International Journal of Pharmaceutics xxx (2014) xxx–xxx

Contents lists available at ScienceDirect

International Journal of Pharmaceutics

journal homepage: www.elsevier.com/locate/ijpharm

Pharmaceutical nanotechnology

Anginex lipoplexes for delivery of anti-angiogenic siRNA

Afrouz Yousefi [a], Meriem Bourajjaj [a], Negar Babae [a], Paula I. van Noort [b], Roel Q. J. Schaapveld [b], Judy R. van Beijnum [c], Arjan W. Griffioen [c], Gert Storm [a], Raymond M. Schiffelers [d], Enrico Mastrobattista [a,*]

[a] Department of Pharmaceutics, Utrecht Institute for Pharmaceutical Sciences, Faculty of Science, Utrecht University, Universiteitsweg 99, 3584 CG Utrecht, The Netherlands
[b] InteRNA Technologies B.V., Hugo R. Kruyt Building, Floor 8, Room O.809, Padualaan 8, 3584 CH Utrecht, The Netherlands
[c] Angiogenesis Laboratory, Department of Medical Oncology, VU University Medical Center, De Boelelaan 1118, PO Box 7057, 1007 MB Amsterdam, The Netherlands
[d] Department of Clinical Chemistry and Hematology, University Medical Center Utrecht, Utrecht, The Netherlands

ARTICLE INFO

Article history:
Received 3 February 2014
Received in revised form 13 June 2014
Accepted 14 June 2014
Available online xxx

Keywords:
siRNA
Nucleic acid delivery
Liposome
Lipoplex
Angiogenesis
Anginex

ABSTRACT

Angiogenesis is one of the hallmarks of cancer which renders it an attractive target for therapy of malignancies. Tumor growth suppression can be achieved by inhibiting angiogenesis since it would deprive tumor cells of oxygen and vital nutrients. Activation of endothelial cells of tumor vasculature is the first step in angiogenesis which is mediated by various factors. One of the major triggers in this process is vascular endothelial growth factor (VEGF) which binds to VEGF receptors on endothelial cells of tumor vessels. This induces a series of signaling cascades leading to activation of cellular processes involved in angiogenesis, and therefore down-regulation of VEGF receptor-2 (VEGFR-2) expression seems a viable option to inhibit angiogenesis. In our investigations, this aim has been pursued by using siRNA interfering with the expression of VEGFR-2. Since the discovery of RNA interference (RNAi) as a gene regulation process, successful delivery of small non-coding RNA has presented itself as a major challenge. In the current study, we have characterized a galectin-1 targeted anginex-coupled lipoplex (Angiplex) containing siRNA against the gene of VEGFR-2 as an angiostatic therapeutic. Angiplex particles had a size of approximately 120 nm with a net negative charge and were stable *in vitro*. These particles were internalized in a specific manner by HUVECs compared to a non-targeted lipoplex system, and their uptake was higher than Lipofectamine 2000. Gene silencing efficiency of Angiplex was shown to be 61%.

1. Introduction

In cancer, growth of tumors is highly dependent on sufficient distribution of oxygen and nutrients to tumor cells, which requires abundant vascular supply. When supply is limited, tumor cells can induce angiogenesis, which is the formation of new blood vessels from pre-existing vasculature, to restore (Folkman, 1995; Griffioen and Molema, 2000; Yoo and Kwon, 2013). Therefore, this process is a crucial factor in cancer pathology and metastasis. Angiogenesis is controlled by the balance between positive and negative regulators (Folkman, 1995). This balance favors angiogenesis during tumor growth (Bergers and Benjamin, 2003). Hypoxia and ischemia are the main triggers of the angiogenic response causing the activation of transcription factors (such as hypoxia inducible factor) and release of growth factors. VEGF is a growth factor which plays an essential role in normal and pathological angiogenesis (Forsythe et al., 1996; Gacche and Meshram, 2013).

Endothelial cells are crucial for angiogenesis, and VEGF has several biological effects on the endothelium. Firstly, it induces proliferation of endothelial cells through the activation of mitogen-activated protein kinases (MAPK) (Yu and Sato, 1999). Secondly, it promotes migration and vascular leakage which are important

Abbreviations: NC siRNA, negative control scrambled siRNA; βpep-28, βpeptide-28; AF488 NC siRNA, Allstars Neg Control Alexa Fluor 488 siRNA; HBG, 20 mM Hepes buffer containing 5% glucose at pH 7.4; AxL: Angiplex, anginex targeted lipoplexes; βpep-28-L, βpep-28-lipoplexes; Bare-L, bare lipoplexes; DL, DOTAP liposomes; LF, Lipofectamine 2000; asp-NCpep, aspecific negative control peptide.

* Corresponding author. Tel.: +31 6 22736567; fax: +31 30 251 7839.
E-mail addresses: a.yousefi@uu.nl (A. Yousefi), M.Bourajjaj@uu.nl (M. Bourajjaj), N.Babae@uu.nl (N. Babae), vannoort@interna-technologies.com (P.I. v. Noort), Schaapveld@interna-technologies.com (R.Q. J. Schaapveld), j.vanbeijnum@vumc.nl (J.R. v. Beijnum), a.griffioen@vumc.nl (A.W. Griffioen), G.Storm@uu.nl (G. Storm), R.Schiffelers@umcutrecht.nl (R.M. Schiffelers), e.mastrobattista@uu.nl (E. Mastrobattista).

http://dx.doi.org/10.1016/j.ijpharm.2014.06.028
0378-5173/

steps in angiogenesis (Dvorak et al., 1995). Thirdly, VEGF has an impact on the differentiation of hemangioblasts to endothelial cells (Eichmann et al., 1997). The main binding site of VEGF on endothelial cells is FLK-1/KDR (VEGFR-2) tyrosine kinase which has high affinity for this growth factor. Interaction of VEGF with VEGFR-2 leads to VEGFR-2 dimerization and autophosphorylation resulting in the activation of certain signaling cascades which induce cellular processes involved in angiogenesis such as proliferation and migration (Olsson et al., 2006). Therefore, intervention in the VEGF signaling axis by targeting VEGF or its receptors seems an attractive therapeutic strategy as it has already been demonstrated in several studies (Hurwitz et al., 2004; Zhang et al., 2013; Mahalingam et al., 2014; Smith et al., 2014). In the study of Hurwitz et al., it was revealed that in patients with metastatic colorectal cancer, combination of chemotherapy and a monoclonal antibody against VEGF (bevacizumab) improves survival remarkably (Hurwitz et al., 2004). In another study, DC101, an anti-VEGF monoclonal antibody, was shown to have an inhibitory effect on tumor growth by preventing new vessel formation (Prewett et al., 1999).

Silencing of the expression of VEGFR-2 is an alternative approach and can be accomplished by introducing a small interfering RNA (siRNA) against VEGFR-2 mRNA into the endothelial cells of tumor vasculature. Since naked siRNA molecules are rapidly cleared and easily degraded by nucleases in blood, target localization is generally poor. Moreover, siRNA cannot be readily taken up by cells because of their relatively large size and negative charge.

Direct delivery of naked siRNA into target organs has been achieved by electroporation but the invasiveness of this method and the dependency on electrode proximity limits its applicability in humans. Gymnotic delivery has been suggested to be efficacious, but this approach requires persistent and high siRNA concentrations in order to result in silencing.

Alternatively, systemic delivery of siRNA by carrier systems improves stability in blood, provides the possibility of active targeting and enhances cellular uptake. However, such systems also have specific advantages and disadvantages. Viral and non-viral carrier systems have been explored for nucleic acid delivery (Elsabahy et al., 2011; Guo et al., 2010; Sliva and Schnierle, 2010; Nimesh et al., 2011; Zhu and Mahato, 2010). Viral vectors lead to high transduction efficiencies but they have raised concerns about immunogenicity and oncogenicity when used in humans and their production is difficult. Non-viral systems, are regarded as safer and easier to produce in large scale. However, these systems are generally cationic, which upon injection can cause serious side effects.

A solution to the problems related to cationic particles would be to deliver nucleic acids by anionic particles. These complexes have been shown to cause low toxicity *in vitro* compared to cationic systems while maintaining good transfection efficiency and longer circulation times *in vivo* (Guo and Lee, 2000; Patil et al., 2005). Koldehoff et al. observed low hematotoxicity when anionic lipoplexes were administered intravenously in a patient with chronic myeloid leukemia (CML) (Koldehoff et al., 2007). siRNA against bcr-abl oncogene was successfully delivered and led to an increase in apoptosis of CML cells. In another study, Zimmermann et al. also reported more than 90% silencing efficiency by siRNA encapsulated in stable nucleic acid lipid particles in non-human primates without toxicity (Zimmermann et al., 2006). The low non-specific binding of these anionic lipoplexes in the blood compartment, prevents aggregation and allows active targeting of these particles.

In the present study, active targeting of anionic lipoplexes was explored by functionalizing the particles using a 33-mer angiostatic peptide called anginex (Griffioen et al., 2001). This peptide has been reported to specifically target the tumor vasculature (van der Schaft et al., 2002) and to use galectin-1 as its cellular receptor (Thijssen et al., 2006). Galectin-1 is a lactoside-binding lectin which plays an important role in cancer. Firstly, it promotes tumor cell-endothelial cell adhesion (Lotan et al., 1994; Clausse et al., 1999; Astorgues-Xerri et al., 2014; D'Haene et al., 2013) and secondly, by inhibiting T-cell activation it helps tumors to escape the immune system (Rubinstein et al., 2004). By including anginex in the formulation of lipoplexes, on the one hand active targeting to tumor endothelial cells of the siRNA containing lipoplexes can be achieved and on the other hand, inhibition of galectin-1 may interfere directly with angiogenesis and immune recognition.

This paper describes preparation, characterization and investigation of *in vitro* efficacy of Angiplex for intracellular delivery of siRNA to HUVECs. Stability, cytotoxicity and internalization of Angiplex in HUVECs have been studied, and gene knock-down efficiency has been evaluated after transfection of HUVECs with Angiplex containing siRNA against VEGFR-2.

2. Materials and methods

2.1. Materials

siRNA against human VEGFR-2 (siVEGFR-2) and negative control scrambled siRNA (NC siRNA) were purchased from Eurogentec Nederland B.V. (Maastricht, The Netherlands) with the following sequences:

siVEGFR-2: sense strand: 5′-GGA-AAU-CUC-UUG-CAA-GCU-AUU-3′, anti-sense strand: 5′-UAG-CUU-GCU-AGA-GAU-UUC-CUU-3′; NC siRNA: sense strand: 5′-CAU-CGU-CGA-UCG-UAG-CGC-AUU-3′, anti-sense strand: 5′-UGC-GCU-ACG-AUC-GAC-GAU-GUU-3′. 1,2-Distearoyl-sn-glycero-3-phosphoethanolamine-*N*-[methoxy(polyethylene glycol)] with PEG M_w of 2000 g mol^{-1} was purchased from Lipoid (Ludwigshafen, Germany). 1,2-Dioleoyl-sn-glycero-3-phosphoethanolamine (DOPE) and 1,2-distearoyl-sn-glycero-3-phosphoethanolamine-*N*-[maleimide(polyethylene glycol)-2000] (DSPE-PEG2000-Mal) were supplied by Avanti® Polar Lipids Inc. (Alabaster, USA). Cholesteryl hemisuccinate (CHEMS) and protamine sulphate salt were obtained from Sigma–Aldrich (St. Louise, USA). Protected N-terminal SATA-modified peptides were synthesized by China Peptides Co., Ltd. (Shanghai, China) with the following sequences:

Anginex peptide: SATA-ANIKLSVQMKLFKRHLKWKIIVKLND-GRELSLD

βpeptide-28 (βpep-28): SATA-SIQDLNVSMKLFRKQAKWK-VIVKLNDGRELSLD

Aspecific peptide cyclic (Arg-Ala-Asp) was purchased from JPT (Berlin, Germany). Quant-iT™ RiboGreen® RNA Reagent and Lipofectamine 2000 were obtained from Invitrogen (Breda, The Netherlands) and Allstars Neg Control Alexa Fluor 488 siRNA (AF488 NC siRNA) was purchased from Qiagen (Venlo, The Netherlands). CytoTox-One Homogeneous Membrane Integrity Assay was obtained from Promega Corporation (Madison, USA). All the other reagents were of analytical grade.

2.2. Cell culture

Human umbilical vein endothelial cells (HUVECs) were purchased from Lonza (Verviers, Belgium) and cultured in endothelial cell growth medium-2 (EBM-2) supplemented with 2% FBS, 0.04% hydrocortisone, 0.4% hFGF-B, 0.1% VEGF, 0.1% R3-IGF-1, 0.1% ascorbic acid, 0.1% hEGF, 0.1% GA-1000, 0.1% heparin which were provided by Lonza (Verviers, Belgium). HUVECs were maintained in a humidified CO_2-incubator at 37 °C, and mycoplasma tests were performed regularly to ensure the absence of mycoplasma in cultures. Cells were used between passages 3–7.

Please cite this article in press as: Yousefi, A., et al., Anginex lipoplexes for delivery of anti-angiogenic siRNA, Int J Pharmaceut (2014), http://dx.doi.org/10.1016/j.ijpharm.2014.06.028

2.3. Preparation and physicochemical characterization of Angiplex

Angiplex was prepared as illustrated in Fig. 1. Briefly, siRNA was first complexed with protamine in 20 mM Hepes buffer containing 5% glucose at pH 7.4 (HBG) at siRNA to protamine ratio of 1:1.2 (w/w). Then DOPE, CHEMS, DSPE-PEG2000, DSPE-PEG2000-Mal were dissolved in chloroform/ethanol (3:2 v/v) in a round-bottom flask at molar ratios of 6:4:0.3:0.3, respectively. A lipid film was prepared by solvent evaporation method, and the film was hydrated by siRNA–protamine complexes. Total lipid concentration was 10 mM, and siRNA concentration was 1 μM. Lipoplexes were extruded repeatedly through polycarbonate membranes (Nuclepore, Pleasanton, CA, USA) with a final pore size of 100 nm by a Lipex™ Extruder (Northern Lipids, Burnaby, BC, Canada).

A solution of 0.5 M Hepes, 0.5 M hydroxylamine and 25 mM EDTA at pH 7.0 was added to SATA-anginex at volume ratio of 1:10 and was incubated for 45 min at room temperature to deprotect the SATA groups. Deprotected peptides were then added to lipoplexes at a concentration of 10 μg peptide per 1 μmol phospholipid and left at 4 °C overnight. Uncoupled peptides were separated from the peptide-modified lipoplexes by ultracentrifugation for 60 min at 200,000 × *g* at 4 °C (Brandwijk et al., 2007). 10-fold molar excess of maleimide groups of DSPE-PEG2000-Mal to SATA groups of SATA-anginex was calculated to ensure complete coupling. Coupling of the negative control peptide (SATA-βpep-28) was performed in the same manner.

Hydrodynamic diameter of lipoplexes was measured at 25 °C by dynamic light scattering on a Malvern 4700 system using an argon-ion laser (488 nm) operating at 10.4 mW (Uniphase) and PCS (photon correlation spectrometry) software for Windows version 1.34 (Malvern Instruments Ltd., Worcestershire, UK). 90° angle was used for all measurements, and viscosity and refractive index of water at 25 °C were applied. System calibration was done by standard polystyrene beads with a diameter of 200 nm (Thermo Scientific, DE, USA). Size distribution of lipoplexes was presented by PDI (polydispersity index) ranging from 0 indicating a monodisperse sample to 1 for a polydisperse sample. ζ-potential was determined at 25 °C by Zetasizer Nano-Z (Malvern Instruments Ltd., Worcestershire, UK). Calibration of the apparatus was done by Zeta Potential Transfer Standard (Malvern Instruments Ltd., Worcestershire, UK) with a known ζ-potential.

2.4. Transmission electron microscopy (TEM)

Morphology of Angiplex was visualized by TEM with a Philips Tecnai 10 equipped with a Biotwin lens and a LaB6 filament (100 kV acceleration voltage). 10 μL of lipoplexes was pipetted on a piece of Parafilm ,and a glow discharged copper grid (200 mesh with a carbon coated thin film) was placed on top of it. After 2 min, the excess liquid was removed by filter paper, and the grid was negatively stained with 2% uranyl acetate (Merck) and left for 5 min before capturing the images with a SIS Megaview II CCD-camera.

2.5. Agarose gel electrophoresis

Lipoplexes were prepared at siRNA concentration of 1 μM (20 pmol per gel slot) in HBG and mixed with Triton X-100 (5% w/v) and dextran sulfate sodium (DSS; 1% w/v). They were then incubated for 20 min at room temperature and applied to the slots of 4% (w/v) agarose gels. Tris-acetate-EDTA was used as running buffer, and electrophoresis was performed at 100 V for 45 min. Gelred™ (Phenix Research Products, NC, USA) was used to stain RNA after electrophoresis according to the manufacturer's protocol and images were made with UV transilluminator (ImaGo compact imaging system (B&L Systems), The Netherlands).

2.6. LDH cytotoxicity assay

HUVECs were seeded at 4000 cells/well in a 96-well plate 24 h prior to transfection. Then, the cells were transfected with formulations containing 38 nM RNA and a lipid concentration of 0.38 mM in EGM-2 complete medium. After 4 h of incubation at 37 °C in CO_2-incubator, LDH assay was performed with CytoTox-ONE™ kit (Promega, Madison, WI, USA) as described by the manufacturer.

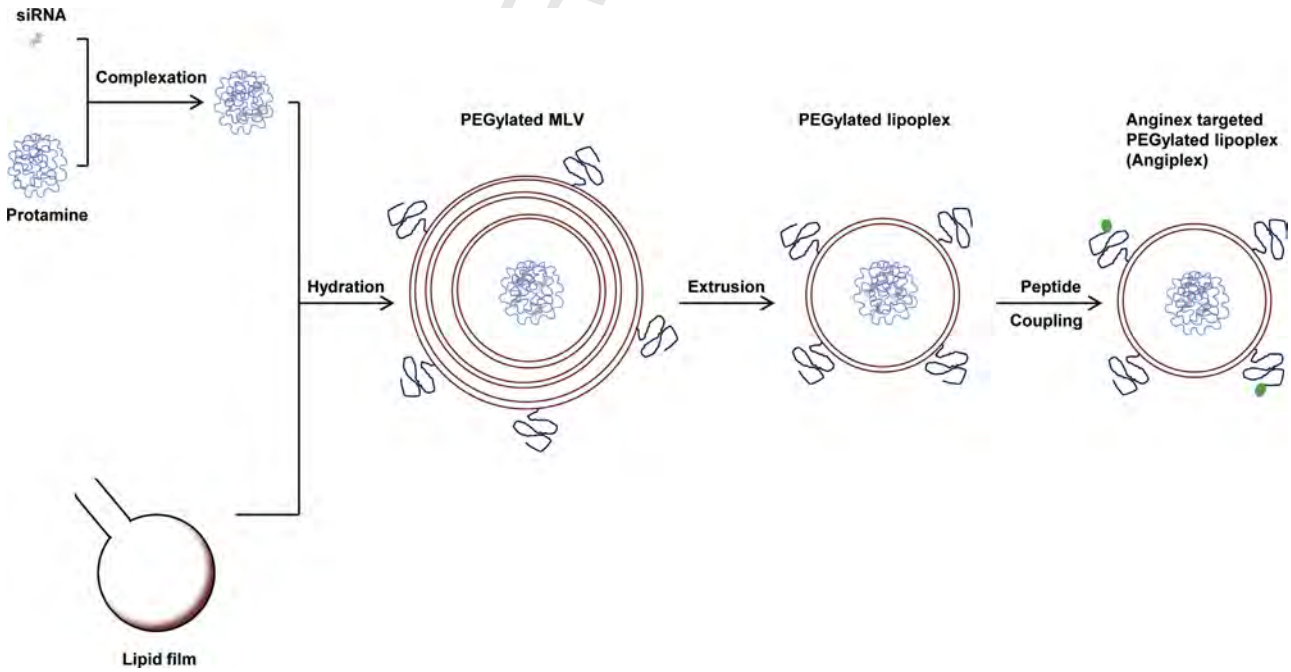

Fig. 1. Schematic representation of preparation of anginex targeted lipoplexes (Angiplex). MLV, multilamellar vesicles.

Please cite this article in press as: Yousefi, A., et al., Anginex lipoplexes for delivery of anti-angiogenic siRNA, Int J Pharmaceut (2014), http://dx.doi.org/10.1016/j.ijpharm.2014.06.028

2.7. Cell association and internalization

For cell association studies, HUVECs were seeded on ice in 96 well-plates at a concentration of 50,000 cells/well. Treatments containing 0.5 μM RNA were added immediately, and the plates were left at 4 °C for 1 h. Then, cell pellets were obtained by centrifugation of plates at 250 × *g* for 5 min at 4 °C. Pellets were washed 3 times with 0.3% BSA in PBS and fixed in 10% formalin.

For cell internalization experiments, HUVECs were seeded in 24 well-plates at concentration of 50,000 cells/well 24 h prior to transfection. Transfections were performed with formulations containing 0.2 μM RNA in EGM-2 complete medium. Cells were incubated for 4 h at 37 °C and then medium was changed with fresh EGM-2 complete medium. After another 44 h incubation time at 37 °C, cells were washed with acid wash solution (0.2 M glycine and 0.15 M NaCl at pH 3), detached and fixed in 10% formalin.

Flow cytometry analysis was performed by Becton & Dickenson FACSCalibur flow cytometer to determine mean fluorescence intensity per cell. 10,000 events were recorded per sample, and data analysis was done by FACSDiva™ software (Becton & Dickenson, Mountain View, CA, USA). GraphPad Prism 4 software was used for statistical analysis, and one-way ANOVA with a Tukey's post-test was used to calculate statistical significance.

2.8. Confocal microscopy

HUVECs were seeded in 16-well chamber slides (Thermo Fisher Scientific, MA, USA) at concentration of 10,000 cells/well 24 h before transfection. Thereafter, medium was removed, and cells were washed with 5% BSA in PBS on ice. Lipoplexes containing DiI (0.1 mol% of total lipid) and AF488 NC siRNA were added to cells at RNA concentration of 152 nM. Chamber slides were left for 1 h at 4 °C in the dark after which they were washed 2 times with PBS and incubated for 1 h at 37 °C in CO_2-incubator. Cells were then fixed in 10% formalin and incubated for 5 min at room temperature with DAPI for nuclei staining. Slides were mounted by FluorSave (Calbiochem, San Diego, CA, USA). A Zeiss Axiovert 200M confocal microscope (Carl Zeiss Microscopy GmbH, Germany) was used for obtaining the images. The microscope had a ×63 oil immersion objective (NA 1.2) plus ×2 digital zoom. An air-cooled argon-ion laser (LASOS, RMC 7812 Z, 488 nm) for FITC and a HeNE (LASOS, SAN 7450 A, 543 nm) laser for DyLight were used for excitation.

2.9. Competition assay

HUVECs were seeded in 24 well-plates at concentration of 50,000 cells/well 24 h before transfection. On the day of transfection, cells were washed once with PBS and free anginex was added immediately followed by addition of treatments. Separate plates were used for each concentration of free anginex (0, 10 or 30 μg/ml), and the experiment was done in triplicates. Treatments included Angiplex, βpep-28-lipoplexes (βpep-28-L), and bare lipoplexes (Bare-L) all containing AF488 NC siRNA. Plates were then incubated for 3 h at 37 °C in CO_2-incubator. After the incubation period, cells were washed with PBS, acid wash solution (0.2 M glycine and 0.15 M NaCl at pH 3) and another time with PBS. Then they were detached and fixed in 10% formalin. Flow cytometry analysis was performed as described in Section 2.7.

2.10. Western blotting

HUVECs were seeded in 6 well-plates at concentration of 50,000 cells/well 24 h prior to transfection. Cells were transfected 4 times with 1 h incubation-intervals with formulations containing 38 nM RNA (siVEGFR-2 or NC siRNA), and finally incubated for 48 h at 37 °C. Cell lysates were prepared using radioimmunoprecipitation assay (RIPA) buffer supplemented with EDTA and protease inhibitors (Thermo Scientific, DE, USA), and protein concentration was determined by MicroBCA Assay (Thermo Scientific, DE, USA). Samples containing 10 μg reduced protein were loaded on NuPAGE® 4–12% Bis–Tris polyacrylamide gel (Life Technologies, NY, USA), and electrophoresis was performed at 150 V for 45 min. Proteins were transferred from the gel to nitrocellulose membrane using the iBlot® Dry Blotting System (Life Technologies, NY, USA). Membranes were blocked with 5% BSA in TBS-T (Tris-buffered saline containing 0.1% Tween20 at pH 7.4) for 1 h at room temperature. After 3 washing steps with TBS-T, membranes were incubated with primary antibody against β-actin (Cell Signaling Technology, OR, USA) or VEGF Receptor2 Rabbit mAb (Cell Signaling Technology, OR, USA) for 2 h at room temperature. Then, the blots were washed 3 times with TBS-T and were incubated with Peroxidase Conjugated Stabilized Goat Anti-Rabbit IgG (H + L) (Thermo Scientific, DE, USA) for 2 h at room temperature. All primary and secondary antibodies were diluted 1:1000 (v/v) in 5% BSA in TBS-T. Visualization of proteins was performed by Super-Signal West Femto Chemiluminescent Substrate (Thermo Scientific, DE, USA) as indicated by the manufacturer, and images were made by Bio-Rad CHEMDOC XRS (Veenendaal, The Netherlands). Bands were quantified by ImageJ 1.45s (NIH, USA). Statistical analysis was performed by GraphPad Prism 4 software. Statistical significance was calculated by one-way ANOVA with a Tukey's post-test.

3. Results and discussion

3.1. Preparation and characterization of Angiplex

Angiplex was prepared in 2 steps. First, siRNA was complexed with protamine. Pre-complexation of siRNA with polycationic molecules is a common strategy to increase the encapsulation efficiency of these small RNA molecules inside neutral or negatively charged vesicles (Li and Huang, 2006a; Chono et al., 2008; Li and Huang, 2006b; Li et al., 2008; Mastrobattista et al., 2001; Wang et al., 2013). Second, siRNA–protamine complexes were coated with lipid bilayers by the lipid film hydration method. In the composition of the lipid bilayers, DOPE (a cone-like fusogenic lipid) was used. It causes transition of the lipid bilayer from lamellar phase (L_α) to hexagonal phase (H_{II} in acidic pH by creating negative curvature (Koltover et al., 1998; Guo et al., 2003). This property is beneficial in endosomal escape which is usually the limiting step in nucleic acid delivery. In presence of DOPE, the lipid bilayer can fuse to the endosomal membrane

Table 1
Mean size and mean zeta potential of lipoplexes ($n = 3$).

Sample name	Size (nm) ± SD	PDI	Zeta potential (mV) ± SD
siVEGFR-2 protamine lipoplex (Bare-L)	104 ± 7	0.1	−13 ± 1
siVEGFR-2 protamine Ax lipoplex (Angiplex)	124 ± 3	0.2	−15 ± 1
siVEGFR-2 protamine βpep-28 lipoplex (bpep-28-L)	124 ± 1	0.1	−15 ± 1
Empty Ax lipoplex	106 ± 1	0.1	−13 ± 1

Please cite this article in press as: Yousefi, A., et al., Anginex lipoplexes for delivery of anti-angiogenic siRNA, Int J Pharmaceut (2014), http://dx.doi.org/10.1016/j.ijpharm.2014.06.028

when the pH drops, and therefore the content is released into the cytosol. Another important phospholipid in the formulation was DSPE-PEG2000 which, due to the PEG-chains, prevents aggregation. In addition, it may provide stealth properties to the particles which is important for circulation time. It has been shown in the literature that PEG decreases the pH-dependent activity of DOPE as a result of stabilizing the particles (Drummond et al., 2000; Venugopalan et al., 2002) but since PEG itself possesses fusogenic properties, it has a net contribution to the efficacy of DOPE liposomes (Simoes et al., 2001). In the composition of the lipoplexes, DSPE-PEG2000-Mal has been used to attach the targeting peptide anginex or control peptide βpep-28 to the distal ends of PEG-chains.

As indicated in Table 1, the resulting peptide-coupled lipoplexes were relatively monodisperse with an average size of ~120 nm and a net negative charge of −15 mV. Coupling of peptides did not change size and charge of the particles significantly. These particles have attractive size for nanomedicine applications and their PEGylation should prevent opsonization by plasma proteins and improve their circulation half-life. The net negative charge of the final preparations creates an advantage over cationic liposomes. Positively-charged particles interact more with plasma proteins than their negatively-charged counterparts which leads to their rapid clearance and undesirable pharmacokinetics (Tros de Ilarduya et al., 2003; Opanasopit et al., 2002; Knudsen et al., 2014). Also, they result in activation of immune response and thereby toxicity (Yasuda et al., 2005; Dow et al., 1999; Liu et al., 1995; Liu et al., 1997).

Encapsulation efficiency of siRNA in Angiplex was determined by quantifying the amount of siRNA present in the formulation using RiboGreen®. For this, particles were first disrupted with 5% (w/v) of Triton X-100 and 1% (w/v) of DSS. Then the percentage of siRNA entrapped in the lipoplexes was calculated by subtracting the amount detected for non-treated lipoplexes from the amount detected for disrupted lipoplexes. Based on the RiboGreen® quantitation it was calculated that 51% of the initial amount of siRNA was entrapped inside the lipoplexes.

As control formulation, lipoplexes with siRNA equipped with βpep28 (βpep-28-L), empty Angiplex, and bare lipoplexes (Bare-L) were prepared in the same way as described above, and there was no considerable difference between size and charge of these formulations and that of Angiplex (Table 1).

Morphology of Angiplex was investigated by TEM (Fig. 2). Vesicular structures and tubular structures were observed. Based on the method of particle preparation it is likely that the vesicular structures represent typical liposomal bilayers encompassing an aqueous core.

3.2. Angiplex is stable and maintains integrity of siRNA

To investigate complexation stability of Angiplex, gel retardation experiments were done in the presence or absence of 5% (w/v) of Triton X-100 and 1% (w/v) of DSS. Angiplex released siRNA only after the formulations were treated with 5% (w/v) of Triton X-100 and 1% (w/v) of DSS (Fig. 3, column 5). Maintaining nucleic acid integrity is essential for an intravenously injected carrier because in stably-complexed siRNA carriers less displacement of siRNA in the formulation with negatively charged serum components occurs (van de Water et al., 2006).

3.3. Cellular toxicity is significantly lower after treatment with Angiplex than with cationic liposomes

In order to evaluate cytotoxicity, HUVECs were transfected with Angiplex, positively-charged DOTAP liposomes (DOTAP/cholesterol at a molar ratio of 1:1) and a commercially available transfecting agent, Lipofectamine 2000. All formulations contained 38 nM NC siRNA. 4 h after transfection, release of LDH was measured and reported relative to that of non-treated cells as measure of cytotoxicity. Release of LDH was 13 times lower in cells treated with Angiplex compared to DOTAP liposomes and 5 times lower than Lipofectamine 2000-treated cells (Fig. 4). This effect could be related to PEGylation and the negative charge at the surface of Angiplex which cause less adsorption to cell-membranes with concomitant reduction of membrane integrity disruption.

3.4. Angiplex binds to HUVECs and is internalized

Cell association of Angiplex to galectin-1 was investigated on HUVECs. To detect only the particles bound to the cell surface and not internalized, we performed our studies at low temperatures. Fluorescently-labeled siRNA (AF488 NC siRNA) was incorporated in the particles to allow detection of cell association by flow cytometry. Results were expressed as mean fluorescence intensity (MFI) relative to that of non-treated samples. Cell association of Angiplex was 10-fold higher than that of Lipofectamine 2000 which could be attributed to

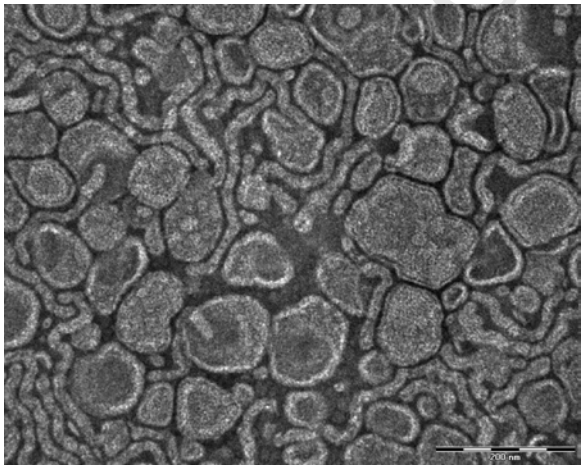

Fig. 2. TEM image of Angiplex. Scale bar shows 200 nm.

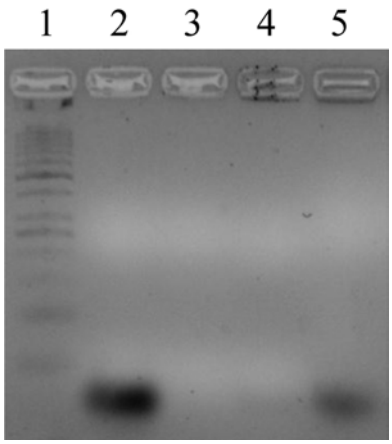

Fig. 3. Gel electrophoresis of Angiplex before and after treatment with Triton X-100 and DSS to evaluate complexation stability (1: marker, 2: naked siVEGFR-2, 3: empty Angiplex, 4: siVEGFR-2-Angiplex, 5: siVEGFR-2-Angiplex + Triton X-100 5% + DSS 1%).

multimeric display and high affinity of anginex to galectin-1 on HUVECs (Fig. 5A) (Thijssen et al., 2006). The association of Lipofectamine 2000 particles is likely not based on receptor interaction but rather on charge-based interactions with negatively charged surface molecules. Although Lipofectamine 2000 is a cationic transfection agent which would interact readily with cell membrane, the lower binding observed in this experiment compared to Angiplex could be due to different quenching of fluorescent siRNA in Angiplex and in Lipofectamine 2000. Binding of βpep-28-L and Bare-L were below detection limit, likely because of lack of specific interaction. These last two controls do show that the surface is indeed little interactive.

In the internalization studies, in order to remove the surface-bound particles the cells were washed with acid wash solution (0.2 M glycine and 0.15 M NaCl at pH 3) prior to fixation and then flow cytometry was performed. Angiplex was internalized by HUVECs 1.5-fold more than Lipofectamine 2000, and fluorescence intensities were noticeably higher compared to βpep-28-L and Bare-L (Fig. 5B). This observation could be related to the fact that Angiplex is taken up specifically via ligand–receptor as well as non-specific interactions whereas Lipofectamine 2000 complexes are internalized via charge interactions in a non-specific way. Uptake of other formulations were hypothesized to be mainly through non-specific endocytosis.

Confocal microscopy images taken after transfection of HUVECs with lipoplexes confirmed the above findings. Cells were first incubated for 1 h at 4 °C and then washed with PBS to remove non-bound particles. Then, they were incubated for 1 h at 37 °C to provide enough time for the attached particles to be internalized. A punctuate pattern of green fluorescence from AF488 dye was seen in the cytosol in case of samples treated with Angiplex which was absent in cells treated with βpep-28-L and Bare-L (Fig. 6). These dots which indicated the presence of siRNA could point to the entrapped particles in the endosomes. The diffused red fluorescence observed in the cytosol of the cells is due to DiI dye which is incorporated in the liposomes as well as autofluorescence since it is also observed in the non-treated samples. The red color is present in all samples although it is the strongest in Angiplex-treated cells.

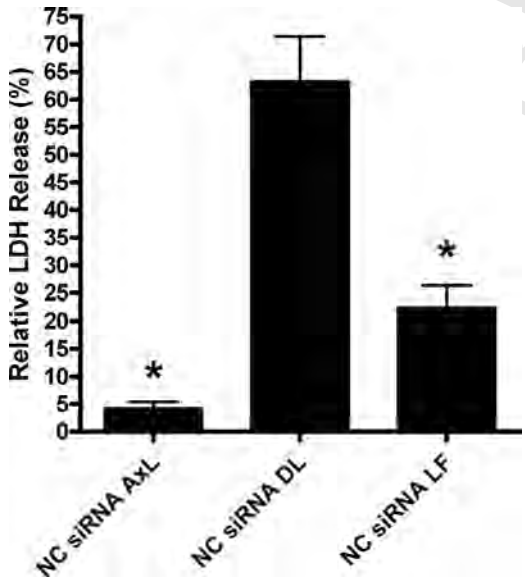

Fig. 4. Toxicity of Angiplex (AxL) compared to DOTAP liposomes (DL) and Lipofectamine 2000 (LF) in HUVECs 4 h after addition of formulations. Lipid concentration: 0.38 mM. Release of cytosolic lactate dehydrogenase (LDH) indicates toxicity ($n = 5$). $p < 0.001$ is denoted by (*).

3.5. Uptake of Angiplex in HUVECs is peptide-dependent

In order to assess whether the uptake of lipoplexes was through specific anginex–galectin-1 interaction, competition assays were performed. HUVECs were incubated with particles competing with free anginex for 3 h at 37 °C. The amount of free anginex added to the treated cells was at the following molar ratios compared to coupled anginex: 0:1, 1:1, 3:1 corresponding to free anginex

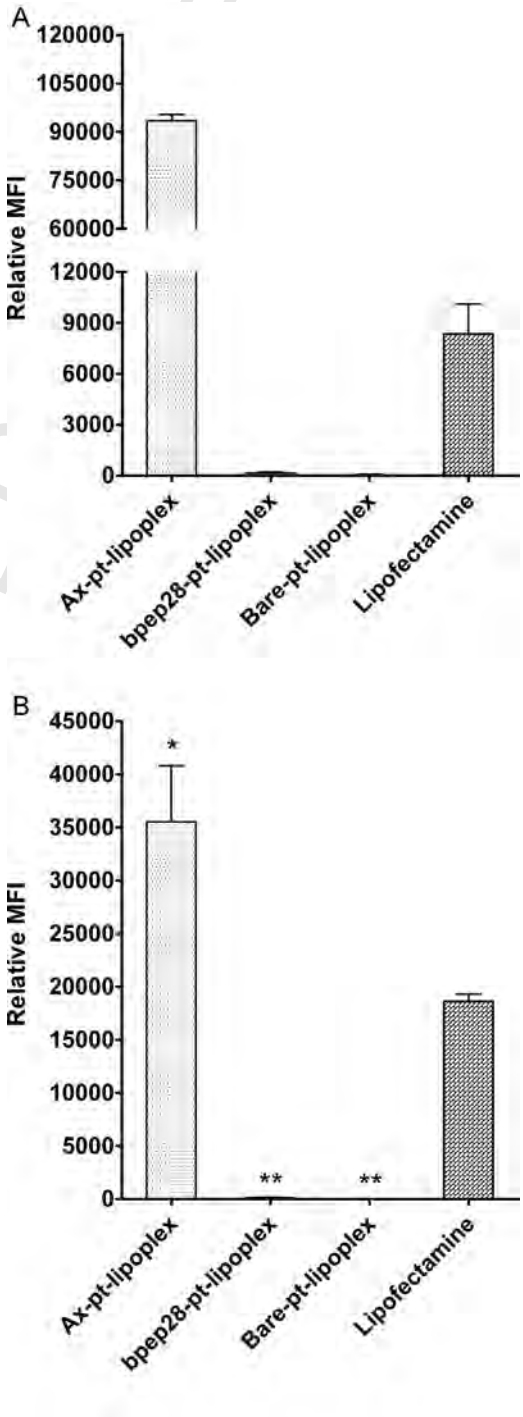

Fig. 5. (A) Cell binding of Angiplex in comparison with βpep-28-L and Lipofectamine 2000 in HUVECs 1 h after transfection. All treatments contained 0.5 μM of AF488 NC siRNA ($n = 3$). (B) Cell uptake of Angiplex in comparison with βpep-28-L and Lipofectamine 2000 in HUVECs 48 h after transfection at AF488 NC siRNA concentration of 0.2 μM ($n = 3$). $p < 0.05$ is denoted by (*) and $p < 0.001$ by (**).

Please cite this article in press as: Yousefi, A., et al., Anginex lipoplexes for delivery of anti-angiogenic siRNA, Int J Pharmaceut (2014), http://dx.doi.org/10.1016/j.ijpharm.2014.06.028

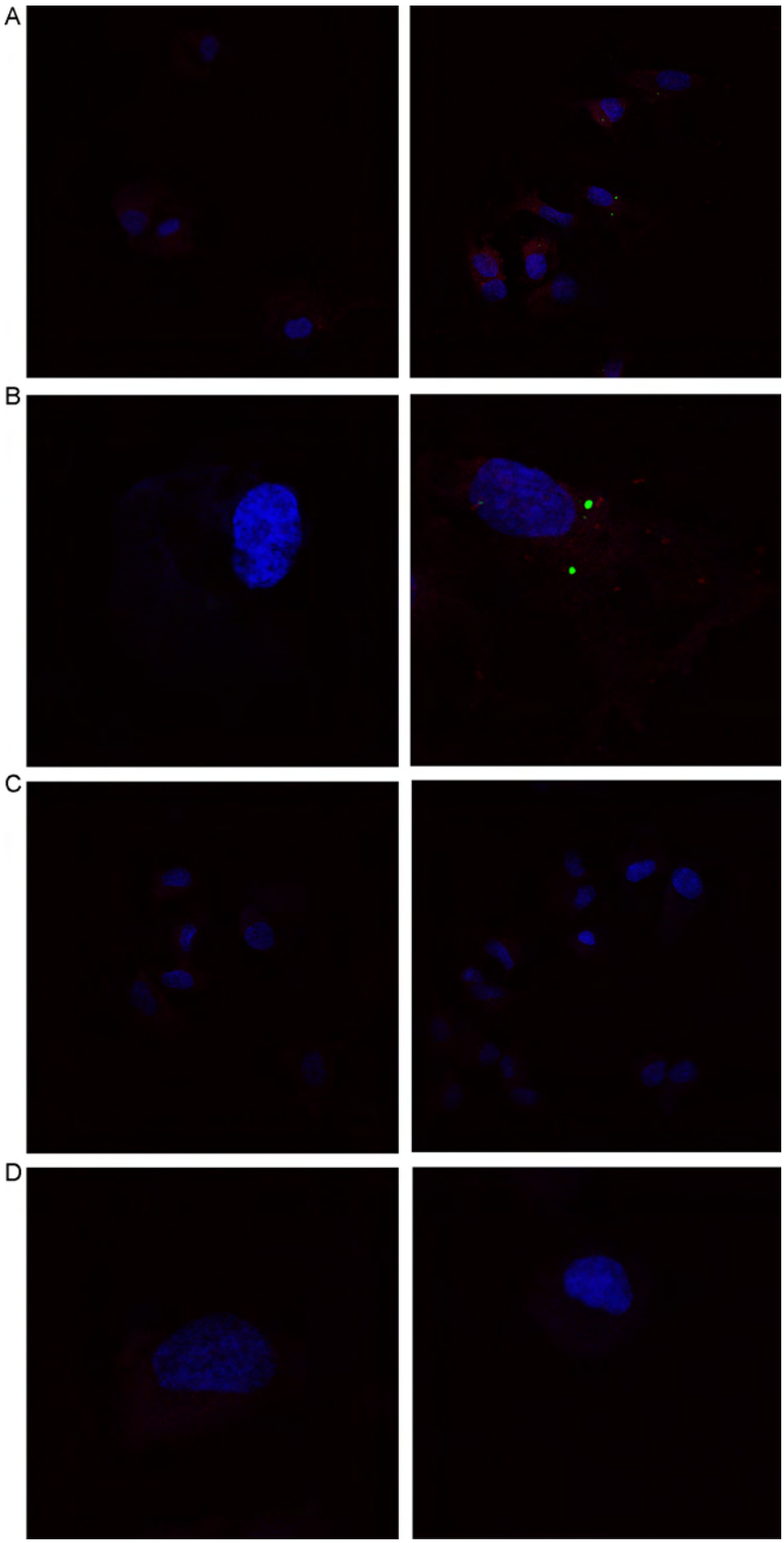

Fig. 6. Confocal microscopy images of HUVECs incubated with treatments for 1 h at 4 °C, washed with PBS and then incubated for 1 h at 37 °C. Panels (B) and (D) show the same cells from panels (A) and (C), respectively, with a higher zoom. Different colors of staining reflect the following: blue for cell nuclei, red for liposomes, and green for siRNA. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

Please cite this article in press as: Yousefi, A., et al., Anginex lipoplexes for delivery of anti-angiogenic siRNA, Int J Pharmaceut (2014), http://dx.doi.org/10.1016/j.ijpharm.2014.06.028

concentrations of 0, 10, and 30 μg/ml. After removing non-bound particles, flow cytometry measurements were done. Fluorescence intensity reflected the level of cell internalization. A drop in fluorescence signal was observed for Angiplex when the concentration of free anginex increased from 10 to 30 μg/ml while this effect was absent for βpep-28-L and Bare-L (Fig. 7). There was no significant difference of fluorescence at free anginex concentrations of 0–10 μg/ml. It was hypothesized that a minimum concentration of free anginex is needed to block enough galectin-1 receptors and result in a measurable effect. These results demonstrate specific uptake of Angiplex through anginex.

3.6. Angiplex reduces expression of VEGFR-2 by delivering siRNA complementary to mRNA of VEGFR-2

Biological activity of delivered siVEGFR-2 was evaluated 48 h after treatment of HUVECs with Angiplex. Transfections were done multiple times with 1 h time intervals to mimic the constant clearance of particles in the blood stream *in vivo*. βpep-28 was used as a non-targeting peptide mimicking anginex since it has the same length of 33 amino acids, 22 of which are identical to anginex. Moreover, an aspecific negative control peptide (asp-NCpep) with no similarities to anginex was used to assess any differences that might occur due to resemblance of βpep-28 to anginex. Down-regulation of VEGFR-2 was 61% when HUVECs were treated with Angiplex (Fig. 8). Angiplex showed effect through RNAi and through the binding of galectin-1 to neuropilin-1. The latter can trigger the down-regulation of VEGFR-2-mediated signaling pathway (Hsieh et al., 2008). Another observation was that treatments containing negative control peptides resulted in similar silencing efficiency to Angiplex. As this effect was not observed in the uptake study (Section 3.5) it could be concluded that the different set-up of the gene knock-down experiment (maintaining the formulations with the cells for 48 h) versus the internalization study (4 h incubation of the formulations with the cells) has led to remarkable non-specific uptake.

Compelling evidence has indicated that simultaneous targeting of different pathways in tumor cells offers more efficiency benefits compared to a single-targeted approach because it can potentially circumvent drug resistance (Nie et al., 2011; Kluza et al., 2012; Kibria et al., 2011; Takara et al., 2012). For example, anti-prostate-specific membrane antigen (PSMA) RNA aptamer conjugated to gelonin toxin, improved toxicity in prostate tumor cells (Chu et al., 2006). In another study, siRNA against signal transducer and activator of transcription 3 (STAT3) was delivered by a B-cell-activating factor (BAFF)-receptor aptamer targeting B-cells. A remarkable combined effect was observed by inhibition of B-cell proliferation via interaction of the aptamer with BAFF-receptor and decrease of STAT3 mRNA levels through RNAi in B-cells (Zhou et al., 2013). EGa1 nanobody-coupled liposomes loaded with the AG538 (inhibitor of IGF-1R) have been shown to downregulate EGFR expression with a higher efficacy than either empty EGa1-coupled liposomes or non-targeted AG538 liposomes. This effect has been attributed to the simultaneous inhibition of EGFR activation by EGa1 nanobody and IGF-1R inhibition by AG538 (van der Meel et al., 2012). Our findings have revealed that Angiplex has caused a double effect on a cellular process through combining a ligand–receptor interaction event and manipulating cellular pathways at different levels. Hsieh et al. have reported that binding of galectin-1 to neuropilin-1

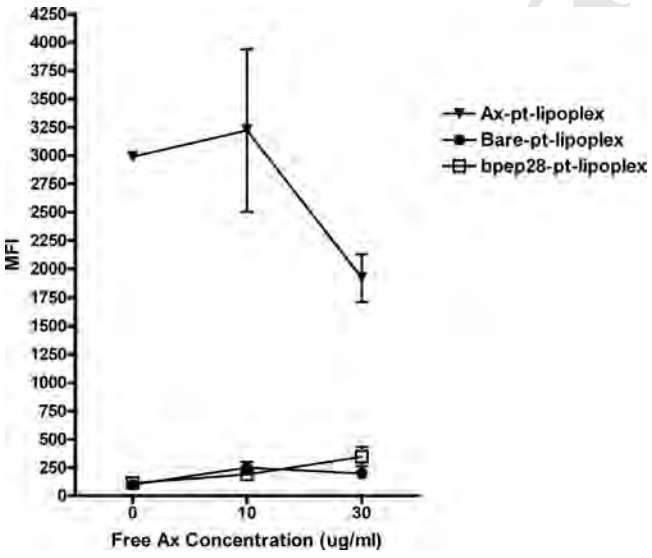

Fig. 7. Competition of Angiplex with free anginex in comparison with βpep-28-L and Bare-L at free anginex concentrations of 0, 10, and 30 μg/ml. MFI represents mean fluorescence intensity ($n = 3$).

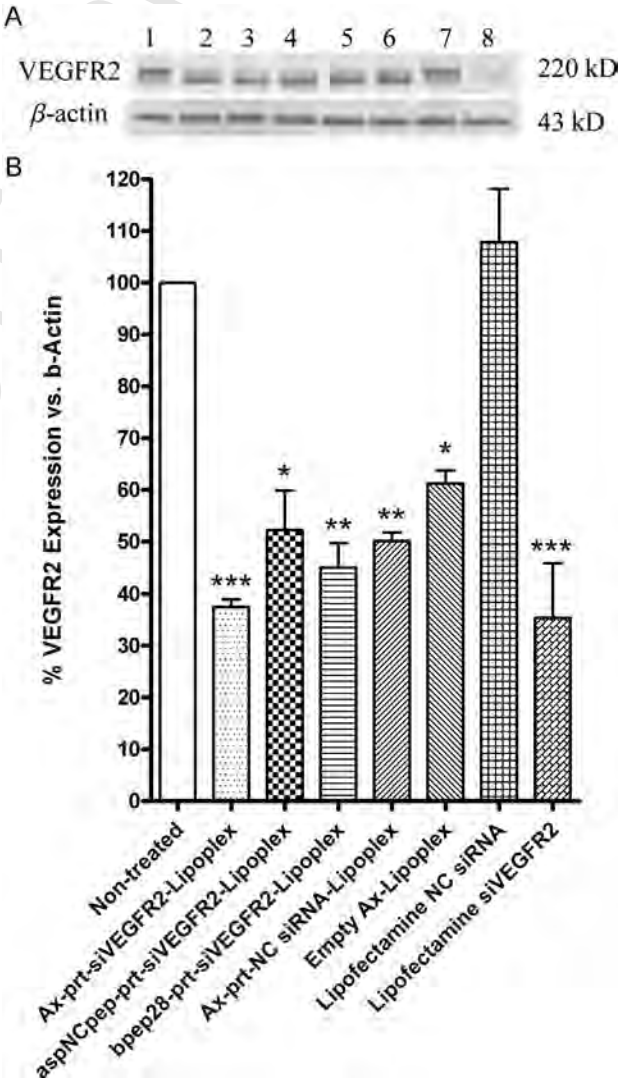

Fig. 8. (A) Western blot of VEGFR-2 48 h after transfection of HUVECs with lipoplexes containing siVEGFR-2, NC siRNA or empty lipoplexes at siRNA concentration of 0.2 μM (1: non-treated, 2: Angiplex-siVEGFR-2, 3: asp-NCpep-L-siVEGFR-2, 4: βpep-28-L-siVEGFR-2, 5: Angiplex-NC siRNA, 6: empty Angiplex, 7: Lipofectamine 2000-NC siRNA, 8: Lipofectamine 2000-siVEGFR-2). β-actin was used as loading control. (B) Quantitation of silencing efficiency ($n = 3$). $p < 0.05$ is denoted by (*), $p < 0.01$ by (**), and $p < 0.001$ by (***).

Please cite this article in press as: Yousefi, A., et al., Anginex lipoplexes for delivery of anti-angiogenic siRNA, Int J Pharmaceut (2014), http://dx.doi.org/10.1016/j.ijpharm.2014.06.028

down-regulates the VEGFR-2 pathway (Hsieh et al., 2008). Moreover, it has been reported that anginex liposomes per se have anti-angiogenic capabilities because of anginex (Brandwijk et al., 2007). The above observations could explain how empty angiplex particles in our study led to silencing of VEGFR-2 gene.

4. Conclusion

In this study, anginex lipoplexes (Angiplex) encapsulating siRNA were prepared with an average size of 120 nm and a net negative charge. These complexes showed low cell toxicity and their high cell internalization resulted in up to 61% gene knock-down. Surprisingly, the lipoplex we developed led to an intrinsic down-regulation of VEGFR-2 irrespective of RNA-payload or targeting ligand. We are currently exploring the underlying mechanism behind this phenomenon. Taken together, Angiplex can be considered as a promising candidate for anti-angiogenic therapy through siRNA delivery and anginex activity.

Acknowledgements

This research was financially supported by and performed within the framework of the Dutch Top Institute Pharma project T 3-301. We thank Raimond Heukers for assistance with confocal microscopy experiments and Joep van den Dikkenberg for TEM experiments.

References

Astorgues-Xerri, L., Riveiro, M.E., Tijeras-Raballand, A., Serova, M., Neuzillet, C., Albert, S., Raymond, E., Faivre, S., 2014. Unraveling galectin-1 as a novel therapeutic target for cancer. Cancer Treat. Rev. 40, 307–319.
Bergers, G., Benjamin, L.E., 2003. Tumorigenesis and the angiogenic switch. Nat. Rev. Cancer 3, 401–410.
Brandwijk, R.J., Mulder, W.J., Nicolay, K., Mayo, K.H., Thijssen, V.L., Griffioen, A.W., 2007. Anginex-conjugated liposomes for targeting of angiogenic endothelial cells. Bioconjug. Chem. 18, 785–790.
Chono, S., Li, S.D., Conwell, C.C., Huang, L., 2008. An efficient and low immunostimulatory nanoparticle formulation for systemic siRNA delivery to the tumor. J. Control. Release 131, 64–69.
Chu, T.C., Marks 3rd., J.W., Lavery, L.A., Faulkner, S., Rosenblum, M.G., Ellington, A.D., Levy, M., 2006. Aptamer:toxin conjugates that specifically target prostate tumor cells. Cancer Res. 66, 5989–5992.
Clausse, N., van den Brule, F., Waltregny, D., Garnier, F., Castronovo, V., 1999. Galectin-1 expression in prostate tumor-associated capillary endothelial cells is increased by prostate carcinoma cells and modulates heterotypic cell-cell adhesion. Angiogenesis 3, 317–325.
D'Haene, N., Sauvage, S., Maris, C., Adanja, I., Le Mercier, M., Decaestecker, C., Baum, L., Salmon, I., 2013. VEGFR1 and VEGFR2 involvement in extracellular galectin-1- and galectin-3-induced angiogenesis. PLoS One 8, e67029.
Dow, S.W., Fradkin, L.G., Liggitt, D.H., Willson, A.P., Heath, T.D., Potter, T.A., 1999. Lipid-DNA complexes induce potent activation of innate immune responses and antitumor activity when administered intravenously. J. Immunol. 163, 1552–1561.
Drummond, D.C., Zignani, M., Leroux, J., 2000. Current status of pH-sensitive liposomes in drug delivery. Prog. Lipid Res. 39, 409–460.
Dvorak, H.F., Brown, L.F., Detmar, M., Dvorak, A.M., 1995. Vascular permeability factor/vascular endothelial growth factor, microvascular hyperpermeability, and angiogenesis. Am. J. Pathol. 146, 1029–1039.
Eichmann, A., Corbel, C., Nataf, V., Vaigot, P., Breant, C., Le Douarin, N.M., 1997. Ligand-dependent development of the endothelial and hemopoietic lineages from embryonic mesodermal cells expressing vascular endothelial growth factor receptor 2. Proc. Natl. Acad. Sci. U. S. A. 94, 5141–5146.
Elsabahy, M., Nazarali, A., Foldvari, M., 2011. Non-viral nucleic acid delivery: key challenges and future directions. Curr. Drug Deliv. 8, 235–244.
Folkman, J., 1995. Angiogenesis in cancer, vascular, rheumatoid and other disease. Nat. Med. 1, 27–31.
Forsythe, J.A., Jiang, B.H., Iyer, N.V., Agani, F., Leung, S.W., Koos, R.D., Semenza, G.L., 1996. Activation of vascular endothelial growth factor gene transcription by hypoxia-inducible factor 1. Mol. Cell Biol. 16, 4604–4613.
Gacche, R.N., Meshram, R.J., 2013. Targeting tumor micro-environment for design and development of novel anti-angiogenic agents arresting tumor growth. Prog. Biophys. Mol. Biol. 113, 333–354.
Griffioen, A.W., Molema, G., 2000. Angiogenesis: potentials for pharmacologic intervention in the treatment of cancer, cardiovascular diseases, and chronic inflammation. Pharmacol. Rev. 52, 237–268.
Griffioen, A.W., van der Schaft, D.W., Barendsz-Janson, A.F., Cox, A., Struijker Boudier, H.A., Hillen, H.F., Mayo, K.H., 2001. Anginex, a designed peptide that inhibits angiogenesis. Biochem. J. 354, 233–242.
Guo, W., Lee, R.J., 2000. Efficient gene delivery using anionic liposome-complexed polyplexes (LPDII). Biosci. Rep. 20, 419–432.
Guo, X., MacKay, J.A., Szoka Jr., F.C., 2003. Mechanism of pH-triggered collapse of phosphatidylethanolamine liposomes stabilized by an ortho ester polyethyleneglycol lipid. Biophys. J. 84, 1784–1795.
Guo, J., Fisher, K.A., Darcy, R., Cryan, J.F., O'Driscoll, C., 2010. Therapeutic targeting in the silent era: advances in non-viral siRNA delivery. Mol. Biosyst. 6, 1143–1161.
Hsieh, S.H., Ying, N.W., Wu, M.H., Chiang, W.F., Hsu, C.L., Wong, T.Y., Jin, Y.T., Hong, T. M., Chen, Y.L., 2008. Galectin-1, a novel ligand of neuropilin-1, activates VEGFR-2 signaling and modulates the migration of vascular endothelial cells. Oncogene 27, 3746–3753.
Hurwitz, H., Fehrenbacher, L., Novotny, W., Cartwright, T., Hainsworth, J., Heim, W., Berlin, J., Baron, A., Griffing, S., Holmgren, E., Ferrara, N., Fyfe, G., Rogers, B., Ross, R., Kabbinavar, F., 2004. Bevacizumab plus irinotecan, fluorouracil, and leucovorin for metastatic colorectal cancer. N. Engl. J. Med. 350, 2335–2342.
Kibria, G., Hatakeyama, H., Ohga, N., Hida, K., Harashima, H., 2011. Dual-ligand modification of PEGylated liposomes shows better cell selectivity and efficient gene delivery. J. Control. Release 153, 141–148.
Kluza, E., Jacobs, I., Hectors, S.J., Mayo, K.H., Griffioen, A.W., Strijkers, G.J., Nicolay, K., 2012. Dual-targeting of alphavbeta3 and galectin-1 improves the specificity of paramagnetic/fluorescent liposomes to tumor endothelium in vivo. J. Control. Release 158, 207–214.
Knudsen, K.B., Northeved, H., Ek, P.K., Permin, A., Andresen, T.L., Larsen, S., Wegener, K.M., Lam, H.R., Lykkesfeldt, J., 2014. Differential toxicological response to positively and negatively charged nanoparticles in the rat brain. Nanotoxicology 8, 764–774.
Koldehoff, M., Steckel, N.K., Beelen, D.W., Elmaagacli, A.H., 2007. Therapeutic application of small interfering RNA directed against bcr-abl transcripts to a patient with imatinib-resistant chronic myeloid leukaemia. Clin. Exp. Med. 7, 47–55.
Koltover, I., Salditt, T., Radler, J.O., Safinya, C.R., 1998. An inverted hexagonal phase of cationic liposome-DNA complexes related to DNA release and delivery. Science 281, 78–81.
Li, S.D., Huang, L., 2006a. Surface-modified LPD nanoparticles for tumor targeting. Ann. N. Y. Acad. Sci. 1082, 1–8.
Li, S.D., Huang, L., 2006b. Targeted delivery of antisense oligodeoxynucleotide and small interference RNA into lung cancer cells. Mol. Pharm. 3, 579–588.
Li, S.D., Chen, Y.C., Hackett, M.J., Huang, L., 2008. Tumor-targeted delivery of siRNA by self-assembled nanoparticles. Mol. Ther. 16, 163–169.
Liu, Y., Liggitt, D., Zhong, W., Tu, G., Gaensler, K., Debs, R., 1995. Cationic liposome-mediated intravenous gene delivery. J. Biol. Chem. 270, 24864–24870.
Liu, Y., Mounkes, L.C., Liggitt, H.D., Brown, C.S., Solodin, I., Heath, T.D., Debs, R.J., 1997. Factors influencing the efficiency of cationic liposome-mediated intravenous gene delivery. Nat. Biotechnol. 15, 167–173.
Lotan, R., Belloni, P.N., Tressler, R.J., Lotan, D., Xu, X.C., Nicolson, G.L., 1994. Expression of galectins on microvessel endothelial cells and their involvement in tumour cell adhesion. Glycoconj. J. 11, 462–468.
Mahalingam, D., Malik, L., Beeram, M., Rodon, J., Sankhala, K., Mita, A., Benjamin, D., Ketchum, N., Michalek, J., Tolcher, A., Wright, J., Sarantopoulos, J., 2014. Phase II study evaluating the efficacy, safety, and pharmacodynamic correlative study of dual antiangiogenic inhibition using bevacizumab in combination with sorafenib in patients with advanced malignant melanoma. Cancer Chemother. Pharmacol. .
Mastrobattista, E., Kapel, R.H., Eggenhuisen, M.H., Roholl, P.J., Crommelin, D.J., Hennink, W.E., Storm, G., 2001. Lipid-coated polyplexes for targeted gene delivery to ovarian carcinoma cells. Cancer Gene Ther. 8, 405–413.
Nie, Y., Schaffert, D., Rodl, W., Ogris, M., Wagner, E., Gunther, M., 2011. Dual-targeted polyplexes: one step towards a synthetic virus for cancer gene therapy. J. Control. Release 152, 127–134.
Nimesh, S., Gupta, N., Chandra, R., 2011. Cationic polymer based nanocarriers for delivery of therapeutic nucleic acids. J. Biomed. Nanotechnol. 7, 504–520.
Olsson, A.K., Dimberg, A., Kreuger, J., Claesson-Welsh, L., 2006. VEGF receptor signalling – in control of vascular function. Nat. Rev. Mol. Cell Biol. 7, 359–371.
Opanasopit, P., Nishikawa, M., Hashida, M., 2002. Factors affecting drug and gene delivery: effects of interaction with blood components. Crit. Rev. Ther. Drug Carrier Syst. 19, 191–233.
Patil, S.D., Rhodes, D.G., Burgess, D.J., 2005. Biophysical characterization of anionic lipoplexes. Biochim. Biophys. Acta 1711, 1–11.
Prewett, M., Huber, J., Li, Y., Santiago, A., O'Connor, W., King, K., Overholser, J., Hooper, A., Pytowski, B., Witte, L., Bohlen, P., Hicklin, D.J., 1999. Antivascular endothelial growth factor receptor (fetal liver kinase 1) monoclonal antibody inhibits tumor angiogenesis and growth of several mouse and human tumors. Cancer Res. 59, 5209–5218.
Rubinstein, N., Alvarez, M., Zwirner, N.W., Toscano, M.A., Ilarregui, J.M., Bravo, A., Mordoh, J., Fainboim, L., Podhajcer, O.L., Rabinovich, G.A., 2004. Targeted inhibition of galectin-1 gene expression in tumor cells results in heightened T cell-mediated rejection; a potential mechanism of tumor-immune privilege. Cancer Cell 5, 241–251.
Simoes, S., Slepushkin, V., Duzgunes, N., Pedroso de Lima, M.C., 2001. On the mechanisms of internalization and intracellular delivery mediated by pH-sensitive liposomes. Biochim. Biophys. Acta 1515, 23–37.
Sliva, K., Schnierle, B.S., 2010. Selective gene silencing by viral delivery of short hairpin RNA. Virol. J. 7, 248.

Smith, D.A., Conkling, P., Richards, D.A., Nemunaitis, J.J., Boyd, T.E., Mita, A.C., de La Bourdonnaye, G., Wages, D., Bexon, A.S., 2014. Antitumor activity and safety of combination therapy with the Toll-like receptor 9 agonist IMO -2055, erlotinib, and bevacizumab in advanced or metastatic non-small cell lung cancer patients who have progressed following chemotherapy. Cancer Immunol. Immunother. .

Takara, K., Hatakeyama, H., Kibria, G., Ohga, N., Hida, K., Harashima, H., 2012. Size-controlled, dual-ligand modified liposomes that target the tumor vasculature show promise for use in drug-resistant cancer therapy. J. Control. Release 162, 225–232.

Thijssen, V.L., Postel, R., Brandwijk, R.J., Dings, R.P., Nesmelova, I., Satijn, S., Verhofstad, N., Nakabeppu, Y., Baum, L.G., Bakkers, J., Mayo, K.H., Poirier, F., Griffioen, A.W., 2006. Galectin-1 is essential in tumor angiogenesis and is a target for antiangiogenesis therapy. Proc. Natl. Acad. Sci. U. S. A. 103, 15975–15980.

Tros de Ilarduya, C., Arangoa, M.A., Duzgunes, N., 2003. Transferrin-lipoplexes with protamine-condensed DNA for serum-resistant gene delivery. Methods Enzymol. 373, 342–356.

Venugopalan, P., Jain, S., Sankar, S., Singh, P., Rawat, A., Vyas, S.P., 2002. pH-sensitive liposomes: mechanism of triggered release to drug and gene delivery prospects. Pharmazie 57, 659–671.

Wang, Y., Zhang, L., Guo, S., Hatefi, A., Huang, L., 2013. Incorporation of histone derived recombinant protein for enhanced disassembly of core-membrane structured liposomal nanoparticles for efficient siRNA delivery. J. Control. Release 172, 179–189.

Yasuda, K., Ogawa, Y., Yamane, I., Nishikawa, M., Takakura, Y., 2005. Macrophage activation by a DNA/cationic liposome complex requires endosomal acidification and TLR9-dependent and -independent pathways. J. Leukoc. Biol. 77, 71–79.

Yoo, S.Y., Kwon, S.M., 2013. Angiogenesis and its therapeutic opportunities. Mediators Inflamm. 2013, 127170.

Yu, Y., Sato, J.D., 1999. MAP kinases, phosphatidylinositol 3-kinase, and p70 S6 kinase mediate the mitogenic response of human endothelial cells to vascular endothelial growth factor. J. Cell Physiol. 178, 235–246.

Zhang, Y., Schwerbrock, N.M., Rogers, A.B., Kim, W.Y., Huang, L., 2013. Codelivery of VEGF siRNA and gemcitabine monophosphate in a single nanoparticle formulation for effective treatment of NSCLC. Mol. Ther. 21, 1559–1569.

Zhou, J., Tiemann, K., Chomchan, P., Alluin, J., Swiderski, P., Burnett, J., Zhang, X., Forman, S., Chen, R., Rossi, J., 2013. Dual functional BAFF receptor aptamers inhibit ligand-induced proliferation and deliver siRNAs to NHL cells. Nucleic Acids Res. 41, 4266–4283.

Zhu, L., Mahato, R.I., 2010. Lipid and polymeric carrier-mediated nucleic acid delivery. Expert Opin. Drug Deliv. 7, 1209–1226.

Zimmermann, T.S., Lee, A.C., Akinc, A., Bramlage, B., Bumcrot, D., Fedoruk, M.N., Harborth, J., Heyes, J.A., Jeffs, L.B., John, M., Judge, A.D., Lam, K., McClintock, K., Nechev, L.V., Palmer, L.R., Racie, T., Rohl, I., Seiffert, S., Shanmugam, S., Sood, V., Soutschek, J., Toudjarska, I., Wheat, A.J., Yaworski, E., Zedalis, W., Koteliansky, V., Manoharan, M., Vornlocher, H.P., MacLachlan, I., 2006. RNAi-mediated gene silencing in non-human primates. Nature 441, 111–114.

van de Water, F.M., Boerman, O.C., Wouterse, A.C., Peters, J.G., Russel, F.G., Masereeuw, R., 2006. Intravenously administered short interfering RNA accumulates in the kidney and selectively suppresses gene function in renal proximal tubules. Drug Metab. Dispos. 34, 1393–1397.

van der Meel, R., Oliveira, S., Altintas, I., Haselberg, R., van der Veeken, J., Roovers, R. C., van Bergen en Henegouwen, P.M., Storm, G., Hennink, W.E., Schiffelers, R.M., Kok, R.J., 2012. Tumor-targeted nanobullets: anti-EGFR nanobody-liposomes loaded with anti-IGF-1R kinase inhibitor for cancer treatment. J. Control. Release 159, 281–289.

van der Schaft, D.W., Dings, R.P., de Lussanet, Q.G., van Eijk, L.I., Nap, A.W., Beets-Tan, R.G., Bouma-Ter Steege, J.C., Wagstaff, J., Mayo, K.H., Griffioen, A.W., 2002. The designer anti-angiogenic peptide anginex targets tumor endothelial cells and inhibits tumor growth in animal models. FASEB J. 16, 1991–1993.

Please cite this article in press as: Yousefi, A., et al., Anginex lipoplexes for delivery of anti-angiogenic siRNA, Int J Pharmaceut (2014), http://dx.doi.org/10.1016/j.ijpharm.2014.06.028