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The neuropilin-1 receptor mediates enhanced tumor delivery of H2K polyplexes

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Abstract

Background—Promising plasmid-based treatments have limited value without an effective delivery system. Recently, the linear H2K with a repeating –KHHK pattern was determined to be an effective plasmid carrier to tumor xenografts *in vivo*. Although unpacking of the H2K polyplex within the tumor may have a role, the mechanism for the enhanced efficacy remains unclear.

Methods—After solid-phase synthesis of linear and branched histidine-lysine (HK) peptide carriers of plasmids, the peptides were compared for their ability to lyse endosomes with a red blood cell model and to transfect MDA-MB-435 xenografts in the presence or absence of neuropilin-1 receptor (NRP-1) antibodies. To examine stability, polyplexes were incubated with trypsin or NaCl and then analyzed by electrophoresis.

Results—After screening peptides with a model for endosomal lysis at 2 pHs, the 33-mer H3K peptide lysed red blood cells effectively at the lower pH. Combining H3K and H2K peptides as carriers of plasmids expressing luciferase were more effective than H2K alone. Based on the repeating –KHHK- sequences of H2K, we studied whether the widespread gene expression in the tumor may be mediated by NRP-1. By blocking NRP-1 in tumor-bearing mice, luciferase activity in tumors delivered by HK polyplexes was reduced by 96% whereas activity in normal tissues was minimally reduced.

Conclusions—Combining an endosomolytic peptide, H3K with H2K polyplexes as a carrier further enhanced transfection *in vivo*. Moreover, the widespread distribution of H2K polyplexes is mediated by NRP-1, suggesting that transcytosis of these polyplexes through the tumor endothelium may lead to efficient transfection.

Keywords

non-viral; plasmid; gene therapy; cancer; neuropilin-1 receptor; peptide

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The authors declare no conflict of interest

Supporting information

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Introduction

The fundamental problem with gene therapy and nucleic acid therapeutics for treatment of cancer is their poor delivery system. This is particularly relevant for non-viral plasmid delivery systems that are relatively safe to administer but deliver the therapeutic nucleic acids inefficiently to the tumor. The HK peptide carriers, comprised of histidine and lysine amino acids, can be tailored to transport different forms of nucleic acids such as plasmids or siRNA into cells by altering their degree of branching and amino acid sequence. In contrast to the effective *in vitro* highly-branched H2K peptide carrier of plasmids, the linear H2K peptide is a poor carrier *in vitro*, yet surprisingly, the linear H2K polyplexes target tumor xenografts with high transfection efficiency and specificity [1]. Although the mechanism that underlies this high specificity *in vivo* remains unclear, evidence supports that binding and release of HK from DNA are important.

Recently, the NRP-1 mediated transport system has garnered significant attention for its ability to transport efficiently small and large molecules, including targeting ligands with and without nanoparticles, through tumor vessels [2], [3]. One of the most studied molecules of this group that binds and activates NRP-1 is the cyclic targeting internalizing RGD peptide (iRGD). After iRGD binds to the $\alpha v \beta 3$ receptor on endothelial cells, trypsin-like enzymes cleave the peptide exposing the $-K/RXXK/R-CO_2H$ motif, enabling the peptide to bind to the NRP-1. The requirement of the K/RXXK/R sequences to have a C-terminal carboxyl end to bind NRP-1 appears to be inviolate and is known as the C-end rule [3], and thus peptides/proteins with this motif at their C-terminal ends with an amide cap bind and activate NRP-1 poorly [4, 5]. Because H2K peptides share a common sequence of $-KXXK-$ with peptides that target the neuropilin-1 receptor (NRP-1) and activate this pathway [3], H2K peptides with reduced binding to DNA (compared to H2K4b) may more readily be enzymatically processed, enabling the transport of the polyplex through the tumor endothelium. Although NRP-1 mediated uptake of macromolecules is by a macropinocytosis-like mechanism, the energy-dependent and rapid trans-tissue penetration of these molecules is not well understood [2, 6]. Nevertheless, at least part of the NRP-mediated transport of macromolecules appears to be due to transcytosis [2, 7]. It has also been postulated that endothelial NRP-1 mediated transcytosis is a mechanism to transport nutrients in bulk to calorie-restricted tissues and that tumors have usurped this transport system to enhance their survival [2]. Indeed, inhibitors of the glucose transport system injected intratumorally markedly increased fluorescently-labeled molecules through the tumor endothelium [2]. Because H2K has a repeating sequence pattern of $-KHHK-$, transcytosis of the polyplex through the tumor endothelium provides a rationale for its enhanced tumor targeting and accumulation.

Partial disruption of the polyplex, together with the associated binding and release of peptide from DNA, may also have an important role in endosomal lysis. If the endosomolytic peptide binds too tightly to DNA and stabilizes the polyplexes within acidic endosomes, the peptide will not be released and may not be particularly effective in lysing endosomes [8, 9]. Thus, with agents or endosomolytic peptides that do not alter significantly the biophysical properties of the H2K polyplex, transfection *in vitro* and *in vivo* should increase. When such endosomolytic agents are incorporated within polyplexes, *in vitro* transfection results may

be more predictive of *in vivo* results. In this report, we examined both endosomolytic and NRP-1 mediated transport mechanisms and further investigated whether modifications of HK peptides based on these mechanisms augment transfection of HK polyplexes *in vivo*.

Materials and methods

Animals

Female athymic mice (4–8 weeks old, body weight; 20 g) were purchased from NCI Frederick. All experiments were performed in accordance with regulations by the Institutional Animal Care and Use Committee of the University of Maryland School of Medicine.

Cell Lines

The malignant cancer cell lines (MDA-MB-435 and MDA-MB-231) were cultured and maintained in Dulbecco's minimal essential medium (DMEM) containing 10% fetal calf serum (FCS) and 20 mM glutamine. HUVEC were cultured and maintained in EGM-2 and 2% fetal calf serum.

Plasmids

Construction of pCpG-Luc and pCpG-tdTomato plasmids were described previously [1]. The plasmids were prepared for systemic injection as described previously [10]. The plasmids were purified with an EndoFree Maxi kit (Qiagen, Valencia, CA, US). An analytical gel of each plasmid (cut and uncut) was examined to verify that there was no contamination with other nucleic acids.

Peptides

The linear and branched HK peptides were synthesized on a Rainin Voyager synthesizer (PTI, Tucson, AZ, US) by the biopolymer core facility at the University of Maryland or by Genscript (Piscataway, NJ, US), as previously described [11]. The sequences of HK peptides are in Table 1. For the branched H2K4b peptide, each of four terminal branches are conjugated to the three-lysine core. Internalizing cyclic RGD (CRGDKGPDC) was synthesized [12] by GenScript. Peptides were analyzed by high-performance liquid chromatography (HPLC) (Beckman, Fullerton, CA, US) to ensure purity of 90% or greater. Further analyses of the peptides were done by matrix-assisted laser desorption/ionization (MALDI-TOF) mass spectroscopy on a Voyager system (Applied Biosystems, Foster City, CA, US) and in selected cases by amino acid analysis (AAA Laboratory Service, Boring, OR, USA).

cRGD-PEG-H3K4b conjugate was synthesized as previously described [12] with an average of 4 ligands per H3K4b molecule.

Red blood cell lysis

We used a red blood cell (RBC) lysis model for examining endosomal membranes as described previously [13, 14]. Fresh sheep RBCs (10^8 cells) in either 1 ml of isotonic buffers of sodium phosphate or MES (0.1 M at appropriate pH between pH 5.0 and 7.4) were

incubated with H2K, H3K, or H2K4b peptides (concentrations between 0 to 100 µg/ml). During the 4-h incubation at 37°C, microfuge tubes containing RBCs with various polymers were rotated continuously. The RBC solution was centrifuged at 13,500 g for 5 min, and absorbance of the supernatant were measured at 541 nm. To determine 100% lysis, 10⁸ RBCs were lysed in distilled water.

***In vitro* transfection and luciferase reporter assay**

Initially, 1×10⁵ cells were plated in 24-well plates in the presence of 500 µl of DMEM with 10% serum; after 24 h, the cells reached 70% confluency. Unless otherwise indicated, increasing amounts of the peptide(s) (0.5 to 12.0 µg) in 50 µl were mixed with 1.0 µg of the plasmid (CPG-Luciferase (Luc) in 42 µl of Opti-MEM or water) and the mixture was allowed to stand for 30 min (1/1, w/w ratio for HK/DNA is approximately equal to 1.3/1, N/P ratio). The transfection protocol was performed as described previously with few modifications [15]. In brief, 50 µl of the transfection complex were added to cells for 24 h. The cells were then lysed with 100 µl of 1x passive lysis buffer (Promega Corp., Madison, WI, US). Protein concentration was measured by using the BCA protein assay kit (Pierce ThermoFisher Scientific, Waltham, MA, US). Luciferase activity was measured and expressed as relative light units (RLU) by the direct current Turner 20/20 luminometer (Turner Design, Sunnyvale, CA, US) as described previously [15] with 3 measurements performed for each concentration.

Inhibition of Endocytosis

Inhibition of endocytosis pathways was done as previously described [16, 17]. In brief, HUVEC (1 × 10⁵ cells) were placed in 24-well plates 24 h before transfection. After the cells were pretreated with a polyclonal clathrin antibody (Sigma-Aldrich, St. Louis, MO, US) (1:100 vol/vol. dilution in cell culture medium), filipin (2.0 µg/ml) (Sigma-Aldrich), polyclonal NRP-1 antibody (50 µg/ml) (R & D, Minneapolis, MN), or cytochalasin D (1 µg/ml) (Sigma-Aldrich) for 30 min, the medium was changed to fresh EGM-2 (Lonza; Basel CH) /2% serum. As discussed previously, the HK polyplexes were then added to each well and luciferase activity was measured approximately 24 h later.

Flow Cytometry

Cells (2 × 10⁶) (MDA-MB-435, HUVEC) were harvested, washed with PBS, and then fixed with 4% formaldehyde for 5 min. Goat anti mouse Neuropilin-1 antibody (R&D company, Minneapolis, MN (which also binds to human NRP-1) was incubated with the cells (2.5ug/ 1×10⁶ cells) at RT for 30 min, then washed with PBS, with the secondary Cy5 labeled rabbit antigoat IgG added (Kierkegaard & Perry Laboratories, Inc., Gaithersburg, MD) (1:100; 10ug/ml) for 30 min at RT; for control, NRP-1 was not added. Flow cytometry (FACS CANTO II, Becton Dickinson, East Rutherford, NJ) and analyses (FlowJo) were performed at the University of Maryland Marlene and Stewart Greenebaum Cancer Center Flow Cytometry Shared Service.

Particle size and zeta potential

For each preparation of plasmid polyplexes, the size and zeta potential were determined with the Zetasizer (Malvern, Westborough, MA) prior to their injection. The size was reported as the average size obtained from unimodal analysis of dynamic light scattering of the particles at a 90° angle with use of software provided by the instrument manufacturer. Each particle size and zeta potential data point represents the mean $\pm$ SD of three measurements.

Gel retardation assay

Various amounts of HK peptides were mixed with 1 μ g of plasmids and incubated for 30 min at room temperature. Specifically, polyplexes with HK/plasmid ratios (w/w) of 1/2 or 4/1 were prepared in water. After 30 min, the formed HK polyplexes were incubated with trypsin (0.025%) or NaCl (1 M) for an additional 30 min, respectively. The HK polyplexes were loaded onto the gel (1% agarose containing ethidium bromide) and electrophoresis was carried out at a constant voltage of 75 V for 45 min in TBE buffer. A gel retardation assay was also done on each *in vivo* HK polyplex preparation before injection into the mice.

Stability of HK Plasmid Polyplexes in Serum

HK (H2K/H3K or H2K4b) plasmid polyplexes were formed by mixing peptides (0.25 μ g) with 1 μ g of plasmid DNA. After the 30 minutes, the polyplexes (2 μ l) were incubated with increasing concentrations (0%, 10%, and 50%) of mouse serum (total volume 10 μ l) for 1 h at room temperature. The HK-plasmid polyplexes were then loaded onto the gel (1 % agarose), and electrophoresis was carried out at a constant voltage of 75 V for 45 minutes in TBE buffer containing ethidium bromide. The plasmid band densities were then visualized under a UV transilluminator at a wavelength of 365 nm. On the basis of fluorescence (UN-SCAN-IT; Silk Scientific, Orem, UT) of plasmids that had migrated into each lane, the stability of HK plasmid polyplex was determined.

Polyplex formation *in vivo*

To the plasmid DNA (36 μ g in 150 μ l of water), H2K (15 μ g in 100 μ l water), H2K/H3K (12.5/2.5 μ g), or H2K/H2KCO2H/H3K (11.25/1.25/2.5 μ g) were added quickly and mixed by pipetting. Forty-five minutes after mixing, cRGD-PEG-H3K4b (1.63 μ g in 30 μ l) was added to polyplex for 20 min prior to the intravenous injection of the polyplex unless otherwise stated. The remainder (40 μ l) of the polyplex preparation was analyzed by gel electrophoresis, size, and zeta potential. *invivojet*-PEI (6 μ l) in complex with plasmid DNA (36 μ g) was prepared according to instructions from the manufacturer (Polyplus, Illkirch FR).

In vivo transfection

When tumors in mice were approximately 100 mm³ in size, the mice were treated by intravenous injection in the tail vein with plasmid polyplexes prepared as described above. After 24 h, the tumors and major organs were homogenized and luciferase activity was measured with a direct current TD 20/20 luminometer (Turner Design, Sunnyvale, CA, US). In selected cases, mice were administered intravenously with NRP-1 antibody (R&D systems) 30 min prior to injection of polyplexes.

pCpG-tdTomato Detection

Twenty-four hours after the HK-pCpG-td (tandem dimer) Tomato polyplexes (w/w, HK/plasmid, 1/2 ratio) were injected intravenously, mice were euthanized and frozen sections of MDA-MB-435 tumor xenografts were prepared. Images of the tumors were obtained with TE2000-S microscope.

Statistical Analysis

The results, reported as means $\pm$ standard error of the mean (SEM), represent at least three separate data measurements, unless otherwise indicated. Results were primarily analyzed using a Student's t-test using SigmaPlot 12.5 software. For the *in vitro* study with endocytosis inhibitors, a one-way ANOVA analysis with Holm-Sidak multiple pairwise comparisons post-hoc test was used. To determine whether there were equal variances between two groups of mice treated with HK peptides with different C-terminal ends, the Levene's test was used. A $p < 0.05$ was considered statistically significant.

RESULTS

Addition of H3K peptide augments transfection of H2K Polyplexes

Although widespread distribution of H2K polyplexes with in tumor xenografts was not predicted from *in vitro* studies, we reasoned from the cell culture data and from our previous data [8] that H2K carriers may not be effective at lysing endosomes and that gene expression could be significantly improved by introducing an endosomolytic agent into the polyplex. After screening several peptides with a model for endosomal lysis at 2 pH values (7.4 and 5.5) [13, 14, 18], the 33-mer H3K peptide lysed red blood cells effectively at the lower pH (the pH of most mid- to late endosomes) (Figure 1). The high histidine content in H3K likely accounts for its increased ability to lyse endosomal membranes, through their protonation and interaction with endosomal membranes in the acidic environment. Similar to the combination of H2K and H3K being more effective than H2K as carriers of plasmids *in vitro* (Figure S1A and S1B), the combination of H2K and H3K peptides (H2K/H3K; wt/wt; 1.0/0.2) was more effective *in vivo* as a carrier of plasmids expressing luciferase (Figure 2) or tdTomato (Figure 3) (Table S1). In contrast to H3K, the combination of H2K and H2K4b (H2K/H2K4b: wt/wt; 1.0/0.2) had reduced transfection in the tumor and reduced specificity *in vivo* than either H2K or H2K:H3K carriers (Figure S2). Moreover, when compared to either H2K or the H2K/H3K combination, H3K alone was not as effective a carrier *in vivo* (Figure S3).

In contrast to the branched polyplex, linear polyplexes have similar stability

After incubating H2K, H2K/H3K, or H2K4b with DNA in water to form polyplexes for 30 min, we examined whether a dilute solution of trypsin (0.025%) could release/degrade the polyplexes at varied rates. Polyplexes formed by linear peptides were degraded more quickly than those formed by the branched peptide (Figure 4). Similar results were observed when polyplexes formed from HK peptides and then were exposed to 1M NaCl. Whereas the H2K and H3K were released from DNA, particularly at lower peptide: DNA ratios, H2K4b was released to a lesser degree. Similarly, we found that linear H2K polyplexes were less stable

when exposed to increased concentrations of serum compared to branched H2K4b polyplexes (Table S2). Higher transfection of the tumor by linear H2K polyplexes may be due to partial degradation by trypsin-like enzymes, thereby exposing –KHHK-CO₂H which can then interact with the neuropilin-1 receptor.

NRP-1 mediated transport of polyplexes

We studied whether widespread intratumoral distribution and/or gene expression of H2K polyplexes may be mediated by NRP-1. Endothelial and most tumor cells including HUVEC (Figure S4) and MDA-MB-435 cells express NRP-1 [19, 20]. Thirty min after injection of NRP-1 Ab, H2K/H3K polyplexes carrying either tdTomato red- or luciferase-expressing plasmids were injected intravenously into MDA-MB-435 tumor bearing mice; 24 h later, mice were euthanized and gene expression in tumors/tissues analyzed. Although luciferase activity in lungs was slightly decreased by 25%, its activity in tumors was decreased by about 96% (Figure 5; Table 2). The decreased gene activity in tumors compared to other tissues in mice pretreated with NRP1 suggests that tumor transcytosis of HK polyplexes underlies enhanced tumor specificity. These findings are consistent with endothelial transcytosis, mediated by NRP-1, being necessary for widespread intratumoral distribution.

H2K/H3K polyplexes are more sensitive *in vitro* to blockade by NRP-1 antibody

Using the optimal HK: ratio for transfection *in vitro*, several inhibitors of endocytosis were used to inhibit uptake and transfection by the polyplexes. Transfection was done using HUVECs, a cell line that expresses NRP-1 (Figure S4). Polyplexes formed in water had average sizes of 100–200 nm (Table S3), which were similar in size to those used for *in vivo* studies. When cells were pre-incubated with NRP-1 antibodies, luciferase activity was reduced to a greater extent with H2K/H3K than H2K4b polyplexes (Figure 6). With polyplexes formed in Opti-MEM that were significantly larger in size (2000–3000 nm), H2K/H3K polyplexes were still significantly more sensitive to NRP-1 blockade compared to H2K4b polyplexes (data not shown). Cytochalasin D inhibited luciferase expression from the H2K4b polyplexes more than H2K/H3K polyplexes, suggesting that H2K/H3K polyplexes were less dependent on macropinocytosis. Other inhibitors did not distinguish transfection between H2K and H2K4b polyplexes. Nevertheless, the antibody blocking clathrin-mediated endocytosis reduced luciferase expression by about 50% from either polyplex.

Addition of H2K-CO₂H reduced variability

Because H2K has a C-terminal sequence of –KHK with an amide cap, enzymatic lysis and activation of this peptide may limit transport of its polyplexes. As a result, we examined whether modification of the C-terminal sequence to end with –KHHK- and a free carboxyl group may enhance transport of the polyplex. Although complete replacement of H2K with H2K-CO₂H did not enhance transfection of the tumor and reduced specificity (i.e., increased lung transfection), the 0.9/0.1/0.2 ratio (wt/wt/wt) of H2K/H2K-CO₂H/H3K appeared to augment transfection although statistical significance was not achieved (Figure 7). More striking, variability in transfection of tumors in mice injected with H2K-CO₂H-containing polyplexes was significantly reduced (Figure 7) (H2K/H3K vs H2K/H2K-CO₂H/H3K, Levene's test, $p < 0.05$). This was particularly evident when comparing luciferase

activities of the two tumors in each mouse that had been administered polyplexes containing the H2K-CO2H peptide. The optimal HK carrier also enhanced tumor delivery and gene expression in MDA-MB-231 xenografts. The gene expression in MDA-MB-231 tumors from the optimal HK polyplexes was about 5-fold greater than that previously reported with H2K polyplexes (Figure S5). Moreover, with the most effective combination of HK peptides (H2K/H2K-CO2H/ H3K; 0.9/0.1/0.2), the lack of the cRGD ligand associated with the polyplex modestly reduced transfection of the tumor (and specificity) *in vivo* by about 20% (data not shown). As a reference, *in vivo* jet-PEI as a carrier was about 25-fold less effective in transfecting tumors *in vivo* (data not shown) than the H2K/H2K-CO2H/H3K carrier.

Modulate transcytosis with WZB117

In addition to sequence variation, NRP-1 mediated transcytosis through the tumor endothelium may be increased by restriction of nutrients. Indeed, transcytosis has been speculated to have an important role in the transport of nutrients to nutrient-deficient tissues such as tumors. Support for this rationale comes from mouse studies in which tumor transcytosis was greatly increased by administering glucose -transport blockers intratumorally [2]. Thus, we investigated whether the approach of restricting nutrients to tumors results in further increases of plasmid delivery within tumors as well as other tissues. Prior to administering the polyplexes, different dosages of the glucose-transport (GLUT1 transporter) inhibitor [21, 22], WZB117, were injected intraperitoneal into MDA-MB-435 tumor-bearing mice (WZB117, 10 mg/kg IP, daily X 3 days); WZB117, 20 mg/kg IP for 3 days) [21]. Two hour after the last injection of WZB117, the polyplexes were injected IV and luciferase activity of the tumor and tissues were measured 24 hours later. With either dose of WZB117, there were increases of about 2-fold in luciferase activity of tumors whereas increases were minimal in normal tissues ($p < 0.05$) (Figure 8). Although there were modest increases in the luciferase activity of tumors treated with the higher dose of WZB117, it was not statistically different from the lower dose.

Discussion

Because specificity toward tumors of non-ligand, non-pegylated polyplexes was highly unusual [1], we considered whether unique mechanisms other than EPR may be involved in the tumor delivery of plasmids by HK peptides. Since peptides containing the -KXXK- sequence where X is any amino acid are known to activate NRP-mediated transport, we considered whether H2K (or H3K) with multiple -KHHK- repeats may similarly augment the NRP-mediated pathway. One or more of the -KHHK- repeats within the H2K polyplex, once enzymatically processed on the C-terminal side of lysine, may then bind to the neuropilin-1 receptor, activating transcytosis with transport of the polyplex through the tumor blood vessel. Based on investigations of Ruoslahti and colleagues [3, 4], H2K and H3K peptides are required to be enzymatically processed to activate NRP since these peptides have a -KHK sequence at their C-terminal ends with an amide cap incapable of binding to NRP (Table 1). Although the protease has not been identified that cleaves the cyclic iRGD or H2K peptides, the enzyme is thought to belong to a family of trypsin-like enzymes, which cleaves peptides on the C-terminal side of lysine or arginine [3, 5]. In contrast to the H2K polyplex, enzymatic processing of H2K4b polyplexes on the endothelial surface may

be reduced due to the higher affinity of H2K4b for plasmid DNA as well differences in their distribution [1].

Blocking of the NRP-1 receptor with an antibody provided strong evidence that transcytosis is an important mechanism in the widespread distribution of the H2K/H3K polyplexes. The marked reduction in luciferase of tumors compared to other tissues in mice administered the NRP-1 antibody suggests that transcytosis has an important role in the specificity of the polyplex. Whereas iRGD significantly augments the accumulation of nanoparticles within tumors [6], preliminary data suggests that administering iRGD prior to, with, or after H2K polyplexes had no obvious effect on the luciferase expression. These lines of evidence suggest that transcytosis, not EPR, is the primary mechanism of tumor targeting and accumulation of HK polyplexes. Although the enhanced specificity of the polyplex may be due to a constitutive increase in tumor transcytosis, an alternative mechanism is that increased enzymatic processing of the H2K peptide occurs in the tumor endothelium. Because D-amino acids do not bind and activate NRP-1 [3], HK peptides that vary in their L- and D-amino acid content may be used to define the optimal number of –KHHK– sequences. Of note, binding and release of peptide from DNA should not be affected by replacement of –KHHK– with –khhk– (D-amino acids) sequences. Further increases in tumor transfection with less variability between the tumors in the same mouse were also observed by modifying the C-terminal end of H2K with –KHHK–CO₂H. Many of the H2K–CO₂H peptides bound to DNA within the polyplex are likely not accessible to interact with the NRP-1. As a result, we anticipate that the multiple repeats of –KHHK– will be necessary for optimal delivery, by increasing the likelihood of enzymatic processing and interaction of HK with NRP-1.

Based on our current data, NRP-mediated transcytosis through the tumor endothelium is essential for widespread distribution of the polyplex. Because of the short period of time (30 min) to block the human receptor present on MDA-MB-435 cells prior to administering the polyplexes, we think that the NRP-1 antibodies primarily block the mouse receptor on the tumor vessel due to easy accessibility. Moreover, the vessel provides a major barrier for efficient transfection of the tumor (even with leaky vessels), and thus, NRP-1 mediated transport through the endothelium is at least necessary if not sufficient for widespread distribution.

Nevertheless, it is likely that tumor cells which express NRP-1 are important for efficient distribution of H2K polyplexes. There are at least 3 lines of evidence supporting that NRP-1 tumor cells are critical for effective transport. First, with NRP-1 positive tumor cells, fluorescently labeled silver nanoparticles showed widespread distribution in cells grown as 3D spheroids compared to monolayers [2]. Second, by restricting nutrients to the tumor and upregulating NRP-1 on the cell surface of the tumor cells [2], WZB117 administered intratumorally enhanced tumor accumulation of a fluorescently labeled ligand targeting NRP-1. Notably, WZB117, an inhibitor of the GLUT1 glucose transporter, has been injected daily intraperitoneal for up to 5 months without observed toxicity to mice [21, 22]. Third, the rapid and widespread distribution of the polyplex in the tumor matrix with no obvious increased accumulation near its blood vessels suggests that the mechanism may involve more than the tumor endothelial cells [1]. Although most tumor cells express NRP-1, tumor

cells that lack NRP-1 (together with NRP-1 antibodies) may provide additional and a more definitive answer to the role that tumor cells expressing NRP-1 may have in the widespread distribution of the HK polyplexes *in vivo*.

As stated previously, we think that binding and release and stability are important to the efficacy of HK polyplexes and have a role in multiple mechanisms in this project. In general, H2K4b polyplexes have been determined to be more stable (i.e., gel retardation, transfection, size of the particle) to the effects of serum, while the H2K polyplexes were significantly less stable (Table S2) [1]. Nevertheless, H2K polyplexes accumulate within the tumor significantly more than branched polyplexes, suggesting that transit time to the tumor for H2K polyplexes is relatively short [1]. Consistent with this interpretation, rapid accumulation (less than 30 minutes) within tumors of NRP-mediated molecules has been reported for iRGD peptides and associated nanoparticles [6]. Since branched H2K4b peptides have the same repeating sequence as the linear peptide (-KHHK-), their enhanced binding to DNA may limit their partial enzymatic degradation, resulting in reduced activation of NRP-1 and transcytosis in tumors. We explored whether differences in linear and branched HK polyplexes existed in their susceptibility to trypsin digests. As expected, polyplexes formed by linear peptides were digested more readily than the branched polymer (Figure 4). Similarly, we expect that branched peptide carriers with shorter terminal branches and therefore decreased DNA binding will have enhanced accumulation and specificity of their polyplexes toward tumors. One potential disadvantage of solid-phase synthesized branched peptides, however, is that because the C-terminal amino acid of each branch is conjugated to the core, the branched peptides have to be enzymatically processed to interact with NRP-1.

Previously we reported that H2K polyplexes had negligible transfection *in vitro* of MDA-MB-435 cells, in marked contrast to their efficient transfection of xenografts *in vivo*. *In vitro* assays that reflect *in vivo* results would facilitate development and understanding of HK carriers. In selected cases, transfection of HK polyplexes *in vitro* and *in vivo* may behave similarly, particularly if the modifier enhances transfection post-uptake. Addition of the endosomolytic H3K to H2K polyplexes enhanced transfection *in vitro* and *in vivo*. In contrast to H3K, addition of endosomolytic branched H2K4b to H2K polyplexes reduced transfection and specificity toward the tumor compared to H2K polyplexes (Figure S2). Consequently, the endosomolytic HK peptide should probably have similar DNA binding and condensing properties as H2K and not affect the biophysical properties of the polyplex. While *in vitro* assays in this study did not examine transcytosis, the role of NRP-1 in transport of polyplexes was investigated by transfecting HUVECs (Figure 7). The finding that H2K/H3K polyplexes were more susceptible to blockade of NRP-1 than H2K4b polyplexes suggests that H2K/H3K polyplexes might similarly be transported more effectively by NRP-1 *in vivo* (as it was). Although NRP-1 has recently been found to be associated with a macropinocytosis-like pathway [4], H2K polyplexes enter cells by a number of endocytosis pathways based on the *in vitro* data.

In summary, the addition of the endosomolytic H3K peptide to H2K augmented transfection of tumors *in vivo*. Because NRP-1 mediated transport (and likely transcytosis) is important in the accumulation of H2K polyplexes within the tumor, we suspect that multiple -KHHK-

embedded within H2K increase the probability of enzymatic processing, partial release of the peptide fragment from the polyplex, and subsequent interaction with the NRP-1 receptor. Moreover, modifications to the C-terminal end of H2K to enhance interaction with the receptor reduced the variability of transfection between tumors. Because enhanced transfection of several solid tumor types has been observed with H2K polyplexes [1], it is likely the modifications of HK to enhance transfection of xenografts in this study will be widely applicable.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Figure 1.

Hemolysis. Bovine red blood cells, exposed to selective peptides (25, 50, 100 µg/ml) at two pHs (7.4 or 5.5) for 4 h, were assessed for lysis (hemoglobin release). Values represent the means and SEM in which each condition was repeated three times. * $P < 0.05$, ** $P < 0.01$, Student's t-test, pH 7.4 vs 5.5.

Figure 2.

Tumor and tissue expression of luciferase delivered by H2K/H3K or by H2K polyplexes in MDA-MB-435 tumor-bearing mice. Twenty-four h after polyplexes were injected, tumors and tissues were analyzed for luciferase activity. After H2K (15 μ g) or H2K/H3K (12.5/2.5 μ g) were mixed with plasmid DNA (36 μ g), the cRGD ligand (1.63 μ g) was added prior to injecting the polyplex into the tail vein of the mice. Experiment was repeated five times with values representing the mean and SEM. *, $p < 0.05$, Student's *t*-test, H2K/H3K vs. H2K.

Figure 3.

Frozen sections of MDA-MB-435 tumor xenografts in which H2K (upper panels) or H2K/H3K combination (lower panels) peptides delivered the tdTomato-containing plasmid. Twenty-four h after mice with tumor xenografts were administered HK polyplexes, the tumors were processed to determine the amount of tdTomato expression in frozen sections of tumor xenografts. Left panels, nuclei stained with Syto-9; Middle panels, tdTomato expression; Right panels, merged. Figure is representative of an experiment repeated three times.

Figure 4. Stability of HK polyplexes

A. After various ratios of HK carriers with plasmid DNA were mixed (HK/DNA, wt/wt, 1/2 or 4/1 (1 μ g of plasmid)) and incubated for 30 min at RT, the polyplexes were incubated with trypsin (0.025%) for an additional 30 min at 37°C. The polyplexes were loaded onto the gel (1% agarose), electrophoresis was carried out at a constant voltage of 75 V for 45 min in TBE buffer containing ethidium bromide. **B.** Similarly, HK polyplexes were incubated with 1 M NaCl at RT instead of trypsin. **C.** HK polyplexes, untreated control. Experiment repeated twice.

Figure 5. Effect of NRP-1 blockade on Gene Expression

MDA-MB-435 tumor bearing mice were injected with IgG or NRP-1 Ab (50 μ g); 30 min later, the mice were injected IV with H2K/H3K tdTomato-expressing plasmid polyplexes. After 24 h, TdTomato expression was examined in frozen sections of tumors. Polyplexes were formed by mixing H2K/H3K (12.5/2.5 μ g) with the TdTomato expressing plasmid (36 μ g) for 45 minutes prior to adding the cRGD ligand (1.63 μ g). Representative images of an experiment repeated three times.

Figure 6. Selective inhibition of endocytosis pathways

After HK polyplexes were formed in water, the specific endocytic pathway by which polyplexes entered HUVEC was examined. Prior to transfection with the polyplexes, the cells were first treated for 30 min with the following endocytic inhibitors: clathrin antibody (1/100), filipin (1.0 $\mu\text{g}/\text{ml}$), NRP-1 antibody (50 $\mu\text{g}/\text{ml}$), or cytochalasin (1 $\mu\text{g}/\text{ml}$). Values represent mean and SEM of the percent untreated cells in which each condition was repeated three times. * $P < 0.05$, *** $P < 0.001$, One-way ANOVA with Holm-Sidak multiple pairwise comparisons, H2K/H3K vs H2K4b.

Figure 7. Effect of the H2K-CO₂H peptide on transfection of tumors *in vivo*

With the most effective ratio by weight, H2K/H2K-CO₂H/H3K (0.9/0.1/0.2) polyplexes were compared to H2K/H3K (1.0/0.2) polyplexes for their ability to transfect tumors *in vivo*.

Twenty-four hours after injection of these polyplexes, luciferase activities of tumors and tissues were measured in mice. Values represent means and SEM of an experiment repeated nine times. Polyplexes were formed by mixing H2K/H2K-CO₂H/H3K (11.25/1.25/2.5 µg) or H2K/H3K (12.5/2.5 µg) with a luciferase expressing plasmid (36 µg) for 45 min prior to adding the cRGD ligand (1.63 µg). *P<0.05, Levene's test, H2K/H2K-CO₂H/H3K vs. H2K/H3K.

Figure 8.

WZB117 augments transfection. Prior to injection of polyplexes, two groups of mice were injected with WZB117 (10 or 20 mg/kg) IP daily for 3 days while the control group did not receive WZB-117. Twenty-four h after injection of polyplex, tumors and tissues of mice were analyzed for luciferase activity. Values represent means and SEM of an experiment repeated three times. * $P < 0.05$, ** $P < 0.01$, Student's t-test, WZB117, treated vs untreated.

Table 1

HK sequences. For the branched peptide, the Lys in bold represents the 3-lysine core from which the terminal branches are attached. Repeating –KHHK– sequences are underlined.

Peptides	Sequence	Number of a.a.in linear/ terminal branch
H2K	K-H- <u>K</u> -H-H-K-H-H-K-H-H-K-H-H- <u>K</u> -H-K	20-mer
H2K-CO₂H	K-H- <u>K</u> -H-H-K-H-H-K-H-H-K-H-H-K-H-H-K-CO ₂ H	21-mer
H3K	K-H- <u>K</u> -H-H-K-H-H- <u>K</u> -H-H-H-K-H-H-H-K-H-H-H-K-H-H-H- <u>K</u> -H-H-K-H-H-H- <u>K</u> -H-K	33-mer
H2K4b	[K-H- <u>K</u> -H-H-K-H-H-K-H-H-K-H-H-K-H-H- <u>K</u> -H-K] ₄ LYS	20-mer

Table 2

Effects of blocking NRP-1 on transfection with HK polyplexes. MDA-MB-435 tumor bearing mice were injected with IgG or NRP-1 Ab (50 µg); 30 min later, the mice were injected IV with H2K/H3K luciferase-expressing plasmid polyplexes. After 24 h, luciferase activity was measured in lysates of tumors and normal tissues. Percent of control equals [(RLU in the specified tissue from mice treated with NRP-1 Ab divided by RLU from corresponding tissue treated with IgG control Ab) X 100]. Values are means and SEM from 3 experiments.

Tissues/MDA-MB-435 xenografts	Luciferase Expression (RLU/tissue)		Percent of Control
	Control Ab	NRP-1 Ab	
Lung	46.5±12.8	34.5±19.2	74.2
Tumor	165±26 ^{**}	5.5±2.4	3.5
Liver	0.54±0.38	0.39±0.23	72.2
Spleen	0.32±0.19	0.24±0.16	76.1

^{**} P<0.01, Student's t-test, Control Ab vs. NRP-1 Ab.