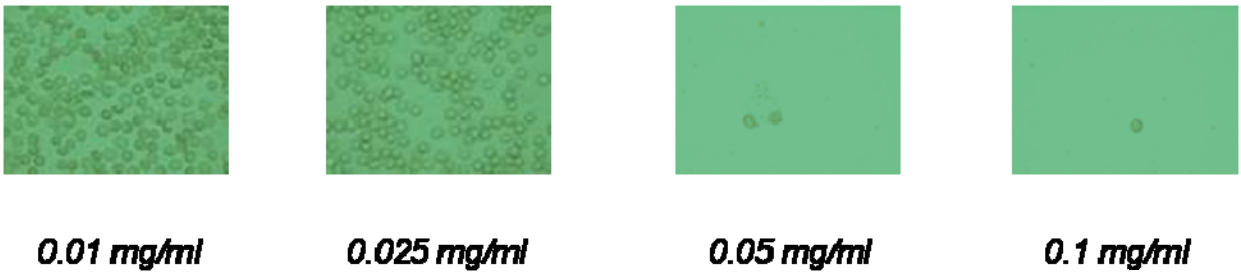


Fig. 3-9: *Effect of OEI-HA10 polymer on erythrocyte aggregation. Murine erythrocytes were incubated with free polymers at different polymer concentration for 45 min at 37°C. Morphology was visualized microscopically with a 51-fold magnification using a Axiovert 200 microscope.*

3.1.2.4 Acute toxicity of OEI-HA10 / Ran siRNA polyplexes

If applied in a w/w ratio of 2 (5 mg OEI-HA10 / kg) in A/J mice animals did not survive for more than 30 minutes. Pathological analysis revealed indications of ischemic stroke in these animals (unpublished data) while all other organs did not show any macroscopically detectable changes. It might be worthwhile to mention that complexes formed in a w/w ratio of 2/1 showed positive surface charges of about 13 mV, while complexes formed at w/w 1 had a negative zetapotential of about - 18 mV. With regard to pathological findings after systemic complex applications, a negative surface charge seems to be much better tolerated *in vivo* than positively charged polyplexes. Importantly, the negatively charged complexes with a w/w ratio of 1 showed optimal transfection efficiency in vitro.

In contrast, polyplexes formed with siRNA in a w/w ratio of 1/1 were tolerated well in A/J mice, even if applied three times every 24 h. No necrotic changes in tail tissues were detectable after polyplex applications.

Since the positive charge of polyplexes is supposed to improve the stability of nucleic acids against degradation as well as the ability to penetrate into cells, the application of positively charged formulations is desired. For enhancement of tolerability of such polyplexes the block-copolymer pluronic F127 was introduced into formulations. Due to its amphiphilic character this copolymer displays surfactant properties and at concentrations above critical micelle concentration (CMC) these copolymers self-assemble into micelles. Additionally, it is able to adsorb on hydrophobic areas and hydrophilize the surfaces.

The tolerated dose of OEI-HA10 was possible to increase by formulating complexes together with pluronic F127 (w/w OEI-HA10 / F127 = 1). This led to formulations that were tolerated much better *in vivo*. Even a w/w ratio of OEI-HA10 / siRNA of 2 was tolerated with this mixture. Nevertheless, one out of six animals showed a paralysed left body side and died within 30 minutes following application. Macroscopic examination of the brain exhibited an polythrombotic stroke in this animal. Another animal showed necrotic tail tissue after complex application (unpublished data).

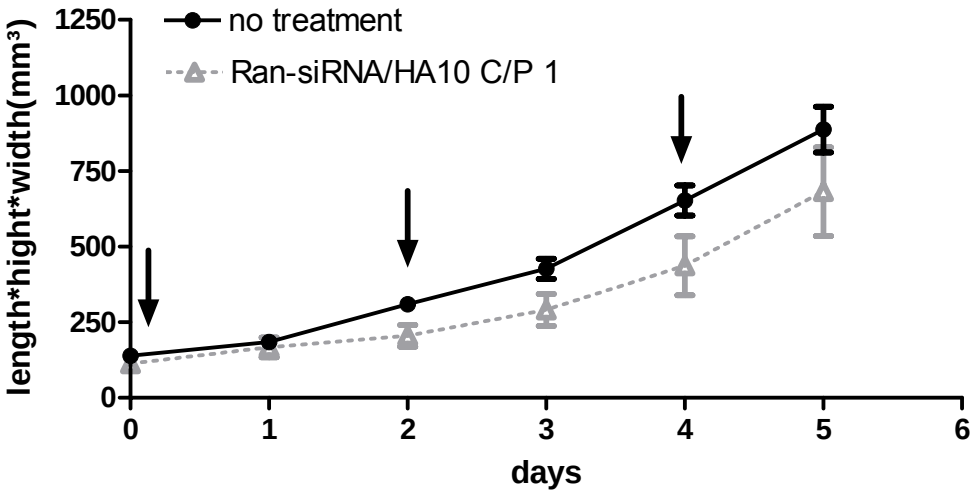
Polyplexes in a w/w of 1 (with and without pluronic F127) were tolerated well in all animals. Complexes could be applied three times, every 48 h without influencing appearance, behavior or body weight of the mice.

3.1.2.5 Tumor growth reduction after polyplex application in vivo

Similarly to therapeutic studies described in chapter 3.1.1 with the polymer OEI-HD, 8 week old female A/J mice bearing a subcutaneous Neuro2A tumor were treated with OEI-HA10 / Ran siRNA complexes or OEI-HA10 / Ran siRNA / pluronic F127 complexes respectively.

Treatment by intravenous tail vein injection (2.5 mg formulated siRNA / kg applied 3 times at 2 days intervals) was started after tumors had reached a size of about 3 mm in diameter. Figures below show tumor growth curves of mice over time (figure 3-10 A, B). Compared to animals without any treatment, mice receiving 3 applications of Ran siRNA complexed to OEI-HA10 in a w/w ratio of 1, showed reduced tumor growth already at day 2 after the first injection. In contrast, pluronic F127 conjugated complexes had no effect on tumor growth.

A



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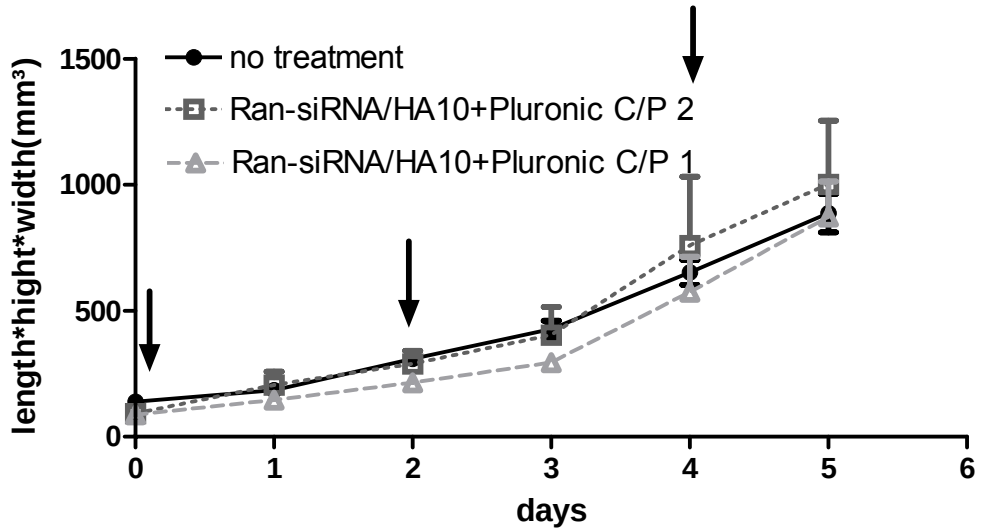


Fig. 3-10: *Tumor growth reduction after systemic treatment of experimental animals by using OEI-HA10 polyplexes of RAN siRNA or non-treated A/J mice. Figure (A) displays results obtained with OEI-HA10 complexes, whereas in figure (B) pluronic F127 in a w/w ratio of 1 was added to the OEI-HA10 complexes. Therapeutic application started after tumors had reached a size of about 3 mm in diameter and was repeated 3 times every 2 days. Tumor volume was measured using a digital caliper. Animals were sacrificed 24 hours following the last application. Mean tumor volume and SD are shown for each day of tumor growth. Arrows indicate days of application.*

3.1.2.6 Liver enzyme levels in treated mice

For determination of liver toxicity, activities of various liver enzymes was analysed 24 hours following the last of three injections. None of the treated groups showed significant differences in alkaline phosphatase (AP), aspartate or alanine transaminase (AST and ALT) or glutamate dehydrogenase (GLDH), indicating tolerability of complexes.

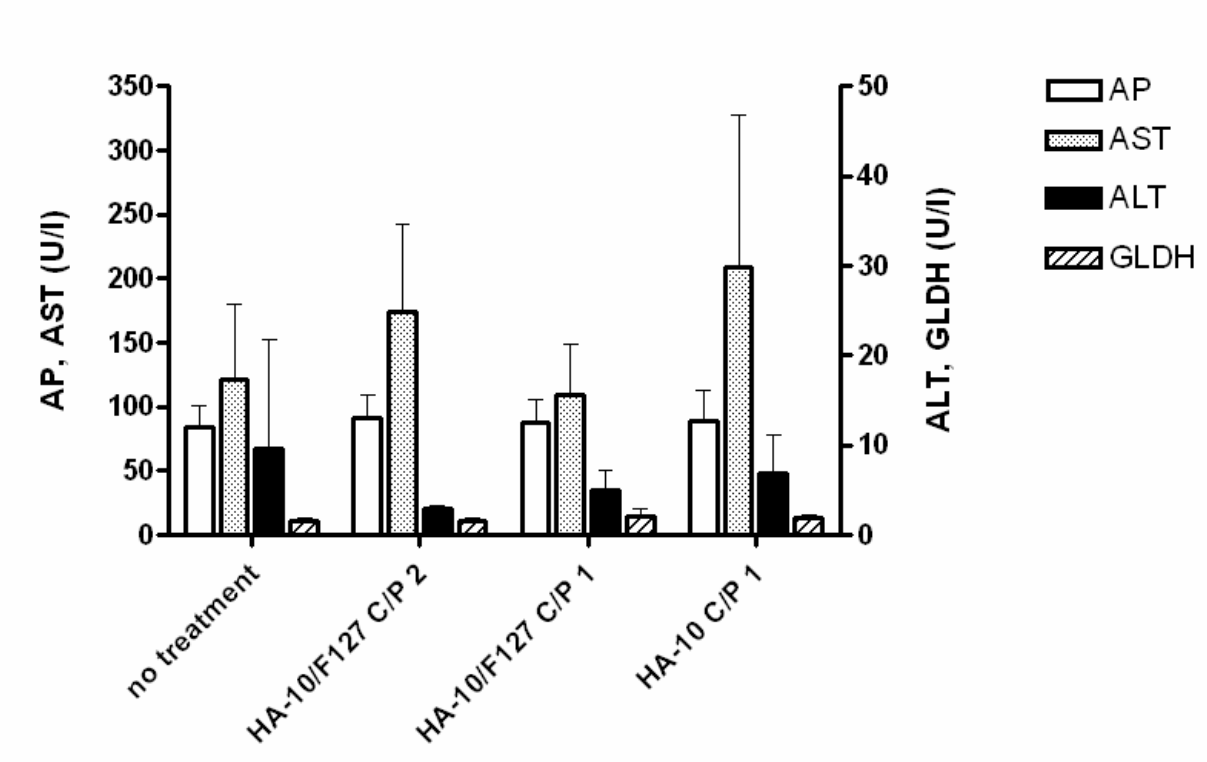


Fig. 3-11: *Liver enzyme activities of HA10 formulation, complexed with Ran siRNA in comparison to non-treated animals. Blood samples were obtained 24 hours following the last of three injections, plasma was separated and measured for liver enzyme activities. Data shown are mean levels ± SD of 5-6 animals.*

3.1.2.7 RAN protein expression in Neuro2A tumors

The effects of systemically applied Ran siRNA formulations were determined by western blot analysis of Ran protein in Neuro2A tumors at 24 hours following the last application (as described in 3.1.2.5) in comparison to non-treated animals. Ran protein content was not affected with any of the applied OEI-HA10 formulations as shown in figure 3-12.

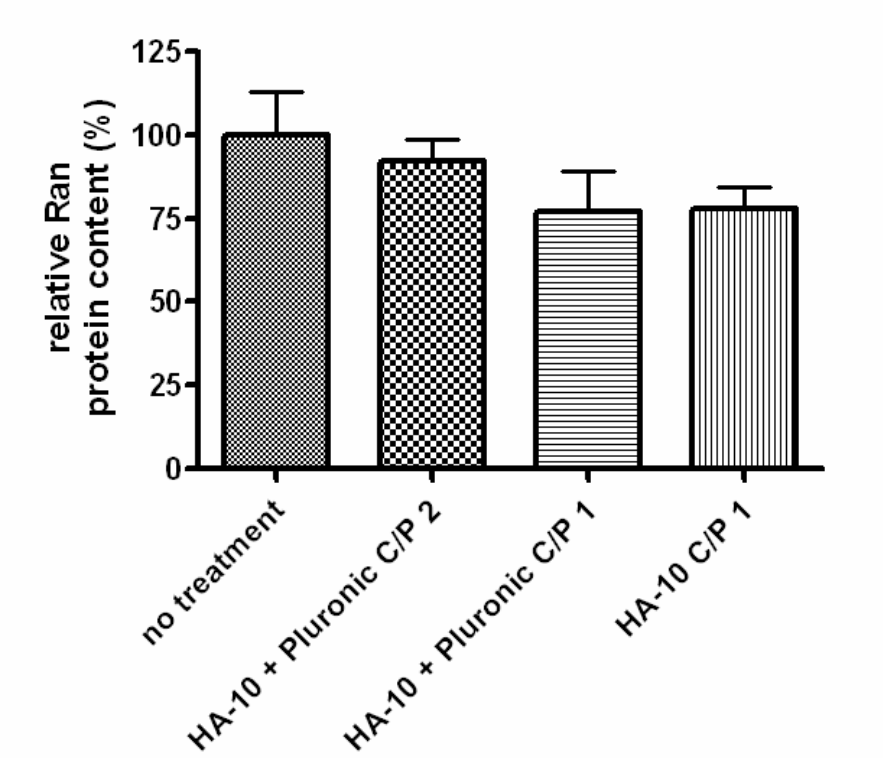


Fig. 3-12: *Analysis of Ran protein expression in Neuro2A tumors in A/J-mice by western blot, following application of Ran siRNA polyplexes, in comparison to non-treated animals. Protein expression levels of each sample were normalized for the expression of α-tubulin set as 100 %. Samples were assessed one day after the last of three systemic applications. Data shown are mean ± SD of at least 5 animals.*

3.1.2.8 Reduction of RAN mRNA expression in Neuro2A tumors

In addition to Western blot results of Ran protein knockdown in tumors, qPCR anaylsis was performed to analyze the reduction of Ran mRNA. For normalization at first the four possible reference mRNAs glyceraldehydes-3-phosphate dehydrogenase (GAPDH), ß-actin (ACTB), cytochrome c-1 (CYC1) and ATP synthase, beta subunit (ATP5ß) were analyzed for minimal variations across siRNA treatment using the geNorm software (Vandesompele et al. 2002). The most stably expressed gene was ACTB, resulting in M = 1.599, M describing the average expression stability (lowest for the most stably expressed genes). The expression of ATP5B (M = 3.525), CYC1 (M = 1.740) and GAPDH (M = 1.636) were less stable. Hence ACTB was the best candidates for normalization of Ran mRNA.

The following dual color qPCR with ACTB as housekeeping gene revealed no knockdown of Ran mRNA with any of the complexes in tumor tissues, confirming previous findings on Ran protein levels by western blot analysis (see 3.1.2.6).

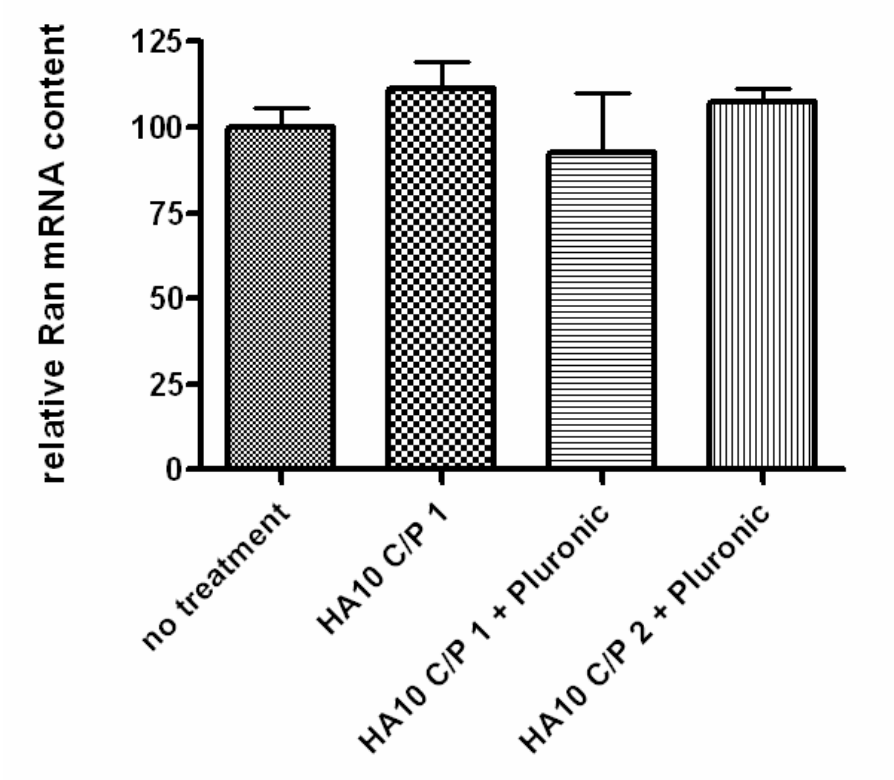


Fig. 3-13: *Detection of Ran mRNA content in tumors of A/J mice, 24 h after the last of three applications. Levels are normalized to ACTB mRNA content. Data are mean levels ± SD from 6 animals per group.*

3.1.2.9 Histopathological analysis of tumors

To analyze the morphology of treated and untreated Neuro2A tumors, 24 h after the last of three injections (see chapter 3.1.2.5) tumors were explanted and embedded in paraffin. After cutting into 5 µm sections and staining with hematoxylin and eosin, pictures were taken for histopathological analysis. In case of OEI-HA10 / RansiRNA treated tumors changes in the histology of tumors were visible. Parts of the tumor tissue showed necrotic areas, being in line with the findings on tumor growth inhibition. No such changes were found for the pluronic F127 modified complexes.

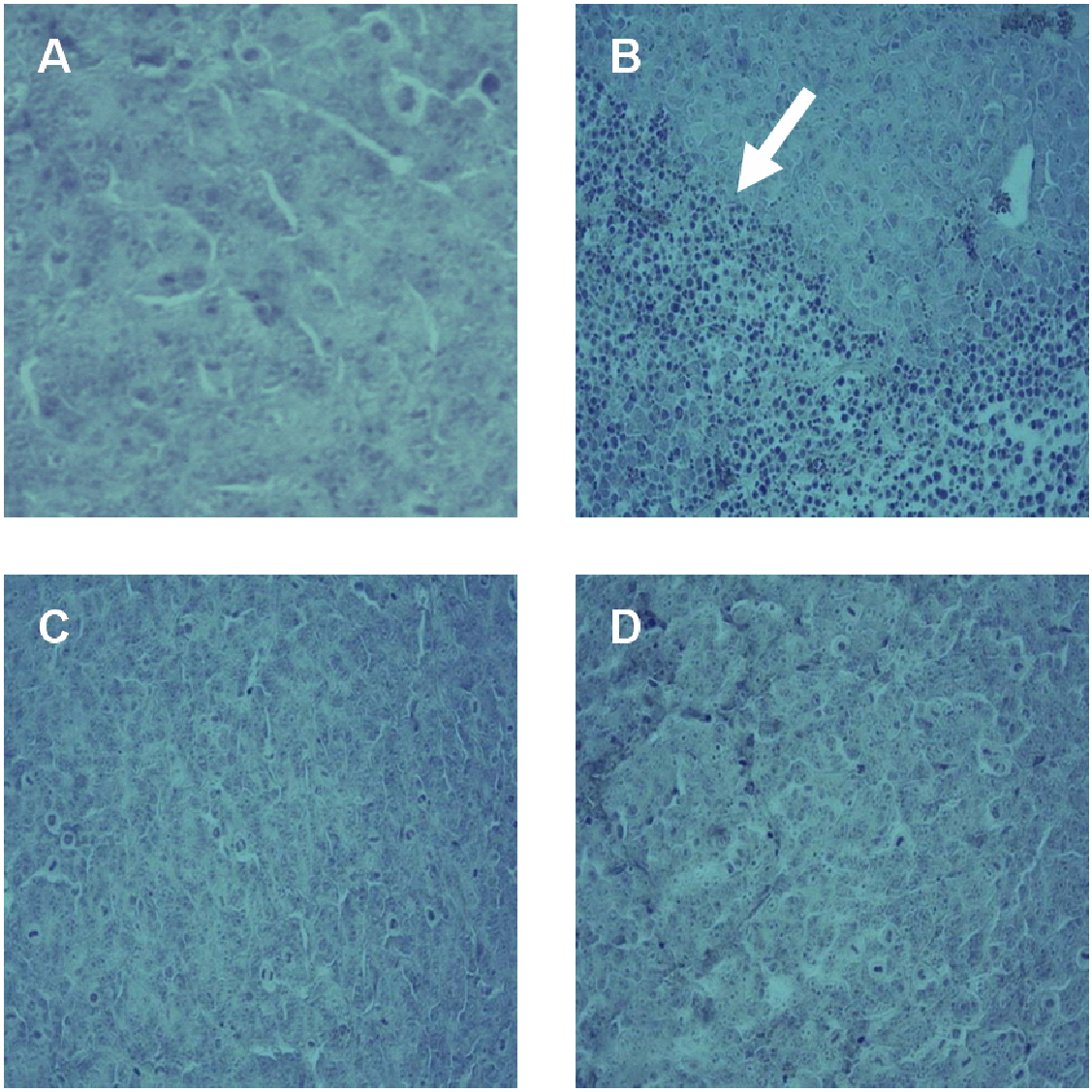


Fig. 3-14: *Histology of Neuro2A tumors after OEI-HA10 therapy. Paraffin embedded sections of untreated (A), OEI-HA10 complex treated (B), OEI-HA10 formulations with pluronic F127 in a w/w ratio of 1 (C) and 2 (D) were stained with hematoxylin and eosin. In case of OEI-HA10 polyplex treatment necrotic areas can be detected.*

3.1.2.10 OEI-HA10 / siRNA complexes formed with helper lipids

In the previous chapters it was shown that masking of hydrophobicity of HA10 by incorporation of lipophilic agents could increase the tolerability of positively charged formulations. A possible reduction of efficiency represents, however, a big hurdle. The incorporation of another lipophilic molecule dioleoyl-phosphatidylethanolamine (DOPE) led even to increase in *in vitro* transfection efficiency of formulations and certain decrease of the toxicity in vitro (Arkadi Zintchenko and Alexander Philipp, unpublished data). Therefore, in a next step the in vivo compatibility of such formulations was tested.

3.1.2.11 Acute toxicity of OEI-HA10 / DOPE

Uncomplexed polymer / DOPE (w/w: 2/1) were injected intravenously into 3 month old female A/J mice in the concentrations 2.5 mg polymer / kg and 5.0 mg polymer / kg. Animals did not survive any of these concentrations and died within one hour due to hemorrhagic extravasation in the intestine and brain liquor as detected by macroscopic evaluation.

3.1.2.12 Acute toxicity of OEI-HA10 / DOPE / siRNA complexes

In a next step OEI-HA10 was mixed with the lipid DOPE and subsequent complexation with siRNA. Three different concentrations were analyzed for tolerability, namely:

2.5 mg siRNA / kg complexed with 5.0 mg OEI-HA10 / kg and mixed with 10.0 mg DOPE / kg (surface charge of ~ +30 mV)

2.5 mg siRNA / kg and the reduced amounts of 2.5 mg OEI-HA10 and 5.0 mg DOPE (surface charge of ~ - 40 mV)

The siRNA amount was decreased to 1.25 mg / kg, complexed with 3.75 mg OEI-HA10 and mixed with 7.5 mg DOPE / kg (surface charge of ~ +30 mV)

The incorporation of DOPE did not enhance tolerability, as all animals died within one hour after intravenous tail vein injection due to hemorrhagic extravasation in intestine and brain liquor (data not shown). Unlike pure HA10 formulations, even negatively charged polyplexes became toxic.

DOPE is known to show membrane destabilizing properties. This could be the origin of high toxicity of such formulations in vivo. Partial substitution of DOPE by colipids might be able to stabilize the lipid membrane and, therefore, improve the toxicity profile of the formulations. Cholesterol, dioleoyl-phosphatidylcholine (DOPC) and dipalmitoyl-phosphatidylcholine (DPPC) were introduced as colipids at different ratios to DOPE.

Unfortunately, all OEI-HA10 formulation mixtures tested in the PhD thesis showing no toxicity *in vivo* did not show efficiency in mRNA knockdown in cell culture experiments.

3.2 Gene Delivery with pDNA

3.2.1 Influence of delivery vectors on toxicity and transfection efficiency

3.2.1.1 DNA condensation

To study polyplex formation between pDNA and polymers with or without incorporation of transferrin-conjugates at different w/w ratios, ethidium bromide exclusion assays were performed (figure 3-15). The condensation efficiencies of L-PEI, OEI-HD and lt-OEI-HD1.2 were comparable. The experiment furthermore showed that exchange of 25 weight % polymer through the corresponding transferrin-conjugate had no negative effect on condensation properties for any of the analyzed polymers.

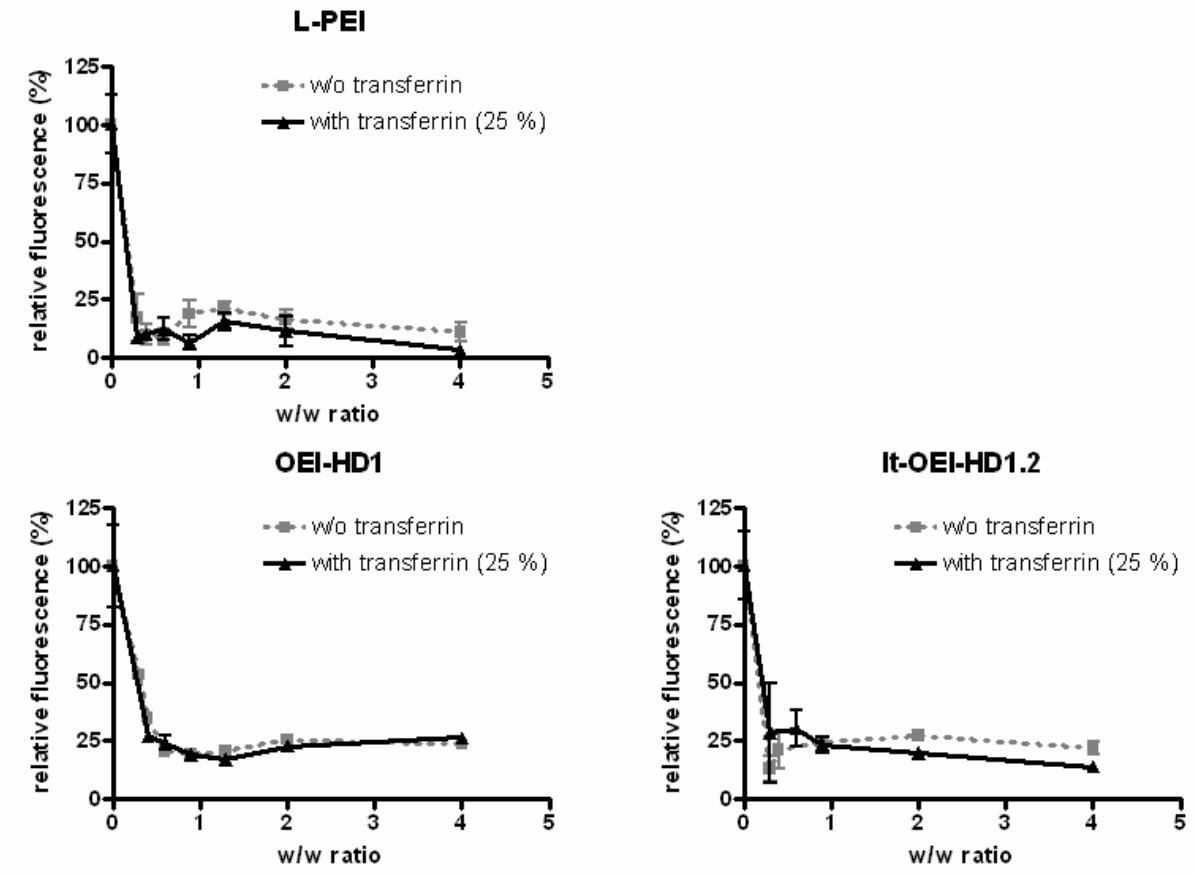


Fig. 3-15: *DNA condensation efficiency of studied by ethidium bromide exclusion assay for OEI-HD1 (A), lt-OEI-HD1.2 (B) and L-PEI (C). Increasing polymer / pDNA ratios (w/w) were analyzed either without or with incorporation of 25 weight % transferrin conjugate (Tf-PEI25 for L-PEI and Tf-PEG-OEI-HD1 for OEI-HD1 and lt-OEI-HD1.2). All experiments were performed in quadruplicate.*

3.2.1.2 Stability against Heparin Displacement

To evaluate polyplex stability against displacement of pDNA by competing polyanions, the polyplexes were challenged with free heparin (figure 3-16). Heparin amounts as low as 0.5 U displaced DNA from the complex in the case of OEI-HD and lt-OEI-HD1.2. OEI-HD and lt-OEI-HD1.2 transferrin complexes showed a slightly decreased stability against heparin displacement. With these polyplexes, free plasmid DNA was observed at heparin concentrations of 0.25 U and above. In contrast, L-PEI / transferrin polyplexes released the pDNA just at heparin concentrations of 0.75 U (at about 1 U without transferrin (Germershaus et al. 2008)).

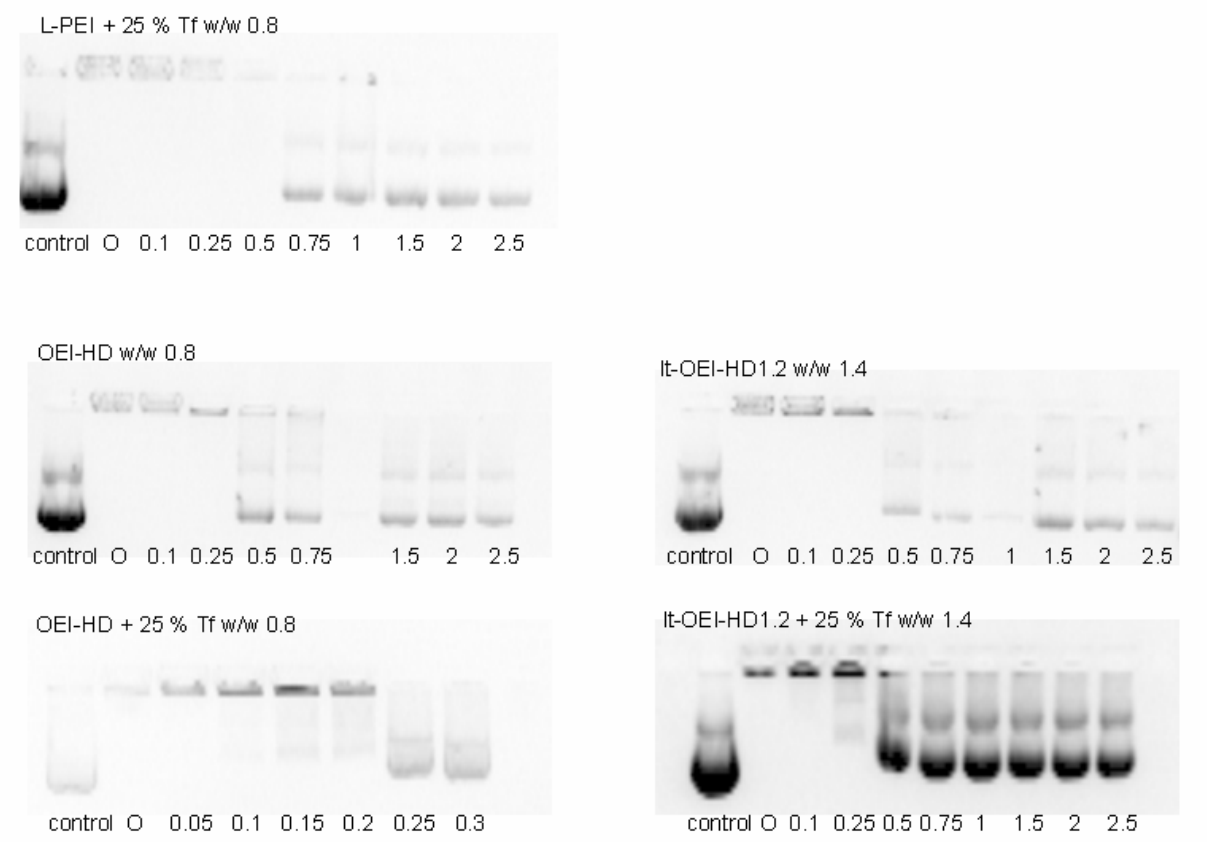


Fig. 3-16: *Heparin displacement assay using L-PEI, OEI-HD and lt-OEI-HD1.2 complexes with and without transferrin. Complexes were incubated for 30 minutes with increasing heparin concentrations. Free plasmid is shown as a control on the left.*

3.2.1.3 Size und Zetapotential

The ability of L-PEI, OEI-HD1 and lt-OEI-HD1.2 polyplexes to condense pDNA was also evaluated by particle size and zetapotential measurements. Hydrodynamic diameters of OEI-HD1 and lt-OEI-HD1.2/pDNA complexes at w/w ratios ranging from

0.5 to 1.4 showed a similar trend of decreased particle size with increased w/w ratios (Figure 3-17). The average particle size is less than 250 nm. Particles sizes tend to be increased after addition of Tf-conjugates, though differences were not significant for L-PEI, OEI-HD or lt-OEI-HD1.2.

As expected, addition of the shielding moiety Tf reduced the zetapotential of complexes.

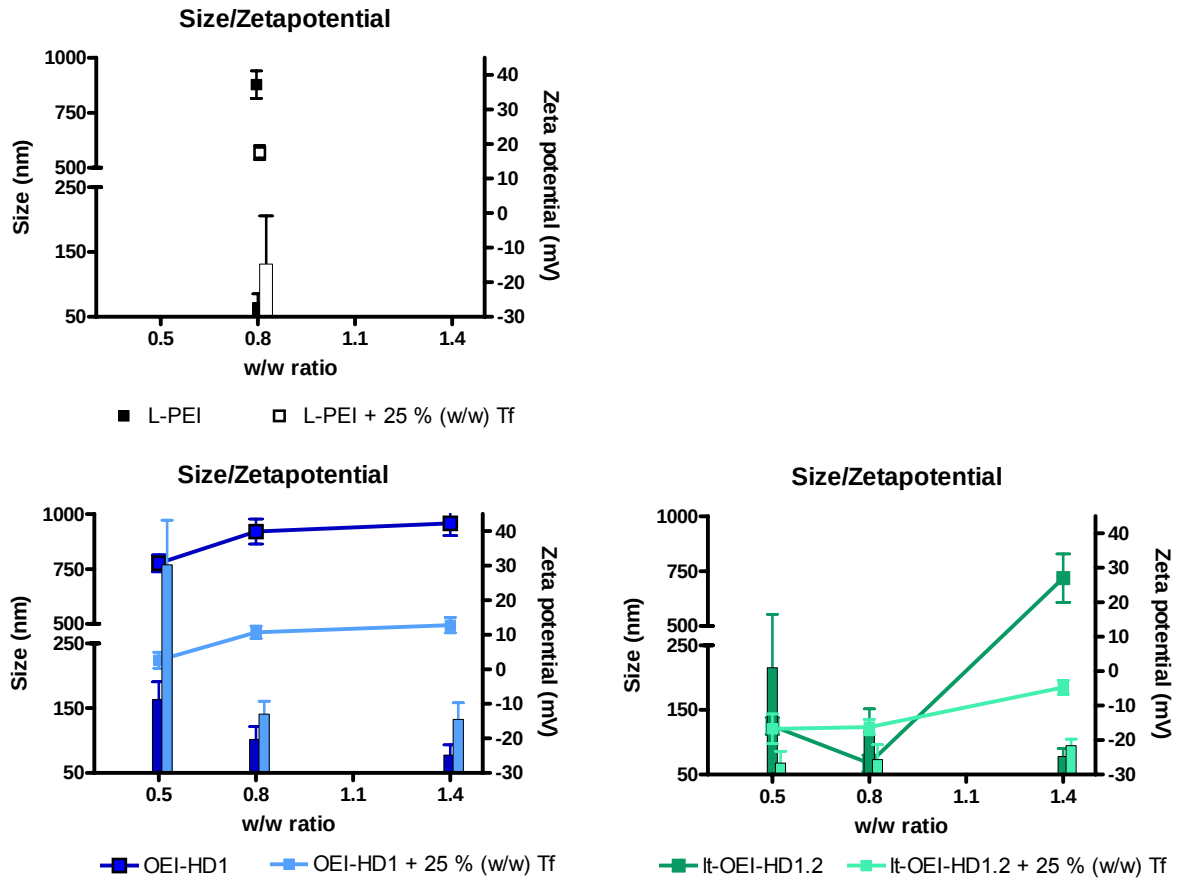


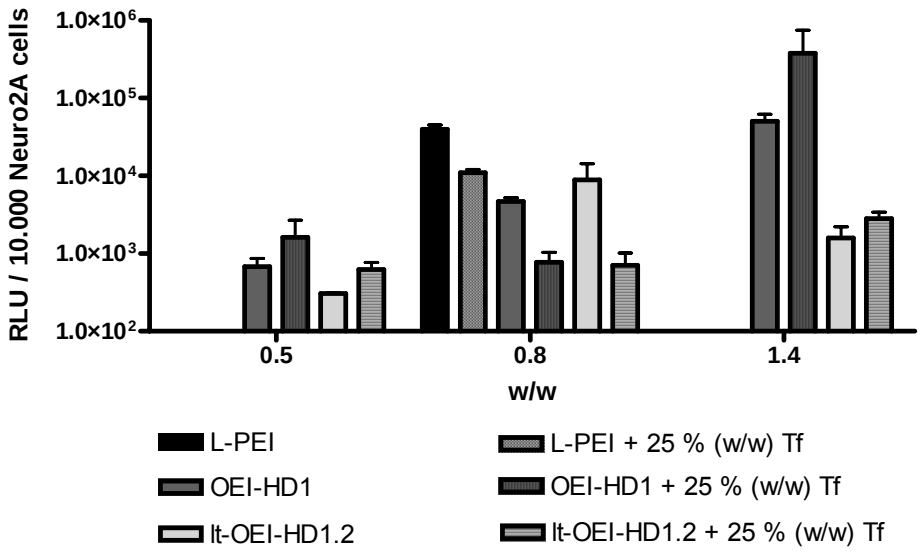
Fig. 3-17: *Size and zetapotential of polyplexes formed with L-PEI (A) , OEI-HD1 (B) and lt-OEI-HD1.2 (C) with and without addition of Tf-conjugate respectively. Values representing size by number are displayed as bars, values representing ζ potential are displayed as dots and connected with a line. All data shown are mean values ± SD from three measurements.*

3.2.1.4 Transfection efficiency in vitro

Plasmid DNA (pCMVLuc) was complexed with L-PEI, OEI-HD or lt-OEI-HD1.2 respectively at w/w ratios of 0.5 to 1.4 to examine the transfection efficiency (figure 3-18A) and metabolic activity in vitro (figure 3-18B). The effect of transferrin incorporation as shielding and targeting moiety was analyzed concurrently. As expected, trans-

fection efficiency increased with increasing polymer ratio. Best results regarding transfection efficiency were obtained with the polymer/DNA ratio 1.4/1.

A



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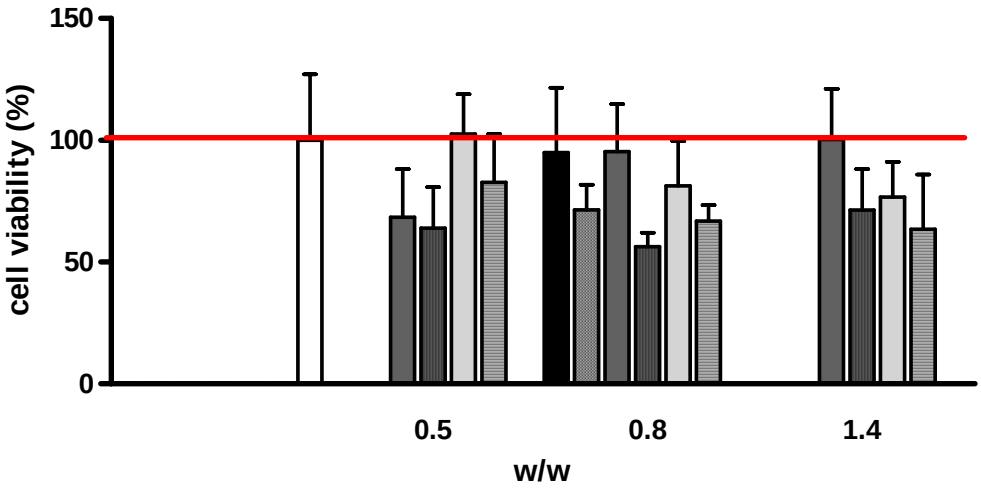


Fig. 3-18: *In vitro transfection efficiency (A) and cell viability after treatment with polyplexes (B) of polyplexes with pCMVLuc. Transfection was carried out on Neuro2A cells and for both assays cells were analyzed 24 hours following transfection. For the cell viability assay all values were normalized against untreated cells (white bar), which were set for 100 % cell viability.*

3.2.1.5 Acute toxicity of polymers and polyplexes in vivo

Free polymers L-PEI (22 kDa), OEI-HD1 and lt-OEI-HD1.2 were applied intravenously into A/J mice (2.5 mg/kg in HBG; total volume 200 µl) and livers of these ani-

mals were examined for histopathological changes 24 hours after administration. In animals receiving L-PEI a microvesicular fatty liver was observed, similar to pathological finding described for acute and chronic intoxications (Ogris et al. 2007) (figure 3-19).

In contrast, after application of OEI-HD and lt-OEI-HD1.2 livers showed no histopathological changes. Images in figure 3-19B show livers of A/J mice 24 hours after i.v. injection of transferrin shielded pDNA complexes formed with the same polymers. Here no changes were observed with any of the used formulations.

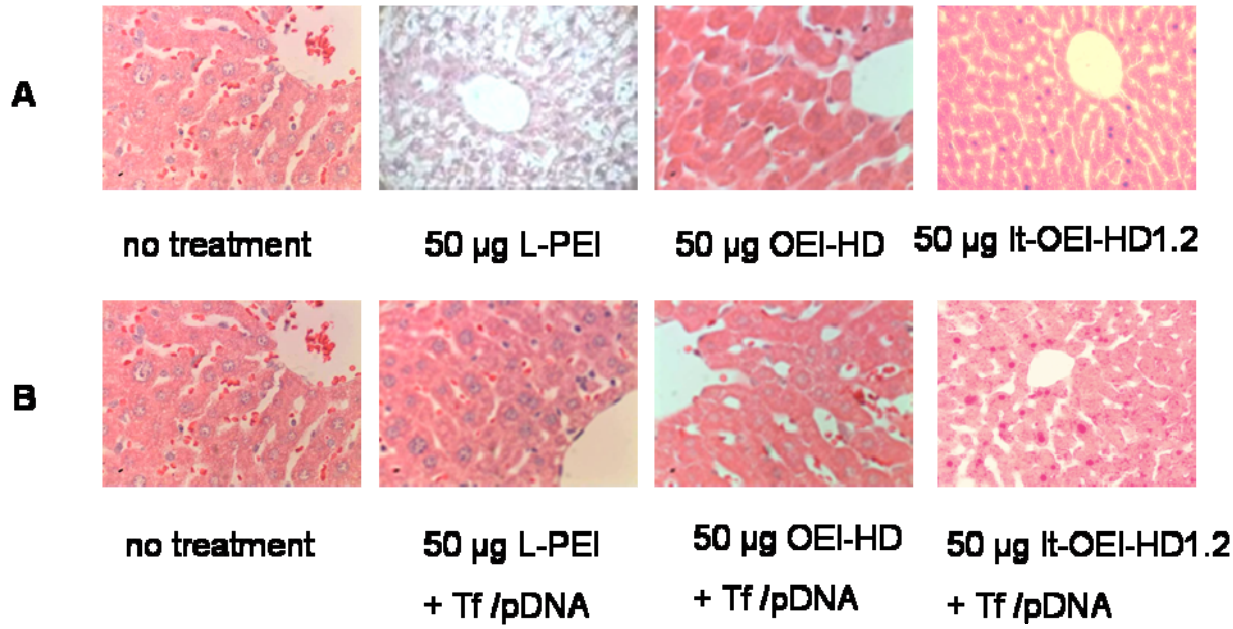


Fig. 3-19: *Liver histology after administration of free polymers (A) and transferrin shielded pDNA polyplexes (B). Livers were explanted 24 hours after i.v. application of polymers and polyplexes (w/w 0.8/1) respectively, embedded in paraffin and cut into 5 µm thick slices. Images were taken after hematoxylin and eosin staining in a 32-fold magnification using an Axiovert 200 microscope, equipped with an Infinity 2 CCD camera.*

Furthermore, the influence on body weight was measured for application of polyplexes. As shown in figure 3-20 complexes without transferrin shielding were less tolerated than those containing 25 % (w/w) transferrin-conjugate, which is in good correlation not only with the histopathological findings shown in figure 3-19 but also with previous findings for PEI by (Ogris et al. 1999).

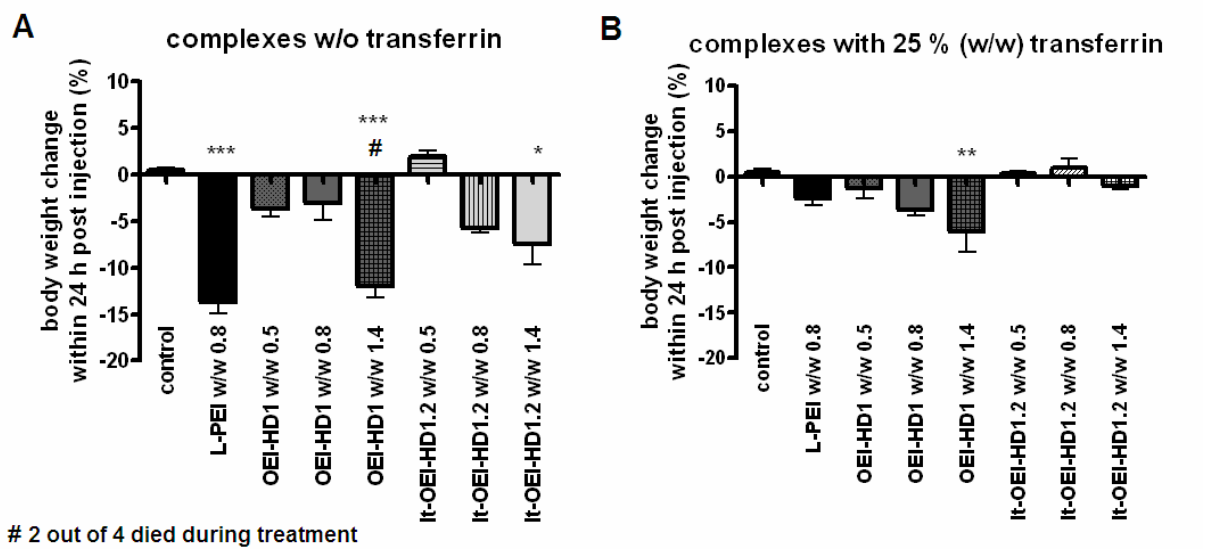


Fig. 3-20: *Influence of systemic polyplex application on body weight of A/J mice. Change in body weight was measured 24 hours after injection of complexes without (A) and with (B) 25 % (w/w) transferrin-conjugate incorporated. Values represent mean levels ± SD of 3-5 animals. Differences between a treated group and the untreated control group a marked with an asterisk; * p<0.05, ** p<0.01, ***p<0.001.*

3.2.1.6 Transfection efficiency in vivo

For in vivo testing of polyplexes the three polymer to pDNA ratios (w/w) 0.5/1, 0.8/1 and 1.4/1 were prepared. Complexes containing 2.5 mg plasmid / kg mouse in a total volume of 250 µl were applied once into the tail vein. Twenty four hours later animals were sacrificed, organs were explanted and analyzed for luciferase expression using a tube luminometer. L-PEI polyplexes in a w/w ratio of 0.8/1 served as a control. In the series of transferrin free complexes L-PEI showed high transfection efficiencies in every organ with by far the highest values in the lung. This effect of L-PEI is commonly known (Zou et al. 2000) and is generally deemed acceptable as a side effect occurring together with good tumor transfection. In contrast, gene expression with OEI-HD and lt-OEI-HD1.2 was lower in all organs. As primarily the tumor is the target for gene expression this is a favored result. However, without transferrin, also in tumors only lt-OEI-HD1.2 in a w/w ratio of 1.4/1 reached levels of L-PEI (w/w 0.8/1) (figure 3-21A). For the series of polymer/transferrin-conjugate polyplexes, almost no differences between the groups was seen in any organ analyzed (figure 3-21B).

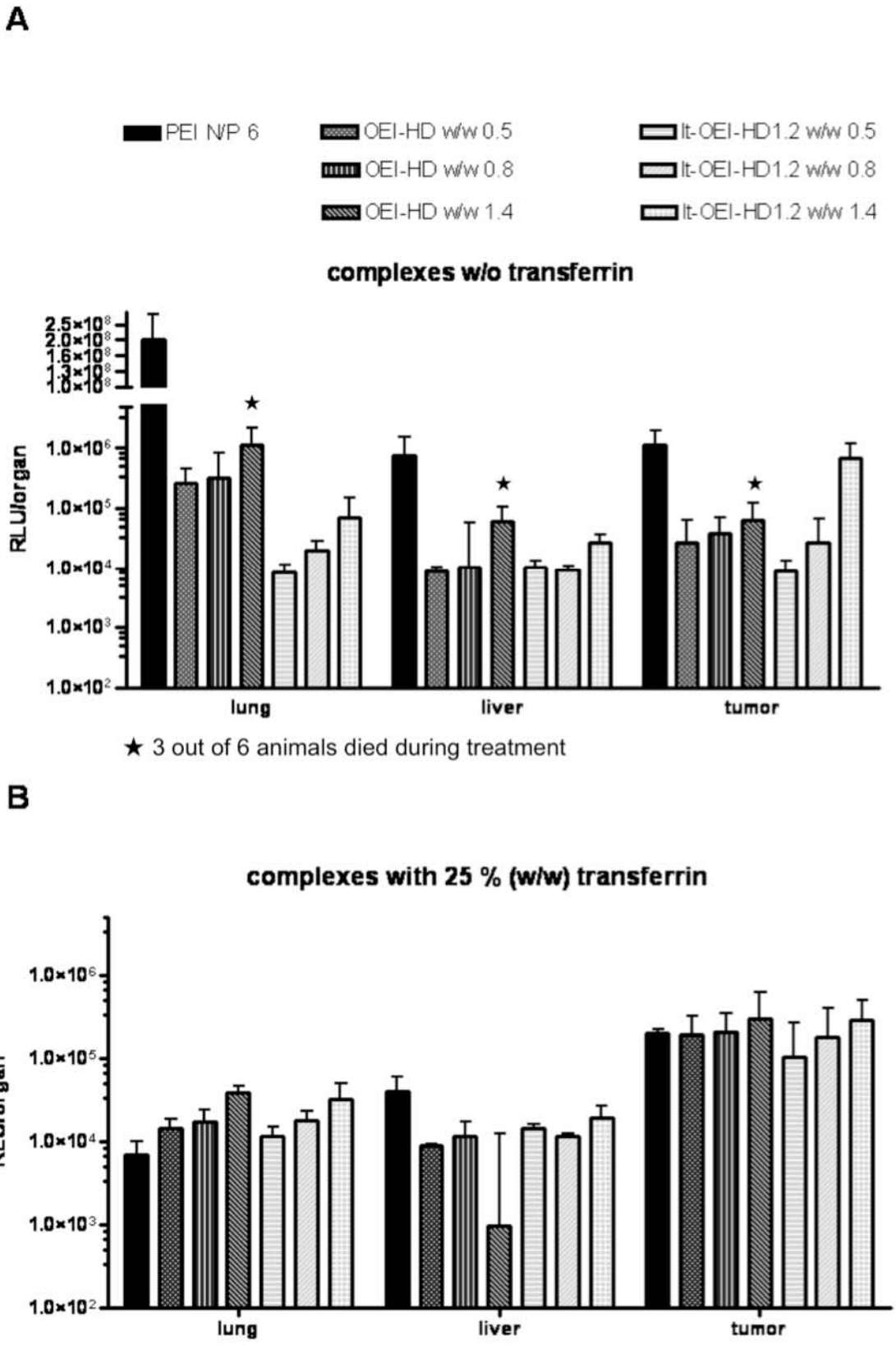


Fig. 3-21: *Gene expression in A/J mice 24 hours after intravenous tail vein injection. Complexes were either used without (A) or with incorporation of 25 weight % of transferrin-conjugate (B). All values represent mean and SD data from 5-6 animals.*

3.2.2 Influence of CpG motifs in pDNA on transfection efficiency

The impact of CpG motifs on transfection efficiency as well as the impact of manufacture-related genomic DNA impurities after normal and hydrodynamic tail vein injection was investigated in the following part.

3.2.2.1 Biophysical properties of L-PEI plasmid complexes

Hydrodynamic diameters of complexes formed at various polymer to pDNA (N/P) ratios prepared in HBG were determined using photon correlation spectroscopy (figure 3-22). All complexes showed particle sizes within the same range of 120 to 150 nm, revealing no differences in complexation characteristics. Complex sizes were 147.0 ± 3.7 nm for pCpGLuc and 153.4 ± 4.4 nm for pCpGLuc ccc at N/P 5, 135.7 ± 9.4 nm and 138.4 ± 5.3 nm for N/P 6, 150.7 ± 5.6 nm and 119.3 ± 29.3 nm at N/P 9 for pCpGLuc and pCPGLuc ccc, respectively.

Zeta potential measurements indicated no major differences between complex charges either (figure 3-22). Zeta potentials of + 30 to + 40 mV for non-shielded L-PEI / pDNA particles are in good correlation with earlier observations made with this polymer in 5 % glucose solution (Zou et al. 2000).

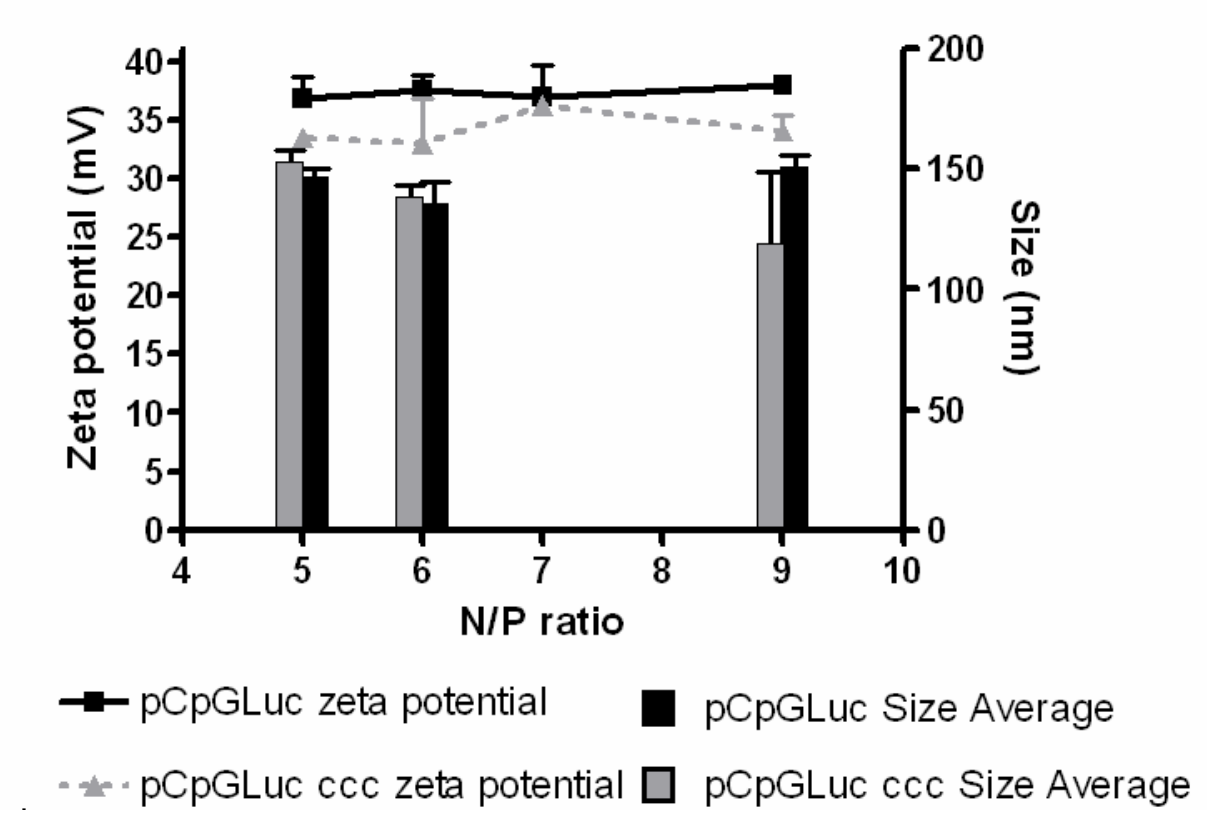


Fig. 3-22: *Surface charge (zeta potential, left y-axis) and hydrodynamic diameter (size, right y-axis) as a function of the N/P ratio. Complexes were prepared in HBG. Values shown represent mean ± SD of three measurements.*

3.2.2.2 L-PEI based pDNA delivery in Neuro2A cells

The pDNA was complexed with linear polyethylenimine (L-PEI) at different N/P ratios commonly used for in vivo transfection. Transfection was analyzed first *in vitro* on Neuro2A cells for its efficiency at the three time points (i) 24 h, (ii) 48 h and (iii) 72 h. Luciferase expression was increased with the supercoiled (ccc) grade plasmid compared to research grade (RG) plasmid for both plasmids with both N/P ratios at any time point. Interestingly the vector containing CpG motifs, pEGFPLuc, showed considerably higher transfection efficiency than the pCpGLuc plasmids (figure 3-23A). Cell viability was influenced similarly by the two analyzed plasmids, showing reduced viability of down to 75 % for all plasmids at N/P 9 at 24 hours after transfection, however completely recovering over the following 24 hours, showing results comparable with untreated control cells (figure 3-23B).

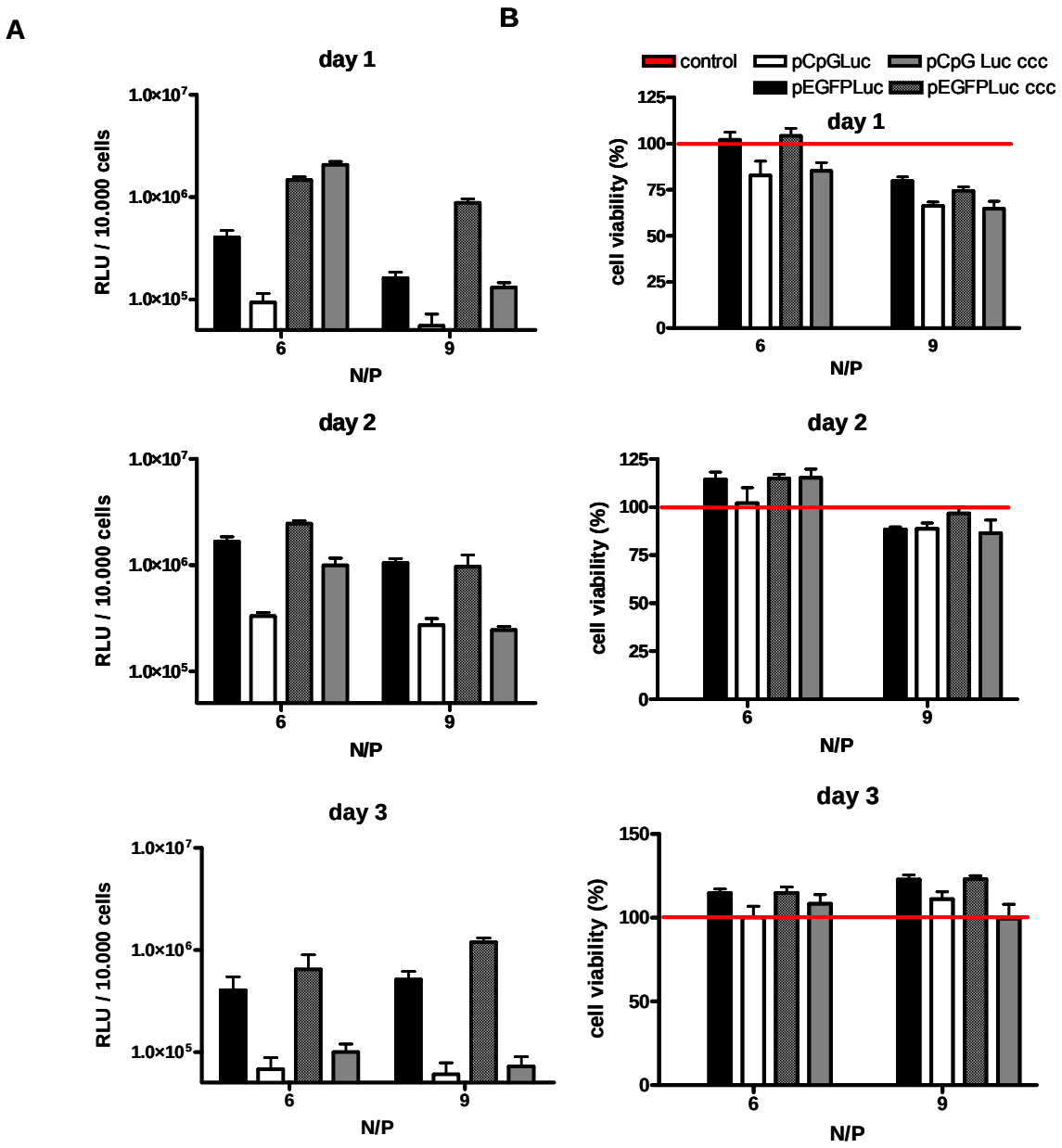


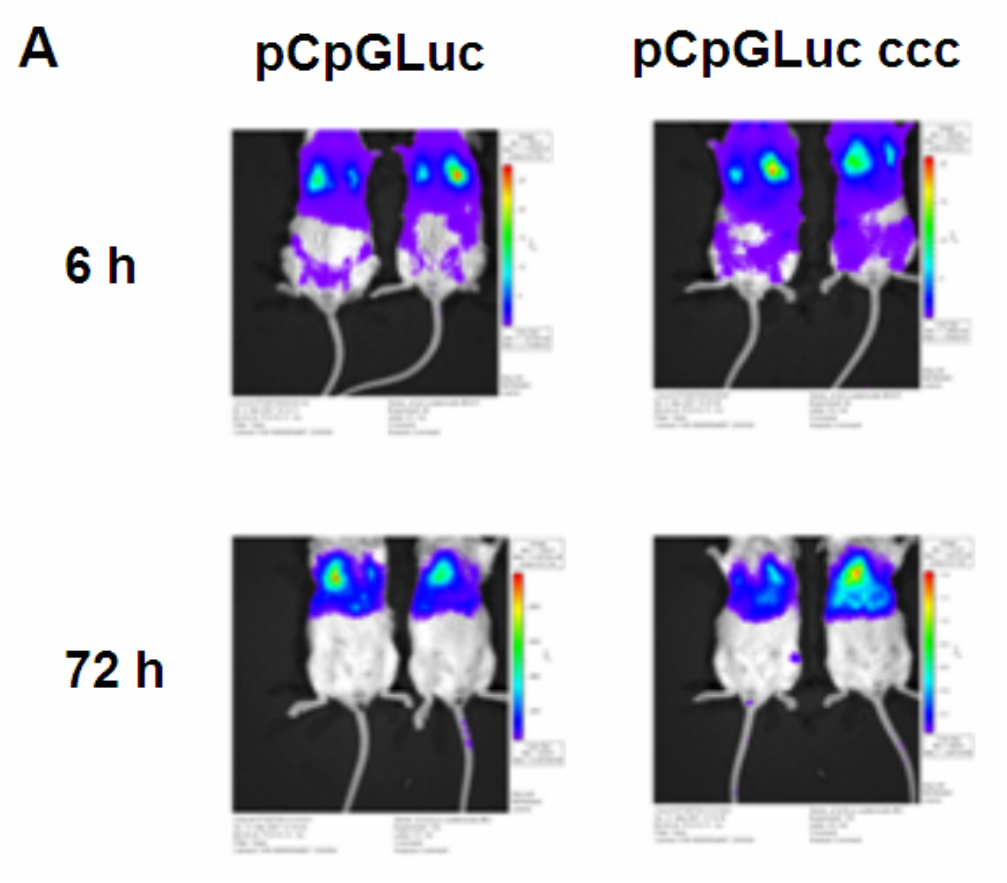
Fig. 3-23: *Transfection efficiency (A) and cell viability (B) over a period of three days. Both experiments were performed on Neuro2A cells. Results display the mean value and standard deviation out of 6 measurements.*

3.2.2.3 In vivo transfection efficiency after standard i.v. application

The three different N/P ratios were tested in vivo in BALB/c mice (n=3-4). Animals received 200 µl of complexes, containing 50 µg plasmid, via the tail vein. Transfection efficiency was measured over time by a CCD camera (XENOGEN IVIS system) 10 minutes after i.p. luciferin injection. For calculation of efficiency a region of interest was drawn over the lung area and luminescence signals integrated from this area were followed over time (figure 3-24).

Minor differences in transfection efficiency between pEGFPLuc and pCpGLuc polyplexes at N/P 6 were monitored, while no significant differences were obtained between the two purification grades, whatever plasmid was used. In contrary starting with similar transfection results for N/P 6, the efficiency of the pCpGLuc ccc plasmid increased with increasing N/P ratio compared to the pCpGLuc RG plasmid polyplex.

This shows that the N/P ratio of complexes as well as the purity of plasmid used influences transfection efficiency in vivo.



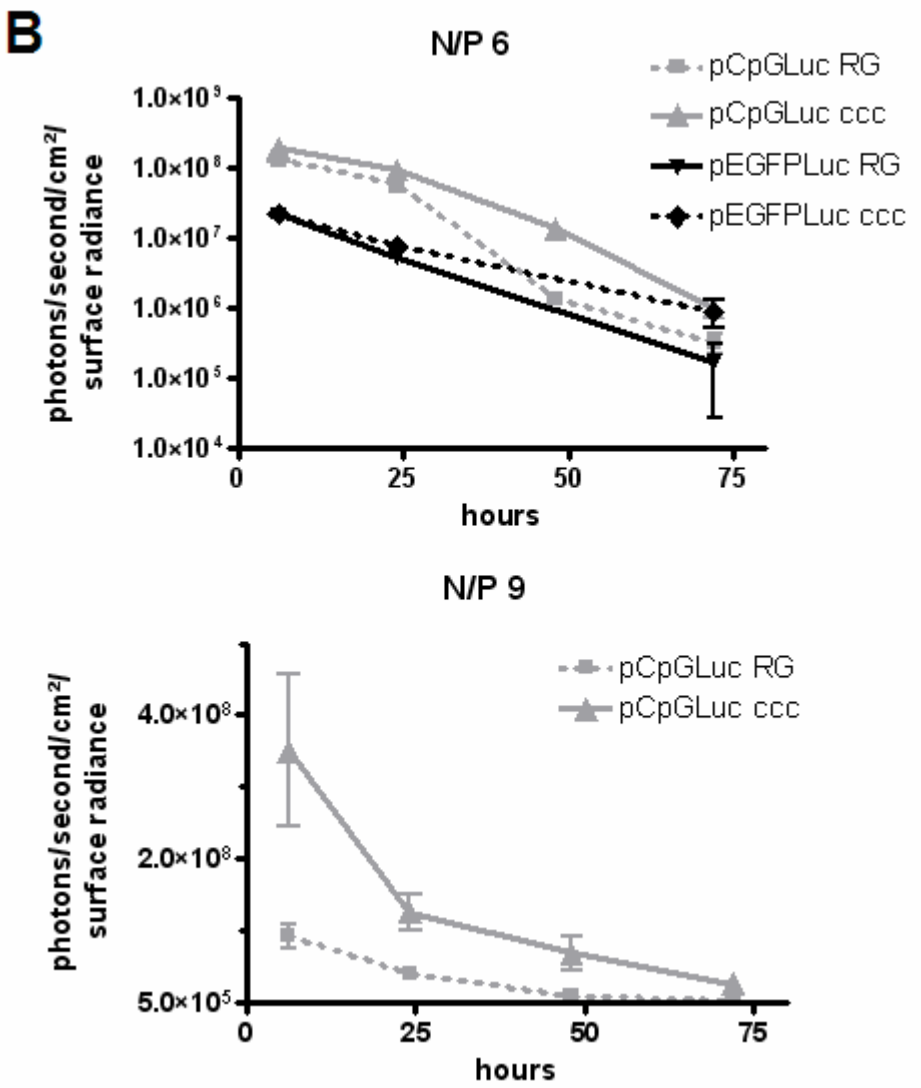


Fig. 3-24: *In vivo transfection efficiency of pCpGLuc and pEGFPLuc. (A) displays representative pictures as seen by the CCD camera 6 and 72 hours after systemic transfection. In (B) lung values of all animals are plotted against time and displayed as mean values ± SD..*

3.2.2.4 Cytokine expression after i.v. polyplex application

In order to examine the possible immunomodulatory activity of our plasmids, we measured expression of 40 cytokines. Cytokine levels were detected in plasma 6 h after injection of plasmid complexes at an N/P ratio of 6. Diagrammed are only cytokines showing measurable distinctions between the non treated control and the polyplex treated groups. The diagrammed cytokines are all associated with inflammatory processes. However, no differences were found between cytokine expression levels after application of RG or ccc plasmid complexes, indicating that the differing effect in transfection efficiency had no influence on cytokine levels of treated animals.

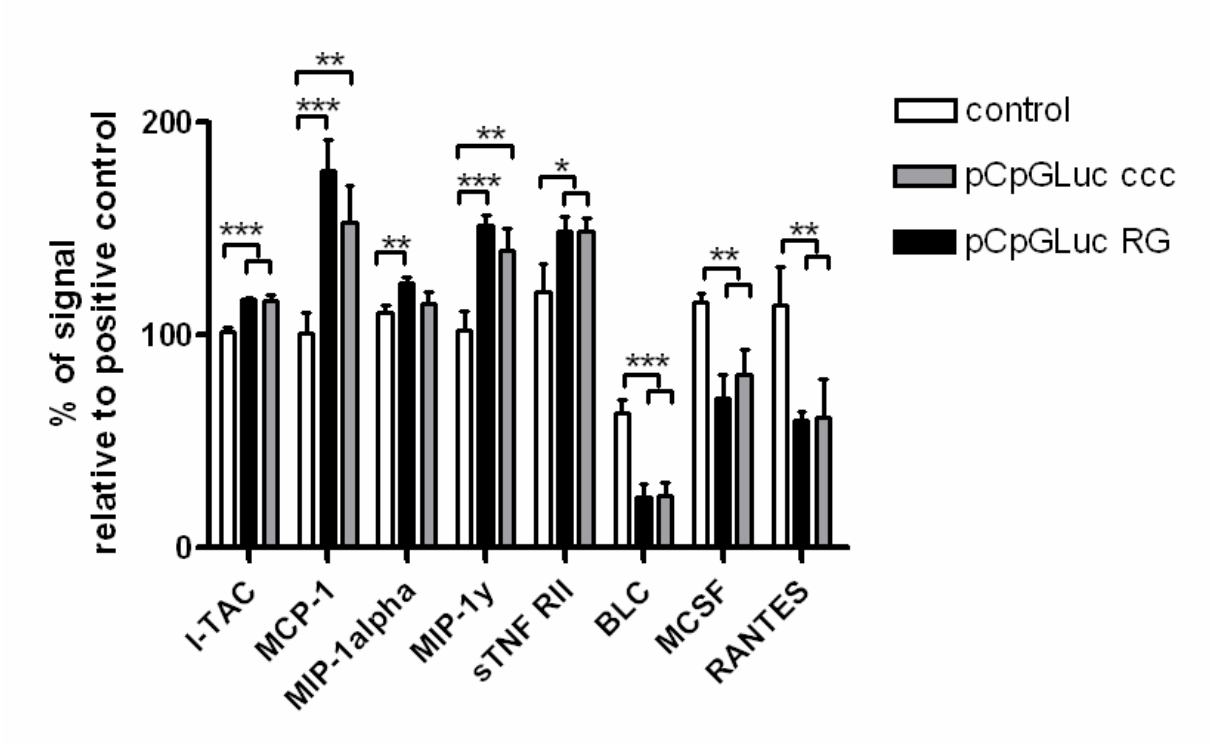


Fig. 3-25: *Cytokine and chemokine expression in mouse plasma, 6 h after polyplex application with pCpGLuc in RG or ccc quality. Values represent mean levels out of 6 animals ± standard deviation. The control group was not treated.*

3.2.2.5 In vivo transfection efficiency after hydrodynamic tail vein application

The influence of CpG motifs as well as of remaining bacterial genomic DNA was analyzed after hydrodynamic delivery of plasmids to the liver. Rapid injection of a relatively large volume of DNA solution (25 µg / 2.5ml) is known to be efficient, safe and induce long term gene expression (Alino et al. 2003, Zhang et al. 2000, Miao et al. 2001). The influence of a carrier can be excluded by this way of application. Luciferase gene expression was measured by in vivo imaging using an IVIS 100 (Caliper LifeSciences) over a time period of 3 weeks.

Only at the first measurement, 24 hours following the hydrodynamic application, could differences be observed between the two purification rates. From day two on diverse expression levels were only detectable between the CpG-containing versus the CpG-free plasmid. While the CpG-free plasmids (pCpGLuc RG and pCPGLuc ccc) showed a relatively constant level of gene expression over a time period of 2 weeks, gene expression levels in CpG-containing plasmid treated animals decreased

dramatically starting already at day two. After 16 days values started to align and no more differences were detectable 3 weeks after the injection.

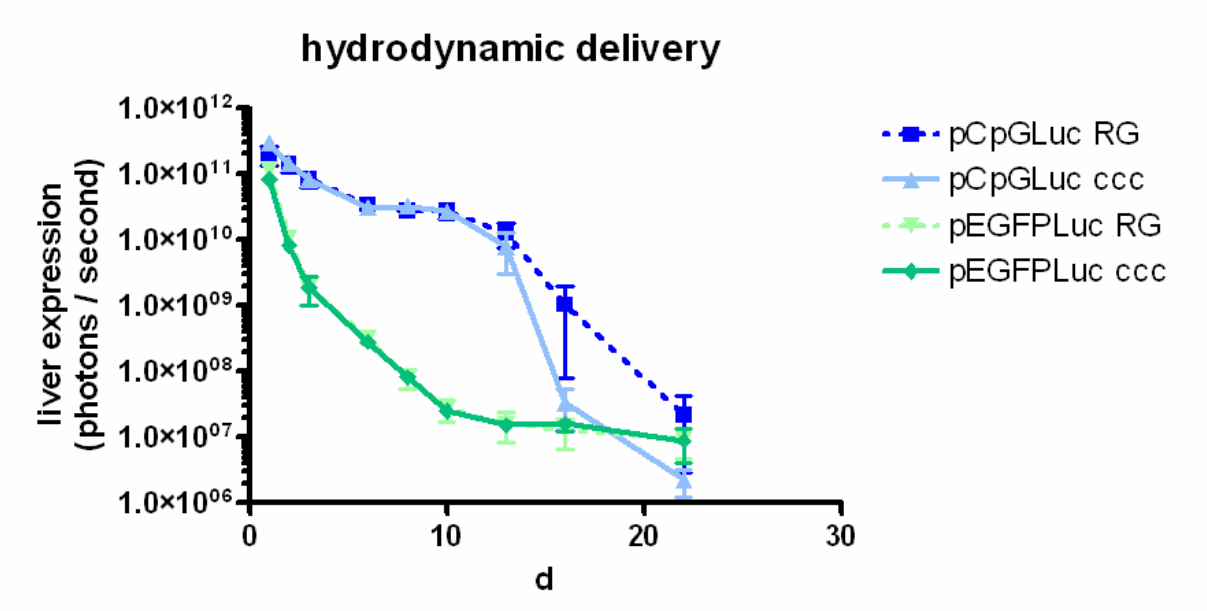


Fig. 3-26: *Luciferase gene expression in livers of BALB/c mice as detected by a CCD camera 10 minutes after i.p. luciferin injection.*

4 Discussion

4.1 Therapy with siRNA

4.1.1 OEI-HD as a delivery vector

High molecular weight polyethylenimine (PEI) is a polycationic carrier for nucleic acids mediating high DNA transfection activity *in vitro* and *in vivo* (Boussif et al. 1995, Zou et al. 2000). *In vivo* application however is limited by significant toxicity (Chollet et al. 2002) and lack of biodegradability of the polymer. In order to develop polycationic carriers that are as effective as the gold standard PEI but less toxic and biodegradable, we and others developed new polymers based on crosslinking of low molecular weight oligoethylenimines (OEI) using various degradable linkages (Kloeckner et al. 2006-1, Kloeckner et al. 2006-3, Gosselin et al. 2001, Forrest et al. 2003, Kim et al. 2005, Hoon et al 2007, Lee et al. 2007). In our studies, a propionamide-crosslinked PEI derivative (OEI-HD) was identified as gene transfer carrier with comparable efficiency as PEI but lower toxicity (Koeckner et al. 2006-3). Interestingly, unlike PEI (Hassani et al. 2005, Grayson et al. 2006), this polymer was found to be also effective for siRNA delivery *in vitro* (Tarcha et al. 2007).

The studies described in this work extended the investigations with OEI-HD formulated siRNA from *in vitro* cell culture to the therapeutically more relevant systemic *in vivo* application in tumor-bearing mice. To convert OEI-HD polyplexes into biocompatible particles which are expected to be shielded from undesired interactions with blood components, our laboratory synthesized and incorporated transferrin-polyethylenglycol-OEI-HD conjugates into the siRNA polyplexes (Alexander Philipp, PhD thesis in progress). Similar strategies have been previously successfully applied by us and others for systemic tumor targeting to the transferrin receptor with DNA lipoplexes (Xu et al. 1999), DNA polyplexes (Ogris et al. 1999, Kursa et al. 2003) and more recently also siRNA /cyclodextrin-conjugate complexes (Hu-Lieskovan et al. 2005).

Optimized transferrin-conjugate/ OEI-HD/ siRNA formulations displayed efficient knockdown efficiency in Neuro2A-EGFPLuc cells which was comparable with non-shielded formulations. Notably, in contrast to non-shielded formulations, transferrin-modified formulations had reduced zeta potential and did not aggregate at low w/w ratios, thus appearing more suitable for *in vivo* applications.

For evaluating the therapeutic potential of the new formulation *in vivo*, the delivery potential of siRNA against RAN in the Neuro2A tumor model was tested in this thesis. RAN encodes a small Ras-related GTPase protein (Dasso 2001, Mattaj and Englmeier 1998, Quimby and Dasso 2003) that is involved in many aspects of nuclear function, such as transport into and out of the nucleus, cell cycle control and post-mitotic nuclear assembly, required for cell survival. The Ran protein was recently identified from an RNAi based screen as possible target in cancer therapy (Morgan-Lappe et al. 2007). A short time later a coherency between Ran overexpression and tumor tissue as well as apoptosis due to Ran silencing was demonstrated in vitro (Xia et al. 2008). OEI-HD/ Ran siRNA formulations were evaluated in our laborator in vitro and showed an antitumor effect in cell culture (A. Phillip and J. Pelisek).

In the current thesis, for systemic application transferrin-free as well as transferrin-shielded OEI-HD/ Ran siRNA formulations were analyzed. Though unshielded complexes tend to aggregate and therefore are less suitable for systemic application (Dash et al. 1999, Ogris et al. 1999, Dash et al. 2000), these complexes were tolerated as well as the shielded complexes in vivo (50 µg siRNA formulated at 0.5/1 ratio, applied intravenously three times at three day intervals). However application of Tf-shielded OEI-HD/ Ran siRNA formulations in the Neuro2A mouse tumor model resulted in a more than 80 % knockdown of RAN protein expression in tumors as compared to non treated control animals and application of unshielded OEI-HD /Ran siRNA formulations only led to about 40 % Ran protein knockdown. Mice treated with the control siRNA formulations did not show significantly reduced Ran expression. The higher efficacy of Tf-shielded particles can not only be explained by the reduced aggregation potential leading to increased circulation times and improved tumor uptake by EPR (enhanced permeation and retention) effect (Iyer et al. 2006). We hypothesize that in addition transferrin-mediated active transport into the tumor cells improves RAN knockdown, as Neuro2A tumor cells are known to over-express Tf-receptors on the cell surface (Kircheis et al. 2001, Kircheis et al. 1999, Bartlett and Davis 2008).

Efficient Ran protein knockdown of Tf-containing formulations was in a next step confirmed on mRNA level by quantitative RT-PCR, giving evidence that knockdown is mediated by RNAi. Furthermore, the knockdown triggered apoptosis up to 12 % in Ran siRNA treated tumors, which was not observed in the control groups. The in-

crease in apoptosis was tumor-specific. This was demonstrated by analysis of liver cells of the same animal that did not reveal any increase in the number of apoptotic cells. Tf-shielded OEI-HD/ RAN siRNA formulations were also able to significantly slow down the tumor growth, which is in good correlation with the findings of protein and mRNA knockdown as well as apoptosis. At this point it has to be mentioned that analyses of blood cells, liver enzymes in plasma, as well as body weight of mice showed no signs of toxicity, giving evidence that the complexes were well tolerated by the animals. However the treatment so far did not lead to the cure of mice. Experiments extending the number of injections of RAN siRNA polyplexes, work with different delivery vectors or siRNA directed against a different target and/or combination with chemotherapy will be future directions to strengthen the therapeutic potency.

4.1.2 OEI-HA10 as novel delivery vector

In an attempt to further optimize *in vivo* Ran siRNA knockdown after systemic complex application, a new delivery polymer, OEI-HA10, was used. In cell culture this polymer displayed even better results regarding knockdown and toxicity than OEI-HD and was therefore seen as a promising candidate for further in vivo studies (unpublished data, Alexander Phillip, Arkadi Zintchenko). For OEI-HA10 800 kDa sized oligoethyleneimine was modified with hexylacrylate chains to improve hydrophobicity and thereby the membrane interaction potential. Due to their small size, renal excreation might be possible for these particles. Chronic side effects due to accumulation in the body therefore can be excluded. In contrast, in OEI-HD OEIs are crosslinked to a longer sized polymer where first degradation of the amid linker formation had to take place until fragments can be excreted.

First experiments on acute toxicity *in vivo* revealed that OEI-HA10 has a very narrow therapeutic window where higher doses lead to necrotic tails, ischemic strokes or hemorrhagic extravasations in brain and intestine. Erythrocyte lysis assays were performed for OEI-HA10, OEI-HD and L-PEI as a control polymer, to investigate hemolytic effects of the polymers. Results showed that OEI-HD and L-PEI were tolerated well, while already low concentrations of OEI-HA10 showed particular high lysis values. The coherency of hemolysis in vitro and in vivo toxicity has already been stated by (Moreau et al. 2002) and indicates a potential reason for the detected side effects. Using Ran siRNA complexes in a w/w ratio of 1 with 2.5mg siRNA / kg systemic application however was tolerated up to three times, with one injection of this dose every second day. With this dosing schedule a significant reduction in tumor growth was observed. However, the formulation did not lead to Ran protein or Ran mRNA knockdown. Histopathological analysis of tumor tissues revealed necrotic areas in OEI-HA10 / Ran siRNA treated tumors. With regards to the results obtained for Ran protein and mRNA knockdown it can be hypothesized that not Ran siRNA downregulation but toxicity of OEI-HA10 polymer itself was responsible for the reduced tumor growth as it was already described for other polycationic vectors (for example by Dufes 2005). For OEI-HA10 this finding can be explained with the hydrophobicity of OEI-HA10 that allows the polymer to strongly interact with cell membranes. Probably due to passive accumulation into the permeable tumor vasculature, based upon the

enhanced permeability and retention (EPR) effect (Aoki et al. 2001), OEI-HA10 was able to reach the tumor tissue, interact with tumor cell membranes or damage tumor vessels and by this lead to necrosis as shown in 3.1.2.9. A similar effect of necrosis after treatment with vascular disrupting agents was described in (Siemann et al. 2005). Looking back at the results obtained with free OEI-HA10 in an erythrocyte lysis assay, were up to 75 % erythrocyte lysis was detected, this effect is likely due to the membrane destabilizing effect of OEI-HA10. Addition of hexylacrylate to OEI was thought to improve transfection efficiency compared to OEI due to a higher membrane interaction potential. However, this property needs to be thoroughly balanced to avoid interaction with erythrocyte membranes leading to hemolysis or lysis of blood vessel membranes leading to hemorrhagic extravasations.

To enhance tolerability as well as efficiency polyplexes were prepared in the presence of pluronic F127, a member of the poloxamer family. This amphiphilic block-copolymers, consisting of hydrophilic polyethylene oxide and hydrophobic polypropylene oxide blocks, were reported as promoting drug penetration across different biological membranes like cell-membranes (Kabanov et al. 1992, Slepnev et al. 1992) and the blood-brain barrier (Kabanov et al. 1989). By mixing with OEI-HA10 hydrophobic hexyl chains of OEI-HA10 were thought to interact with hydrophilic parts of pluronic and by this reducing the hydrophobicity (and toxicity) of complexes. For therapeutic studies on Ran mRNA knockdown with OEI-HA10 in Neuro2A tumor bearing A/J mice, polyplexes with and without the incorporation of pluronic F127 were applied systemically. Administration of pluronic containing particles was tolerated up to a w/w ratio of 2 (6.25 µg OEI-HA10 / kg), while complexes without pluronic could only be administered up to a w/w ratio of 1 (3.1 µg OEI-HA10). However only application of polyplexes without pluronic led to significantly reduced tumor growth, indicating that pluronic not only improved tolerability but in the same step reduced efficiency.

Therefore, we tried to find a lipid that is also able to reduce OEI-HA10's hydrophobic character, however only down to a point where an effective membrane interaction potential still persists. To reach this goal, different neutral lipids, varying in their hydrophilic-lipophilic balance (HLB) were formulated together with OEI-HA10 and Ran siRNA and analyzed for their acute toxicity potential. Complexes formed with dioleoylphosphatidylethanolamine (DOPE) and cholesterol showed reduced tolerability

compared to lipid free OEI-HA10 polyplexes. Dipalmitoylphosphatidylcholine (DPPC) in contrast needed to be formulated together with DOPE as a helper lipid to get tolerated by the animals, whereas Dioleoylphosphatidylcholine (DOPC) on its own was able to form tolerable complexes with OEI-HA10 and siRNA. However, none of the complexes that were tolerated in the first acute in vivo toxicity study showed any knockdown efficiency in cell culture (A. Philipp, unpublished results). These findings signify that further improvement of this vector is necessary to find complex formulations that are not only well tolerated in vivo but also mediate efficient transport of siRNA into the desired cells. Possible alternatives might be

(i) the reduction of hydrophobic units in the OEI-HA polmer

(ii) the surface modification of OEI-HA10 complexes with neutral fragments like e.g. transferrin to provide secure transport within the blood stream

(iii) or as an improvement on the second idea, modification of the complex surface with acid-labile PEG chains similar to (Knorr et al. 2008) is feasible. Here PEG would act as a neutral surface charge agent for secure blood circulation. Due to the EPR effect, polyplexes would be transported to the tumor tissue. As tumor tissue is known to have a higher pH than healthy tissue (Thistlethwaite et al. 1985, Vaupel et al. 1989, Gerweck et al. 1996, Wike-Hooley et al. 1997), PEG chains here would be separated and leave behind the hydrophobic polymer. Indeed, the hydrophobicity is a clear advantage as it might lead to an increased uptake into the tumor cells or into vascular endothelial cells within the tumor. Here OEI-HA10 could act as vascular disrupting agent (VDA), resulting in tumor necrosis as it did in 3.1.2.9. The future potential of this therapy approach can be appraised by the fact that a number of VDAs are already tested in clinical trials (Hinnen and Eskens 2007).

4.2 Gene Delivery with pDNA

4.2.1 Influence of delivery vectors on toxicity and transfection efficiency

PEI is one of the most potent synthetic vectors used for gene delivery. However, especially *in vivo* its utilizability is limited due to toxic effects. These toxic effects are caused by (i) the huge amount of positive charges, leading to complex-mediated aggregation with cellular blood components, especially erythrocytes, and through this to pulmonary embolism (Ogris et al. 1999). (ii) Chronic toxicity can be observed due to lack of biodegradability, leading to accumulation in the liver which itself leads to microvesicular fatty liver (Ogris et al. 2007). Both effects represent major hurdles for its *in vivo* use.

To date a major challenge is to create a synthetic vector which combines efficient gene delivery and minimal *in vivo* toxicity (Kloeckner et al. 2006-2, Forrest et al. 2003, Fischer et al. 1999). The main purpose of the study presented herein was to analyze the influence of the structure of two different polycationic synthetic delivery vectors on transfection efficiency and toxicity especially *in vivo*. Linear PEI (22kDa) served as a gold standard concerning transfection efficiency in all experiments. The two studied vectors only differed in their potential to be degraded into smaller particles under physiological conditions. As described in chapter 4.1.1 and by (Tarcha et al. 2007), OEI-HD consists of 800 Dalton OEI crosslinked by amid bonds, which are slowly degraded in vivo. After biodegradation only low molecular weight fragments are left over, which are well below the excretion limit of the kidneys. Synthesis of lt-OEI-HD1.2 was similar to that of OEI-HD but reaction took place at a lower temperature, thus avoiding aminolysis of ester bonds (Kloeckner et al. 2006-3). This results in a much faster in vivo degradation by esterases.

Experiments on *in vitro* transfection efficiency were carried out at different plasmid / polymer w/w ratios. Furthermore, the effect of incorporation of 25 weight % Tf-conjugate as shielding and targeting moiety was analyzed. Transfection efficiency increased with increasing polymer/DNA ratio for polyplexes with and without transferrin. The highest transfection efficiency was reached by using the polymer/DNA ratio of 1.4/1. Analysis of the biophysical characteristics of the polyplexes showed similar

results regarding DNA condensing ability (with and without the incorporation of a Tf-conjugate) and virtually similar results on stability against heparin displacement.

In vivo toxicity studies with the free polymers showed that the biodegradable polymers were much better tolerated than L-PEI. A dosage that leads to microvesicular fatty liver with L-PEI did not show any signs of liver toxicity with OEI-HD or lt-OEI-HD1.2. However, it turned out that a w/w ratio of 1.4 (using 2.5 mg pDNA / kg) marks the LD_{50} dose for OEI-HD. By incorporation of transferrin as a shielding vector no histopathological changes in the liver were observed after intravenous injection of Tf/L-PEI-complexes. Similarly, incorporation of transferrin also lead to better tolerability of OEI-HD and lt-OEI-HD1.2, regarding weight loss of animals after polyplex injections. Similar to *in vitro* transfection results, transfection efficiency *in vivo* increased with increasing w/w ratios. Furthermore it was demonstrated that undesired transfection of lung and liver was significantly reduced, compared to L-PEI. Using lt-OEI-HD1.2 formulations in a w/w ratio of 1.4 tumor transfection reached the same level as with L-PEI. If transferrin-containing complexes were applied, increasing w/w ratio and increasing transfection efficiency tended to be corrrelated, however, differences were no longer significant. In summary, we have found that the biodegradable polymers OEI-HD and lt-OEI-HD1.2 can reach similar transfection efficiencies as L-PEI in vivo, while showing an improved toxicity profile. Long-term treatment with L-PEI is restricted due to its non-biodegradability and accumulation in the body. In contrast, OEI-HD and lt-OEI-HD1.2 are degradable under physiological conditions (Kloeckner et al. 2006-3) and therefore enable long-term therapy with repeated applications.

4.2.2 Influence of CpG motifs in pDNA on transfection efficiency

The primary signal for the mammalian innate immune system to recognize incoming foreign DNA is triggered by unmethylated CpG motifs present in the DNA sequences of various disease-causing pathogens. These motifs are rare in mammalians, but abundant in bacterial DNAs. As gene therapy typically involves delivery of bacterial derived DNA, an acute inflammatory response and silencing effects may influence results. Furthermore, bacterial genomic DNA containing nonmethylated CpG sequences represents a typical impurity of plasmid preparations which might also trigger inflammatory responses (Li et al. 1999, Krieg et al. 1995, Yew et al. 2000, Krieg 2003, Klinman 2004, Chevalier-Mariette et al. 2003).

For use of pDNA in pharmaceutical products, the FDA has set the following requirements for final specifications:

	Analytical method	***Specification***
Endotoxins	LAL assay	< 40 E.U./mg pDNA
Genomic DNA	RT-PCR	preferably < 1 % /mg pDNA
RNA	Agarose-gelelectrophoresis	preferably < 1 % /mg pDNA
Proteins	BCA assay	preferably < 1 % /mg pDNA
pDNA/cccDNA	Agarose-gelelectrophoresis	> 80 %

Table 4-I: *FDA requirements for pDNA used in pharmaceutical products (source: FDA considerations for plasmid DNA vaccines for infectious disease indications: http://www.fda.gov/cber/gdlns/plasdnavac.htm)*

In our studies two different plasmids which differed in their CpG motif content were used. Both plasmids were purified using different methods resulting in impurity levels of ~ 10 % genomic DNA (research grade (RG)) and ~ 1 % genomic DNA (covalently closed circular plasmid grade (ccc)).

The study included two different ways of administration: (a) standard intraveneous injection of pDNA / L-PEI polyplexes and (b) hydrodynamic application with free plasmids.

L-PEI is widely used in in vivo gene delivery and known for its versatility and comparatively high transfection efficiency in the lung and therefore was chosen as a standard in this study (Dif et al. 2006). Moreover, since the beginning of 2008 L-PEI is available in GMP-quality by Polyplus-transfection (jetPEI^{TM}) and used as delivery reagent in clinical trials for cancer therapy in Israel and the USA (Ohana et al. 2004) and for HIV immune therapy in Germany and Sweden (Lisziewicz et al. 2005-1, Lisziewicz et al. 2005-2). Furthermore, it was shown that injection of L-PEI alone did not induce any pro-inflammatory response (Bonnet et al. 2008). Effects on the innate immune system could therefore be linked to pDNA itself.

Systemic administration of polyplexes indicated that transfection efficiency with pCpGLuc polyplexes lead to increased results compared to pEGFPLuc polyplexes. However, the differences between the two purification grades of one plasmid displayed only minor variations in transgene expression. In a second step it was shown that the N/P ratio chosen for complex formation plays a critical role in transfection efficacy. By forming complexes at a N/P ratio of 9 instead of 6, a significant difference between transgene expression of purified and research grade pCpGLuc polyplexes was detected.

For hydrodynamic delivery of the free plasmids a dose of 1.25 mg plasmid / kg was applied (Hibbitt et al. 2007, Woodell et al. 2008) . No difference was detected between the purification grades of the plasmids. Interestingly, gene expression after application of pEGFPLuc plasmid rapidly decreased, while application of pCpGLuc plasmid resulted in an almost constant gene expression over a time-period of about two weeks. Gene expression of the two plasmids (pCpGLuc and pEGFPLuc) decreased to similar levels after 3 weeks. It was reported that CpG motifs can provoke gene silencing (Chevalier-Mariette et al. 2003, Bird 2002). Since both pEGFPLuc plasmid grades contain CpG sequences gene silencing is a potential explanation for the observed difference. A second aspect which might be taken into consideration are genomic DNA impurities since these contain CpG motifs as well. The difference in impurity levels between RG and ccc plasmids might therefore result in different levels of gene silencing. However, no significant difference was found between the different purification grades. Other points to consider are the different promoters of the two plasmids, as it has been recently shown that plasmids containing the EGF1 promotor

(as the pCpGLuc plasmid) show an increased liver transfection efficiency compared to plasmids with the hCMV promoter (pEGFPLuc) (Nguyen et al. 2008).

A potential reason for the differing results in duration of gene expression obtained with classical systemic and hydrodynamic intravenous application, might be due to the different circulation time of plasmid in the body. Intravenous injection of polyplexes results in short circulation of the particles in the blood. During this short period particles get in contact with a high surface area and might lead to cytokine expression (Merdan et al. 2005, Kawakami et al. 2006), followed by gene silencing. In contrast, hydrodynamic tail vein application of plasmid leads to rapid uptake into liver cells. Due to the high volume injected, an increased pressure in the vena cava is created, pushing the plasmid solution in the large hepatic vein and from there out of the capillaries directly into the liver tissue. This procedure leads to an immediate uptake of about 90 % of injected plasmid in the liver (Herweijer and Wolff 2007). Cytokine induction in the blood therefore is minimized.

5 Summary

For the development of potent nucleic acid (NA) delivery systems different items have to be optimised. Depending on the treatment strategy it might be useful to work with plasmid DNA or siRNA as a vector. For effective transport to the desired cells of action, targeted vectors have to be developed that ideally are non toxic, can be excreted from the body, protect the NA in blood flow and transport them securely and predominantly to the favoured cells. This thesis describes *in vivo* studies on the influence of delivery systems on pDNA and siRNA delivery as well as the influence of pDNA structure on efficient gene transfer. A second goal was to clarify potential toxic effects of delivery systems as well as of NAs following systemic application.

Part 1 describes the effective delivery of a therapeutic RAN siRNA to subcutaneous tumors in mice. The polymer OEI-HD, based on beta-propionamide-cross-linked oligoethylenimine and its chemical transferrin conjugate were evaluated for siRNA delivery into murine Neuro2A neuroblastoma cells in vivo. The Ras-related nuclear protein RAN was selected as a therapeutically relevant target protein. Systemic delivery of transferrin-conjugated OEI-HD/RAN siRNA formulations (three intravenous applications at 3 days interval) resulted in apoptosis, >80% reduced Ran protein expression, 40 % Ran mRNA expression and a reduced tumor growth in Neuro2A tumors of treated mice. The treatment was not associated with signs of acute toxicity or significant changes in weight, hematology parameters, or liver enzymes (AST, ALT, or AP) of mice. All our results demonstrate that OEI-HD/siRNA formulations are well tolerated and can knockdown genes in tumor cells in vivo in mice without unspecific toxicity.

In **part 2** a novel polymer for siRNA delivery was investigated. Initial in vivo experiments with the therapeutic Ran siRNA led to a significantly reduced tumor growth. However, in a following detailed investigation it could be shown that this effect was not due to the siRNA but to the polymer itself. As the analyzed polymer is hydrophobically modified, it easily interacts with cell membranes, as for example tumor vessels thereby leading to necrosis. In a next step poloxamer containing formulations with reduced toxicity were tested, but were ineffective. Further investigations on this field are inevitable.

In **part 3** the comparison of different gene delivery vectors for pDNA delivery in vivo was accomplished. Three different cationic polymers were analyzed concurrently with regards to their ability to degrade within the in vivo circulation. It was hypothesized

that cationic vectors can be developed to show in vivo transfection efficiency that is comparable with that of the gold standard L-PEI, however, due to their biodegradable structure, these vectors should confess the ability to be excreted, thus would be well tolerated after repeated application. When complexed together with pDNA and transferrin-conjugates to target Neuro2A tumor cells, efficient transfection of tumor tissue was obtained with all polymers, showing no significant difference between efficiency of L-PEI and the two degradable polyplexes. Biophysical characterizations regarding DNA complexation and complexation ability, size and surface charge were investigated and did not show differences between L-PEI and the degradable polymers either. However, the polymers with potential to degrade indeed showed better results in vivo regarding acute toxicity.

Part 4 deals with the question how pDNA should ideally be designed to give long lasting gene expression. Analyzed were (i) the influence of CpG sequences within the plasmid and (ii) the influence of remaining genomic DNA. Furthermore it was examined whether these influences varied after changing routes of *in vivo* application. To clear this question, in one setting pDNAs were applied to BALB/c mice by hydrodynamic tail vein injection and in the other setting plasmid DNAs were complexed to L-PEI to lead to efficient lung transfection after low pressure tail vein injection. Measurement of transgene expression by in vivo imaging revealed a strong influence of CpG motifs regardless of the way of administration. Nevertheless only with low pressure polyplex application, a positive influence on transfection efficiency of the purification from genomic DNA from 10 down to 1 % was significant. In the hydrodynamic application setting a reduction of genomic DNA content down to 1 % did not result in significant differences. These results highlight the influence of the plasmid for the efficiency of long-term gene expression for systemic application of free and complexed pDNA. The influence of up to 10 % genomic DNA in a plasmid solution however seems to be dependent on the method of application.

6 Appendices

6.1 Abbreviations

BBB	blood brain barrier
ccc	covalently closed circular (supercoiled)
DAPI	4',6-diamidino-2-phenylindole, dihydrochloride
DOPC	dioleoylphosphatidylcholine
DOPE	dioleoylphosphatidylethanolamine
DPPC	dipalmitoylphosphatidylcholine
pDNA	plasmid DNA
EtBr	ethidium bromide
FDA	US food and drug administration
GMP	good manufacturing practice
HBG	HEPES buffered glucose
HBS	HEPES buffered saline
HE	hematoxylin-eosin
HEPES	2-[4-(2-Hydroxyethyl)-1-piperazinyl]ethanesulfonic acid
LAL	Limulus Amebocyte Lysate
L-PEI	linear polyethyleneimine
OEI	oligoethylenimine
MW	mean value
Mw	molecular weight
N/P	nitrogen / phosphate
NA	nucleic acid
PBS	phosphate buffered saline
PEG	polyethylene glycol
RG	research grade
RLU	relative light units
mRNA	messenger RNA
siRNA	short interfering RNA
SD	standard deviation

TUNEL	TdT-mediated dUTP-biotin nick end labeling
VDA	vascular disrupting agent
w	weight

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6.3 List of publications

6.3.1 Articles

Tietze N, Pelisek J, Philipp A, Roedl W, Merdan T, Tarcha P, Ogris M, Wagner E (2008). Induction of Apoptosis in Murine Neuroblastoma by Systemic Delivery of Transferrin-Shielded siRNA Polyplexes for Downregulation of Ran. **Oligonucleotides**, 18 (2): 161-174.

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Wang D, Sima M, Mosley RL, Davda JP, Tietze N, Miller SC, Gwilt PR, Kopeckova P, Kopecek J (2006). Pharmacokinetic and biodistribution studies of a bone-targeting drug delivery system based on N-(2-hydroxypropyl)methacrylamide copolymers. **Molecular Pharmaceutics,** 3 (6): 717-725

6.3.2 Poster presentations

Tietze N, Schleef M, Schmeer M, Rudolph C, Wagner E, Ogris M. (2007). Extended Nonviral in vivo Gene Expression Using CpG Depleted Specially Purified Plasmid DNA. **Summer School of Nanomedicine,** Cardiff, UK.

Tietze N, Merdan T, Pelisek J, Cheung K, Ogris M, Wagner E, Tarcha P. (2006). A Novel Cationic Polymer for in vitro and in vivo siRNA Delivery. **Gordon Research Conference on Drug Carriers in Medicine in Biology**, Big Sky, Montana, USA.

Tietze N, Klöckner J, Ogris M, Wagner E. (2006). In Vivo Characteristics of Novel Transferrin-Targeted Polycation/DNA Complexes after Systemic Administration in Mice. **Controlled Release Society**, Annual Meeting, Vienna, Austria.

6.3.3 Reviews

Tietze N (2008). Oberbauchbeschwerden. Auf den Magen geschlagen. **Pharmazeutische Zeitung**, 10: 16-24.

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Tietze N (2007). Morbus Crohn. Hilfe für den entzündeten Darm. **Pharmazeutische Zeitung**, 5: 18-22.

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6.5 Curriculum Vitae

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Date of birth: 6th August 1978 in Hannover, Germany

Work experience:

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Education and training:

08/2005 to 09/2008	PhD thesis at the Department of Pharmaceutical Biology-Biotechnology, Ludwig-Maximilian-Universität, Munich, Germany; group leader: Prof. Ernst Wagner
08/2005	Licensure as pharmacist
01/2005 to 07/2005	Internship at the Stern pharmacy Wilhelmshaven, Germany
07/2004 to 12/2004	Internship at the Department of Pharmacy, University of Utah; group leader: Prof. Jindrich (Henry) Kopecek
08/2001to 10/2001	Internship at Knoll AG Ludwigshafen, Germany Pharmaceutical Development, bioanalytics division
04/2000 to 04/2004	Studies of pharmacy, Philipps-University Marburg, Germany