Targeted Lysosome Disruptive Elements for Improvement of Parenchymal Liver Cell-specific Gene Delivery*

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The transfection ability of nonviral gene therapy vehicles is generally hampered by untimely lysosomal degradation of internalized DNA. In this study we describe the development of a targeted lysosome disruptive element to facilitate the escape of DNA from the lysosomal compartment, thus enhancing the transfection efficacy, in a cell-specific fashion. Two peptides (INF7 and JTS-1) were tested for their capacity to disrupt liposomes. In contrast to JTS-1, INF7 induced rapid cholesterol-independent leakage (EC$_{50}$ 1.3 μM). INF7 was therefore selected for coupling to a high affinity ligand for the asialoglycoprotein receptor (ASGPr), K(GalNAc)$_2$, to improve its uptake by parenchymal liver cells. Although the parent peptide disrupted both cholesterol-rich and -poor liposomes, INF7-K(GalNAc)$_2$, only induced leakage of cholesterol-poor liposomes. Given that endosomal membranes of eukaryotic cells contain <5% cholesterol, this implies that the conjugate will display a higher selectivity toward endosomal membranes. Although both INF7 and INF7-K(GalNAc)$_2$ were found to increase the transfection efficiency on polyplex-mediated gene transfer to parenchymal liver cells by 30-fold, only INF7-K(GalNAc)$_2$ appeared to do so in an ASGPr-specific manner. In mice, INF7-K(GalNAc)$_2$ was specifically targeted to the liver, whereas INF7 was distributed evenly over various organs. In summary, we have prepared a nontoxic cell-specific lysosome disruptive element that improves gene delivery to parenchymal liver cells via the ASGPr. Its high cell specificity and preference to lyse intracellular membranes makes this conjugate a promising lead in hepatocyte-specific drug/gene delivery protocols.

The development of a viable nonviral gene delivery system continues to be an important theme in gene therapy (1). The packaging of DNA into compact particles, the cellular uptake, the endosomal escape, and unpacking of these particles as well as the subsequent transfer of DNA to the nucleus are considered important steps in this regard (2). A number of DNA-packaging compounds have been reported, including cationic lipids, polymers and/or peptides that were designed to self-assemble with DNA to form intermolecular complexes (3–8). Upon internalization, the packaged DNA is transported to the lysosome, which subsequently degrades its content, making lysosomal escape a key step in gene delivery. To facilitate the intracellular transport of the packaged DNA to the nucleus and thus to enhance the transfection capacity of the nonviral gene delivery vehicles, lysosome disruptive elements (LDEs) have also been successfully applied, including amphipathic peptides (9–16).

The majority of the amphipathic peptides are derived from viral elements that promote cellular entry and correct intracellular handling. Their membrane permeabilizing capacity generally depends on the lipid composition and the pH. Although they are random coil at pH 7.0, these peptides undergo a conformational change into an amphipathic α-helix at pH 5.0 and aggregate into multimeric clusters (11, 12). Subsequently, the clustered helical peptides associate with and/or penetrate endosomal membranes, thereby destabilizing the membrane. Apart from complete virus capsids and purified capsid proteins, hemagglutinin (HA)-derived peptides and synthetic analogues have also been shown to induce pH-sensitive membrane disruption, leading to improved transfection of DNA-polymer complexes in vitro (9, 17). Although several groups have studied the stimulatory effect of LDEs on nonviral gene transfer (18–20), the use of targeted LDEs, which concomitantly improve the cellular delivery of DNA and its translocation to the nucleus, is rather unexplored.

Previous studies have shown that coupling of a homing device to liposomes or a universal carrier leads to a higher uptake of liposome-encapsulated drugs by the target cell (6, 23–25). The same strategy was applied to generate a targeted LDE. Two fusogenic peptides, INF7, a 23-mer peptide from HA, and JTS-1, an artificial INF7 mimic designed for pH-sensitive helix formation, were studied (10, 21, 22). INF7, the most promising peptide in terms of cholesterol dependence and disruption kinetics, was equipped with a homing device for the asialoglycoprotein receptor (ASGPr), K(GalNAc)$_2$ (1, 9, 23–26) on parenchymal liver cells.

In this report, we show that the glycoconjugated peptide, INF7-K(GalNAc)$_2$, displays a high affinity for the ASGPr and

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1 The abbreviations used are: LDE(s), lysosome disruptive element(s); ASGPr, asialoglycoprotein receptor; BHK, baby hamster kidney; Boc, N-(5-(2-acetamido-2-deoxy-D-glucopyranosyloxy)pentanomido) lysine; Luc, luciferase; MALDI, matrix-assisted laser desorption/ionization; MTI, 3,4,5-trimethoxythiainol-2-y1/2,5-diphenyl-tetrazolium bromide; TOF, time-of-flight.
possesses high lytic activity in cholesterol-poor liposomes only, making it eminently suitable for targeted fusogenic activity in parenchymal cells. Moreover, INF7-K(GalNAc)₃, unlike the parental INF7, accumulates efficiently in the liver after in vivo administration and strongly improves the transfer of polyplexed genes to parenchymal liver cells in an ASGPr-dependent fashion.

**EXPERIMENTAL PROCEDURES**

**Materials**—Egg yolk phosphatidylcholine was purchased from Fluka (Buchs, Switzerland). Cholesterol (>99%), calcein, trypsin inhibitor (bovine origin), orosomucoid, Triton X-100, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT), and BSA were obtained from Sigma. Precipitation L was from Roche Molecular Biochemicals. Sepharose G50 and G10 were from Amersham Biosciences. Agarose was from Eurogentec (Seraing, Belgium), and dimethyl sulfoxide was obtained from Baker. All solvents were of analytical grade. Dry solvents were stored over molecular sieves of 4 Å. Kieselgel 60 F₂₅₄ plates were from Merck. Polyethylene glycol-PS resin was purchased from PerkinElmer Life Sciences. Fmoc amino acids were purchased from Nova Biochem (Bad Soden, Germany). K(GalNAc)₃ was synthesized as described by Valentijn et al. (26). The spacer dithiothreitol in NH₄HCO₃ buffer) for a period of 1.5 h during which the latter lysine residue with trifluoroacetic acid/dichloromethane resulted in 95% purity as was assessed by MALDI-TOF spectrometry. The product was analyzed by reversed phase HPLC using a LiChrospher® 100 RP-18 column (Merck, 5 µm, 4.6 × 250 mm). Automated purification was performed using BIOCAD VISION. Electrospray mass spectra were recorded with a PerkinElmer SCIEX API 165 single quadrupole LC-MS instrument. Matrix-assisted laser desorption/ionization (MALDI) time-of-flight (TOF) mass spectrometry was performed on a PerkinElmer/PerSeptive Biosystems Voyager-DE-RI MALDI-TOF mass spectrometer.

**Peptide Synthesis**—JTS-1 (GLEEALLFLLESWE) was a generous gift from Dr. Tagliaferri (Valentis, Inc., Burlingame, CA). INF-7 (GLFEAIEFGIVGFENGWEGMIWDYG) was prepared by solid phase synthesis on an ABI 433 peptide synthesizer (Wang resin, loading (L) 0.64 mmol/g) analogous to the procedure described by Planc et al. (19). A 50-μmol scale synthesis was performed using Fmoc/tertiary-butyl or Fmoc/trityl-protected amino acids with a BOP/HOBt activation strategy. Deprotection and cleavage of the peptide from the resin in a solution of 95% trifluoroacetic acid, 2.5% H₂O, and 2.5% triisopropylsilane to give resin immobilized cluster galactoside (Scheme 1) (26). The obtained resin was treated with a 20% piperidine solution in N,N-dimethylformamide to remove the Fmoc protecting group, and the resulting free α-amino group was elongated with the SGSC amino acid spacer, followed by assembly of the INF-7 peptide as described above. Final deprotection and cleavage from the resin of the glycoconjugated INF7 4 (Fig. 1) was performed as follows. Removal of the acid-labile Tbu and Trrt side chain protecting groups was achieved by treatment of the resin with trifluoroacetic acid, 2.5% H₂O, 2.5% triisopropylsilane, and 2.5% ethanedithiol (27), followed by washing with trifluoroacetic acid, 5% H₂O (twice), and H₂O (three times). Cleavage of the INF7 derivative 4 (Fig. 1) from the resin and simultaneous debenzylation of the galactosamine moieties were achieved by uptake of the resin in an aqueous 0.4 M NaOH solution at 4 °C. The cytoeine residue was depleted using tributylphosphine in isopropyl alcohol. The obtained glycoconjugated peptide was analyzed by analytical HPLC (CH₃CN gradient in 10 mM NH₄HCO₃ and LC-MS (CH₃CN gradient in 10 mM NH₄Ac) assessed the presence of compound 4 (Fig. 1) as the main (95%) product. Further purification by RP-HPLC using a CH₃CN gradient (5–50% CH₃CN) in 10 mM NH₄HCO₃ buffer gave conjugate 4 (Fig. 1) in an overall yield of 26% (99% pure). The product was analyzed by MALDI-TOF spectrometry (UV mass weight: 2340 dpm/ng (free [¹₂⁵I]I)). Further purification was achieved by usage of a Sephadex G-50 column. Liposomes were prepared using a Sephadex G-50 column. Liposome size was measured routinely by photon correlation spectrometry (Malvern Instruments, Malvern, UK) to exclude small and large particles. Measurements were performed at 27 °C and at a 90-degree angle. The average liposome size was 100 nm. The cholesterol content of the purified calcine laden liposomes was determined using a Roach enzymatic kit for cholesterol using Precipath L as a reference (28).

Leakage assay—Phosphatidylcholine/cholesterol vesicles containing calcine were prepared by sonication. Briefly 100 mg/ml egg yolk phosphatidylcholine and cholesterol in methanol/chloroform (10 and 100 mg/ml) were dried under a stream of nitrogen. The lipid mixture was resuspended in sonication buffer (375 mM NaOH, 50 mM NaCl, pH 7.4) containing 100 µM calcine and sonicated with amplitude of 10 µm for 15 min (2.6% cholesterol) to 20 min (40% cholesterol) at 54 °C with a probe sonicator (Beun de Rode, Abcoude, The Netherlands). Free calcine was separated from liposome-entrapped calcine by using a Sephadex G-50 column. Liposome size was measured routinely by photon correlation spectrometry (Malvern Instruments, Malvern, UK) to exclude small and large particles. Measurements were performed at 27 °C and at a 90-degree angle. The average liposome size was 100 nm. The cholesterol content of the purified calcine laden liposomes was determined using a Roach enzymatic kit for cholesterol using Precipath L as a reference (28).

Calcine release was measured at 535 nm (λex = 485 nm) using a fluorescence spectrophotometer (PerkinElmer Life Sciences). For the leakage assay the calcine-laden liposomes were diluted in citrate-buffered saline (200 mM NaCl, 20 mM sodium citrate), pH 7.0 or pH 5.0, and peptide was added at a concentration of 0–20 µM. Fluorescence was measured 0–60 min after the addition of the peptide. Complete lysis was achieved by adding Triton X-100 to a final concentration of 0.25%. Leakage of Liposome-entrapped Trypsin Inhibitor—Liposomes were loaded with [¹₂⁵I]I-trypsin inhibitor (molecular weight 20,000) as follows. Trypsin inhibitor was radiiodimated according to McFarlane (29) to a specific activity of 2340 dpm/µg (free [¹₂⁵I]I < 5%). Liposomes were sonicated as described above in buffer (5 mM Hepes, 40 mM NaCl, pH 7.4) containing [¹₂⁵I]I-trypsin inhibitor (33.8 × 10⁶ dpm). Free [¹₂⁵I]I-trypsin inhibitor was removed by density ultracentrifugation (40,000 rpm) for 20 h. Liposomal suspensions were resuspended in sonication buffer and liposome size was measured. [¹₂⁵I]I-Tr ispsin inhibitor leakage was measured at 27 °C and at a 90-degree angle. Liposomes were prepared using a Sephadex G-50 column. Liposome size was measured routinely by photon correlation spectrometry (Malvern Instruments, Malvern, UK) to exclude small and large particles. Measurements were performed at 27 °C and at a 90-degree angle. The average liposome size was 100 nm. The cholesterol content of the purified calcine laden liposomes was determined using a Roach enzymatic kit for cholesterol using Precipath L as a reference (28).
16–18 h at 4°C (30). Particles were characterized by size and cholesterol concentration.

For the leakage assay, the liposomes were diluted in citrate-buffered saline, pH 7.0 or pH 5.0. Fusogenic peptide was added to a concentration of 0–20 μM, and the amount of leakage was monitored by 0.75% w/v agarose gel electrophoresis in Tris/hippuric acid buffer, pH 8.8. Radioactivity was visualized using the PhosphorImager (Molecular Dynamics, Sunnyvale, CA).

Isolation of Mouse Parenchymal Liver Cells—10–12-week-old male C57BL/6K mice weighing 22–27 g (Broekman Institute BV, Someren, The Netherlands) were used for parenchymal cell isolation. Hepatocytes were isolated from anesthetized mice by perfusion of the liver with collagenase (type IV, 0.05% w/v) for 10 min at 37°C according to the method of Seglen (31). Cells were >99% pure as judged by light microscopy.

In Vitro Binding Assay—Displacement of 125I-ASOR binding to mouse hepatocytes was determined as follows (32). Freshly isolated mouse parenchymal liver cells (1 × 10^6 cells/vial) viability >90% as determined by 0.2% trypan blue exclusion) were incubated in 0.5 ml of Dulbecco's modified Eagle's medium (Biowhittaker, Verviers, Belgium) containing 2% BSA with 5.5 nM 125I-ASOR in the presence or absence of 50 nM-5 μM displacer. After incubation for 2 h at 4°C under gentle agitation, the medium was removed by aspiration, and the cells were washed twice with 0.2% BSA in medium and once with medium lacking BSA. Subsequently cells were counted for radioactivity. Cell binding was corrected for protein content. Non-specific binding was measured in the presence of 100 nM GalNAc. Displacement binding data were analyzed according to a single site model using a computerized nonlinear fitting program (Pism) to calculate the K_i.

Transfection of Mouse Parenchymal Cells—The pCMV-luciferase, containing the firefly luciferase cDNA insert, was kindly provided by Crucell BV (Leiden, The Netherlands). The preparation of the K8-DNA polyplexes (N/P charge ratio 4:1; 1 DNA/well in HBS buffer) was done as described by Gotteskal et al. (10). Mouse parenchymal liver cells were transfected 3 h after seeding (2 × 10^5 cells/well) with K8-condensed DNA. After a 30-min incubation of the K8-DNA complexes at room temperature, fusogenic peptides (INF7, INF7-SGSC, and INF7-K(GalNAc)2) were added to a final concentration of 1–2,000 nm. After an additional incubation of 30 min at room temperature, the complexes were added directly to the parenchymal cells in 250 μl of Dulbecco's modified Eagle's medium + 0.2% BSA. After incubation for 4 h, 1 ml of medium was added, and the cells were incubated for 44 h. After harvesting of the cells the lysate was analyzed for luciferase activity as described (33) and corrected for protein content (BCA) using BSA as reference.

In analog, BHK cells (seeded at 2 × 10^5 cells/well) were transfected with preformed K8-DNA condensates in the absence or presence of the fusogenic peptides.

MTT Cytotoxicity Test—Parenchymal liver cells were transfected as described above. After a 48-h incubation the medium was replaced by fresh medium, and MTT was added to a final concentration of 0.5 mg/ml. Cells were incubated for 30 min at 37°C, the medium was removed, and dimethyl sulfoxide was added to the cells. Extinction was measured at 550 nm.

Liver Uptake and Serum Decay of INF7 and INF7-K(GalNAc)2 in Mice—Male C57BL/6K mice (19–21 g) were anesthetized, and the abdomen were opened. 125I-INF7 and 125I-INF7-K(GalNAc)2 were injected via the inferior vena cava. At the indicated times blood samples of 50 μl were taken from the inferior vena cava and allowed to clot for 30 min. Serum samples of 10 μl were counted for radioactivity after centrifugation for 5 min at 2,500 × g. To determine liver uptake, liver lobules were tied off at the indicated times, excised, and weighed. At 30 min, mice were sacrificed, and organs were excised and weighed. Radioactivity in serum, liver, and other tissue samples was counted in a gamma counter (minaxi γ-counter 5000, Packard) and corrected for radioactivity in entrapped serum as described by Rensen et al. (28). The weight of muscle, bone, and skin was calculated from the whole body weight of the mouse and the average contribution of the organ to the body weight by the formula: % tissue in standard mouse (muscle = 22.5%, bone = 16.71%, skin = 14.74%)/100 × weight of muscle/weight of tissue sample.

RESULTS

Lytic Activity of JTS-1 and INF7—To improve the intracellular trafficking of nonviral DNA vehicles in a cell-specific fashion, we wanted to target a LDE to the ASGPr on parenchymal liver cells. In the first step, we synthesized two fusogenic peptides with known lysosome disruptive activity, JTS-1 and INF7.

The membrane-disruptive properties of JTS-1 and INF7 were determined by a liposome leakage assay, in which the release of calcein from phosphatidylcholine liposomes was measured. Liposomes with various cholesterol contents were prepared to address whether the lytic capacity of the peptides depends on the cholesterol content of the membranes. Eukaryotic plasma membranes generally contain between 20 and 25% cholesterol, whereas the cholesterol content of endosomal membranes ranges from 0 to 5% (34, 35). Cholesterol dependence is therefore an important criterion in the development of a targeted LDE because it should act specifically on endosomal membranes, leaving cell membranes unaffected.

Leakage of INF7 and JTS-1 was monitored in time at pH 5.0 and pH 7.0. Both peptides appear to be disruptive at acidic pH only (Fig. 2). In agreement with previous studies (10, 20), a kinetic study of calcein release revealed that in cholesterol-rich (40%) liposomes the INF7-induced leakage was much more rapid than that of JTS-1 (t1/2 = 0.24 min versus 33.53 min, respectively; p < 0.001) (Fig. 3B). The kinetics of INF7-induced leakage of cholesterol-poor (2%) liposomes was found to be quite similar (t1/2 = 0.27 min, p < 0.01), whereas that of JTS-1 was accelerated considerably compared with that of cholesterol-rich liposomes (t1/2 = 4.9 min) (Fig. 3C). Moreover JTS-1 induced partial leakage of liposomes containing 2.6% cholesterol (Fig. 3C).

Further study confirmed the differential cholesterol dependence of JTS-1- and INF7-induced lysis. Whereas INF7 was equally fusogenic in liposomes with high and low cholesterol content, JTS-1 was only able to disrupt cholesterol-rich liposomes completely (Fig. 3A).

From these results we concluded that INF7 caused a rapid, cholesterol-independent disruption with an EC50 of 1.3 μM, whereas JTS-1-induced leakage is much slower and cholesterol-dependent. Given that the lysosomal membrane is cholesterol-poor, we selected INF7 for subsequent design of a targeted fusogenic peptide, by conjugation to the -K(GalNAc)2 ligand (Fig. 1).
In Vitro Binding Studies—The affinity of INF7-K(GalNAc)_2 for the ASGPr was monitored by an in vitro competition assay of 125I-ASOR total binding to mouse parenchymal liver cells. As shown in Fig. 4, INF7-K(GalNAc)_2 inhibited the binding of ASOR to the ASGPr (K_d 87 nM), which is comparable with that of K(GalNAc)_2 (K_d 32 nM). We previously used the K(GalNAc)_2 with comparable affinity (32, 41). This indicates that attachment of the INF7 peptide to K(GalNAc)_2 did not affect the affinity of K(GalNAc)_2 for the ASGPr considerably. To exclude nonspecific binding caused by the presence of the SGSC linker arm between the GalNAc cluster and the peptide, an INF7-SGSC was synthesized and tested for the affinity of the ASGPr. INF7 and INF7-SGSC, the peptide with the SGSC linker arm between the peptide and GalNAc, did not show any affinity for the ASGPr (Fig. 4), confirming the importance of K(GalNAc)_2 in the binding to ASGPr.

Lytic Activity of INF7 and the INF7 Glycoconjugate—The membrane-disruptive potency of INF7-K(GalNAc)_2 was determined in the liposome leakage assay, similar to INF7 and JTS-1. INF7-K(GalNAc)_2 displayed only lytic activity at acidic pH (data not shown). The leakage kinetics of INF7-K(GalNAc)_2 were comparable with that of INF7 (Fig. 5). In cholesterol-poor (2.6% liposomes, the fusogenic capacity of the glycoconjugate appeared to be five times lower than that of the parental INF7 (Fig. 5B) (EC_50 1.3 and 6.1 μM, respectively). A closer look at the cholesterol dependence of liposome leakage showed that the fusogenic activity of INF7-K(GalNAc)_2 was impaired markedly in liposomes with cholesterol contents above 20% (Fig. 5C). When aiming at lysosome-specific membrane disruption, this is a clear advantage because lysosomal membranes are known to contain low cholesterol concentrations, whereas plasma membranes are generally rich in cholesterol. This implies that INF7-K(GalNAc)_2 may be even more specific for lysosomal membranes, while leaving the cell membrane intact.

Mechanism of Leakage—We argued that the differential capacity of INF7 and INF7-K(GalNAc)_2 to lyse cholesterol-rich membranes might be related to the actual mechanism of membrane disruption. Previous studies have shown that amphipathic peptides are disruptive either by facilitating pore formation after multimeric assembly of the peptide or by inducing a more detergent-like solubilization of the liposome leading to complete disruption of the liposome. It was reasoned that pore formation might restrict the leakage of entrapped compounds with a low molecular weight, whereas the molecular weight of entrapped compounds would not be a limiting factor after complete disruption of the liposomes (36). For that reason we studied the size-dependent leakage of INF7 and INF7-K(GalNAc)_2 in liposomes containing either calcein (molecular weight, 622) or 125I-trypsin inhibitor (molecular weight, 20,000). Liposomes were incubated with INF7 or INF7-K(GalNAc)_2 (0–20 μM). At pH 7.0, no leakage of calcein or trypsin was observed for up to 60 min of incubation. However, under acidic conditions, INF7 and INF7-K(GalNAc)_2 promoted the release of calcein but not of entrapped 125I-trypsin inhibitor (Fig. 6). This suggests that the observed leakage caused by INF7 and INF7-K(GalNAc)_2 is likely caused by pore formation.
Effect of the Fusogenic Peptides on Polyplex Gene Transfer to Mouse Parenchymal Liver Cells—Next, we assessed whether the glycoconjugated INF7 was able to promote the release of lysosomally entrapped DNA to the cytosol, thus enhancing the transfection level in a receptor-dependent manner. Mouse parenchymal liver cells were transfected with polyplexed DNA in the absence or presence of fusogenic peptide. For gene transfer we made use of the cationic peptide (K8)-based polyplex protocol described by Gottschalk et al. (10). Cells were transfected with plasmid DNA (pCMVLuc), containing a CMV promoter-driven luciferase reporter gene insert, which was condensed with plasmid DNA (pCMVLuc), containing a CMV promoter-driven luciferase reporter gene insert, which was condensed with K8 (at a N:P ratio of 4:1). To exclude artifacts caused by the presence of the SGSC linker arm between the GalNAc cluster and the peptide, INF7-SGSC was included as a control. Preincubation of the polyplexes with INF7 increased the transfection efficiency in a dose-dependent manner up to 30-fold (Fig. 7A). At the concentrations measured, INF-K(GalNAc)2 displayed a capacity to enhance the transfection efficiency similar to that of INF7.

Receptor-specific Recognition of INF7-K(GalNAc)2—INF7-K(GalNAc)2 was not able to stimulate gene transfer to BHK cells, which do not express the ASGPr, whereas INF7 and INF7-SGSC were found to improve the transfection efficiency of the K8 polyplexes considerably (Fig. 7B). To confirm that INF7-K(GalNAc)2 promoted nonviral gene transfer of K8-DNA in an ASGPr-specific fashion, the effect of INF7-K(GalNAc)2 on the transfection efficiency was examined in the presence of GalNAc, which blocks the ASGPr. As shown in Fig. 7C, GalNAc did not influence the transfection ability of INF7 or glycoconjugated INF7, in the presence of the ASGPr, by contrast, had no effect on INF7-K(GalNAc)2-stimulated gene transfer. This indicates that INF7-K(GalNAc)2 exerts its fusogenic activity through the ASGPr, whereas INF7 has a more general nonspecific fusogenic effect.

Toxicity—It has been suggested that disruption of endocytic and lysosomal vesicles might lead to the release of proapoptotic and cytotoxic proteases. Therefore, we mapped the toxic side effects of the targeted LDEs by evaluating the effect of INF7 or glycoconjugated INF7, in the presence of the K8-DNA polyplexes, on the viability of parenchymal liver cells. INF7 and INF7-K(GalNAc)2-equipped polyplexes did not show significant toxicity in the applied relevant concentration range (0–2 μM) (data not shown).

In Vivo Behavior of INF7 and INF7-K(GalNAc)2 in Mice—To study the biodistribution profile of INF7 and INF7-K(GalNAc)2 in mice, both peptides were iodinated and intravenously injected into the vena cava of C57BL/6 mice. INF7 was cleared rapidly from the circulation at a half-life of ~2 min (Fig. 8B), and only 5% of the injected dose could be recovered in the liver after 30 min (Fig. 8A). Fig. 8C shows that hepatic uptake of
The main objective of this study was to improve nonviral gene delivery by the use of targeted LDEs. As a first step we have tested two HA-derived fusogenic peptides, JTS-1 and INF7, for their lysosome disruptive capacity in calcein-laden liposomes (10). INF7 was found to induce a rapid and cholesterol-independent leakage of liposomes, whereas JTS-1-induced leakage was much slower and cholesterol-dependent.

Moreover, JTS-1 was unable to disrupt cholesterol-poor (<20%) liposomes completely. These findings concur with the studies of Gottschalk et al. (10), who showed complete leakage of cholesterol-poor liposomes by INF7 compared with only partial leakage by JTS-1 (35%). The differential fusogenic profile of JTS-1 and INF7 is not the result of differences in net negative charge or hydrophobicity of the peptides because the peptides are very similar in that respect. Possibly, INF7 and JTS-1 have a different orientation in liposomal membranes (12, 37). The conjugate appeared to be slightly less fusogenic than the parent peptide (1.3 μl versus 6.1 μl). However, the lytic activity of the conjugate was cholesterol-dependent in that it only disrupted cholesterol-poor liposomes. This may be an advantage as K(GalNAc)2-conjugated INF7 is designed to act specifically on the cholesterol-deficient lysosomal membranes of ASGPr-expressing cells. INF7, by contrast, may also be fusogenic at the level of the plasma membrane.

The intriguing observation that INF7-K(GalNAc)2-induced leakage depends on the lysosome cholesterol content, in contrast to INF7 alone, prompted further study. Lysosome disruptive peptides may facilitate leakage, through pore formation and through a detergent-like solubilization of the membrane (36). The differential lytic profile of INF7 and its glycoconjugate suggests that INF7 disrupts membranes via both pathways, one of which predominates in cholesterol-rich membranes and is blocked by the presence of the bulky glycoside moiety. However, the lack of leakage of 125I-trypsin inhibitor from INF7 (glycoconjugate)-treated liposomes points to pore formation as the major pathway of lysosome disruption for both INF7 and the glycoconjugated INF7. Rather, steric hindrance of the exposed lysosome group might interfere with the ability of the fusogenic peptide to form pores. This also explains the reduced lytic activity of INF7-K(GalNAc)2 in cholesterol-rich liposomes because the liposomal cholesterol may hamper pore formation by INF7-K(GalNAc)2 caused by steric hindrance of the glycoconjugate.
side group. To confirm this hypothesis, the orientation and distribution of the peptides in the liposomal membrane need to be addressed.

The glycoconjugated peptide was designed for ASGPR-directed delivery. Indeed, glycoconjugated INF7 bound to the ASGPr with an affinity of 87 nM, which is about two times lower than K(GalNAc)2 itself. Earlier studies have shown that an affinity of 87 nM for the ASGPR should be sufficient for effective targeting of INF7-K(GalNAc)2 (32, 42).

The final goal of this study was to elaborate a targeted LDE for improving nonviral gene transfer. To this end, we have evaluated the effect of the fusogenic peptides on the gene transfer efficiency of an established nonviral gene delivery protocol based on the cationic peptide K8 in mouse parenchymal liver cells (10). DNA polyplexed with small sized synthetic oligonucleotides (like K8) may be better for systemic application because the derived condensates generally are smaller and less immunogenic, nonaggregating, and are readily unpacked intracellularly (43, 44). INF7, INF7-SGSC, and INF7-K(GalNAc)2 led to nonaggregating, and are readily unpacked intracellularly (43, 44). INF7, INF7-SGSC, and INF7-K(GalNAc)2 led to a substantial, 30-fold increase in the transfection efficiency of K8-DNA complexes in freshly isolated parenchymal cells. This stimulatory effect was concentration-dependent. Even though in the leakage assay, INF7-K(GalNAc)2 appeared to be 6-fold less potent than INF7, the lower intrinsic activity is compensated for by the enhanced uptake of the targeted peptide, by parenchymal liver cells. However, it should also be kept in mind that liposomal and cellular assays are not fully comparable.

The fusogenic activity of INF7-K(GalNAc)2 was abolished completely in the presence of excess GalNAc, which blocks ASGPr-mediated uptake, whereas GlcNAc had no effect on the transfection efficiency. Moreover, INF7-K(GalNAc)2 did not affect the transfection yield in ASGPr-deficient BHK cells, whereas INF7 and INF7-SGSC were equally potent in BHK and mouse parenchymal cells. This underlines that the stimulatory effect of glycoconjugated INF7 is mediated by the ASGPr and will have fewer side effects. In agreement with previous studies, primary parenchymal liver cells are more difficult to transfect than continuous cell lines; the intrinsic transfection activity of parental INF7. We envision that INF7-K(GalNAc)2 could be applied to improve the transfection efficacy of hepatic nonviral gene transfer vehicles (25) and of antisense drugs for hepatic genes (42, 41, 48) by facilitating the escape from the lysosomal circuit. This would benefit from application of targeted LDEs.

In conclusion, we present a targeted fusogenic peptide, INF7-K(GalNAc)2, which induces lysosomal escape in a receptor-dependent fashion. Its favorable pH- and cholesterol-dependent activity profile makes it even more lysosome-specific than the parental INF7. We envision that INF7-K(GalNAc)2 could be applied to improve the transfection efficacy of hepatic nonviral gene transfer vehicles (25) and of antisense drugs for hepatic genes (42, 41, 48) by facilitating the escape from the lysosomal pathway, which appears to be a major drawback in both therapies (49). Not only gene medicines, but also other drugs that accumulate in the lysosomal circuit might benefit from application of targeted LDEs.

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