Published in final edited form as:

Oral Oncol. 2017 September ; 72: 123–131. doi:10.1016/j.oraloncology.2017.07.004.

Dual peptide-mediated targeted delivery of bioactive siRNAs to oral cancer cells in vivo

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Abstract

Objectives—Despite significant advances in cancer treatment, the prognosis for oral cancer remains poor in comparison to other cancer types, including breast, skin, and prostate. As a result, more effective therapeutic modalities are needed for the treatment of oral cancer. Consequently, in the present study, we examined the feasibility of using a dual peptide carrier approach, combining an epidermal growth factor receptor (EGFR)-targeting peptide with an endosome-disruptive peptide, to mediate targeted delivery of small interfering RNAs (siRNAs) into EGFR-overexpressing oral cancer cells and induce silencing of the targeted oncogene, cancerous inhibitor of protein phosphatase 2A (CIP2A).

Materials and methods—Fluorescence microscopy, real-time PCR, Western blot analysis, and in vivo bioimaging of mice containing orthotopic xenograft tumors were used to examine the ability of the dual peptide carrier to mediate specific delivery of bioactive siRNAs into EGFR-overexpressing oral cancer cells/tissues.

Results—Co-complexation of the EGFR-targeting peptide, GE11R9, with the endosome-disruptive 599 peptide facilitated the specific uptake of siRNAs into oral cancer cells overexpressing EGFR in vitro with optimal gene silencing observed at a 60:30:1 (GE11R9:599:siRNA) molar ratio. Furthermore, when administered systemically to mice bearing xenograft oral tumors, this dual peptide complex mediated increased targeted delivery of siRNAs into tumor tissues in comparison to the 599 peptide alone and significantly enhanced CIP2A silencing.

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Conflict of interest

None declared.

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Conclusion—Herein we provide the first report demonstrating the clinical potential of a dual peptide strategy for siRNA-based therapeutics by synergistically mediating the effective targeting and delivery of bioactive siRNAs into EGFR-overexpressing oral cancer cells.

Graphical Abstract

Keywords

Peptide; Homing/targeting; Cell-penetrating; Endosome-disruptive; Oral cancer; OSCC; siRNA; RNAi; EGFR; CIP2A

Introduction

Oral and pharyngeal cancer is the ninth most common cancer worldwide [1] and in the U.S.A. it is estimated that about 50,000 new cases and approximately 10,000 deaths occur annually due to cancer of the oral cavity and pharynx [2]. Although significant advances have been made in cancer treatment, the prognosis for oral cancer remains poor in comparison to other cancer types, including breast, skin, and prostate [2]. Moreover, the five-year survival rate for all stages of oral cancer is 64% and has only modestly improved over the past few decades [2,3]. Therefore, new approaches to oral cancer therapy are necessary to improve outcomes of treatment for this disease.

Since the discovery that exogenous siRNAs could be delivered intracellularly and result in specific gene silencing [4], RNA interference (RNAi) has been widely investigated as a new therapeutic strategy to treat many diseases, including cancer, by regulating gene expression. Although RNAi therapy is promising, siRNAs delivered in vivo face significant barriers, including serum instability, renal excretion, rapid uptake by the reticuloendothelial system, lack of cell/tissue-type specificity, poor intracellular delivery, and endosomal entrapment [4–6]. Therefore, strategies using direct chemical modification [7–9] and viral [10–12] / non-viral [13,14] delivery systems have been explored to enable intracellular accumulation of siRNAs. In particular, peptide carriers have been widely investigated for siRNA delivery, and several classes of peptides have shown potential to overcome efficacy-limiting barriers, including cell-penetrating peptides (CPPs) [15–17], pH-induced membrane-active (i.e. endosome-disruptive) peptides [18–20], and homing/targeting peptides [21–24].

In our studies, we previously designed a novel chimeric peptide, termed 599, containing the cationic CPP sequence, R9 (nine repeating units of Arg residues), and the influenza virus-derived fusogenic peptide sequence, INF-7, to enable self-assembly of the peptide-siRNA complex (by binding with negatively charged siRNAs), as well as, cellular uptake and endosomal escape of siRNAs [25]. In fact, we found that the 599 peptide enhanced the

intracellular delivery and bioavailability of siRNAs targeting the CIP2A oncogene (siCIP2A) into oral cancer cells and consequently inhibited oral cancer cell invasiveness and anchorage-independent growth in vitro [25]. Furthermore, our more recent work found that intratumoral dosing of the 599 peptide complexed to siCIP2A could induce CIP2A silencing in vivo, consequently resulting in the significant inhibition of tumor growth [26]. Though the 599 peptide was an effective siRNA delivery vehicle, it was not cell/tissue-type specific, which is essential, since systemic delivery remains the standard method of administering drugs for the treatment of solid tumors. Therefore, to overcome this limitation, the current study examined whether co-complexation of the 599 peptide with a cancer cell-targeting peptide would synergistically mediate effective targeting and delivery of bioactive siRNAs into oral cancer cells.

Materials and methods

Peptide synthesis

The 599 (GLFEAIEGFIENGWEGMIDGWYGGGGRRRRRRRRRK) and GE11R9 (YHWYGYTPQNVIGGGRRRRRRRRRK) peptides were synthesized and purified (> 95% purity) by high-performance liquid chromatography at GenScript USA, Inc. (Piscataway, NJ). Both peptides were biotinylated on the lysine residue and the nine arginine residues were D-arginine.

siRNAs

siCIP2A, its DY547/Cy5.5 fluorescently-labeled derivatives, and the negative control siGENOME Non-Targeting siRNA #5 (siNT) were previously described [25–27] and synthesized by GE Healthcare Dharmacon, Inc. (Lafayette, CO).

Cell culture

The human tongue squamous cell carcinoma cell lines CAL 27 and SCC-15 (ATCC, Manassas, VA) were cultured in ATCC-specified complete growth media in a 37°C incubator with 5% CO₂.

In vitro cell targeting assay

High EGFR-expressing CAL 27 cells were either cultured alone or co-cultured with low EGFR-expressing SCC-15 cells on a Collagen Type I coated 8-chamber slide (Fisher Scientific, Waltham, MA) in Opti-MEM® I Reduced Serum Media (Life Technologies, Grand Island, NY) containing 10% fetal bovine serum (FBS) and allowed to attach overnight. The following day, 100 nM of DY547-siCIP2A was complexed with either GE11R9 peptide at a 60:1 or 100:1 (GE11R9:siRNA) molar ratio, 599 peptide at a 50:1 (599:siRNA) molar ratio, or the dual peptide complex at molar ratios of 60:5:1, 60:10:1, 60:20:1, 60:30:1, 60:40:1, and 60:50:1 (GE11R9:599:siRNA). Peptides and siRNA were incubated for 20 minutes in Opti-MEM® at room temperature (RT) to allow complex formation. Subsequently, cells were washed with Opti-MEM® and incubated for four hours with the peptide-siRNA complexes; after which, the cells were prepared for fluorescence microscopy analysis (see Supplementary Material for a detailed description of this method).

Peptide-mediated siRNA transfection in vitro

Peptide-mediated siRNA transfection experiments were performed as previously described [25]. Briefly, siNT or siCIP2A complexed with the GE11R9 peptide at a 60:1 or 100:1 molar ratio, the 599 peptide at a 50:1 molar ratio, or the dual peptide at various molar ratios was added to CAL 27 cells and incubated for 48 hours. Afterwards, total RNA or protein was extracted from the cells for subsequent real-time PCR or Western blot analyses (see Supplementary Material for a detailed description of these methods).

Cell viability

Cell viability of CAL 27 cells treated with siNT alone or in complex with the dual peptide at various molar ratios was examined using the CellTiter 96® AQueous One Solution Cell Proliferation Assay (Promega, Madison, WI), as previously described [25].

Particle size and zeta potential

The hydrodynamic diameter and zeta potential of the dual peptide complex formulated at the 60:30:1 (GE11R9:599:siRNA) molar ratio in water was measured using a Beckman Coulter DelsaMax PRO Light Scattering Analyzer.

Gel-shift and ribonuclease (RNase)/serum-protection assays

Naked siCIP2A or siCIP2A complexed with the dual peptide at a 60:30:1 (GE11R9:599:siRNA) molar ratio, the GE11R9 peptide at a 60:0:1 molar ratio, or the 599 peptide at a 0:30:1 molar ratio were subjected to a gel-shift assay, as previously described [25]. For RNase/serum-protection assays, the stability of the dual peptide-siCIP2A complex was compared to naked siCIP2A based on an established protocol [26]. All images were captured using a BIO-RAD ChemiDoc XRS system.

In vivo delivery of the dual peptide-siCIP2A complex

Orthotopic tumor generation was performed as previously described [26]. Briefly, based on protocols approved by the Institutional Animal Care and Use Committee of MUSC, CAL 27 cells were injected submucosally in the floor-of-mouth of anesthetized five-week-old nude mice (NCI Athymic Ncr-nu/nu strain; Charles River, Bar Harbor, ME) and allowed to grow for 10–16 days to generate oral tumors. Upon tumor formation, mice were randomly assigned to four groups (n = 4) and then treated with dual peptide-complexed Cy5.5-labeled siCIP2A at a 60:30:1 (GE11R9:599:siRNA) molar ratio using 0.5 mg/kg siRNA in 100 µl saline solution that was administered intravenously into anesthetized mice via tail-vein injection. As controls, mice were also either left untreated or treated with Cy5.5-labeled siCIP2A complexed to 599 peptide at a 0:30:1 molar ratio or with the dual peptide complexed to siNT at a 60:30:1 molar ratio. Subsequently, mice were imaged using a PerkinElmer Maestro 2 In Vivo Imaging System 1, 6, 24, and 48 hours post-injection, as previously described [26]. Afterwards, mice were euthanized and tumor tissues were harvested and preserved at –80°C in RNeasy® (Life Technologies) for real-time PCR analysis (see Supplementary Material for a detailed description of this method).

Statistical analysis

All results are presented as mean $\pm$ standard error of the mean (SEM). Statistical significance was determined using either a Student's t-test or one-way ANOVA (Tukey's or Dunnett's Multiple Comparison Test) for comparisons between groups. A p-value < 0.05 was considered statistically significant. Statistical analyses for all studies were performed using GraphPad Prism 6 software (La Jolla, CA).

Results

The GE11R9 peptide targets delivery of siRNAs to cells with high expression of EGFR, but is not capable of delivering bioactive siRNAs

Although the 599 peptide was an effective siRNA delivery vehicle [25,26], it was not cell/tissue-type specific. Consequently, because EGFR is frequently overexpressed in oral squamous cell carcinomas (OSCCs) [28], and its increased expression has been associated with an aggressive phenotype and poor prognosis [28–30], we designed and synthesized a targeting peptide, termed GE11R9, that comprised a non-mitogenic EGFR-targeting sequence, GE11 [31], and the R9 sequence (which enables self-assembly with siRNAs through electrostatic interactions) to facilitate targeted delivery of siRNAs to oral cancer cells. Examination of the ability of GE11R9 to mediate specific delivery of siRNAs to cells overexpressing EGFR by fluorescence microscopy demonstrated that GE11R9 could indeed induce uptake of DY547-conjugated siCIP2A (DY547-siCIP2A) into high EGFR-expressing CAL 27 cells with high specificity at 100:1 and 60:1 (GE11R9:DY547-siCIP2A) molar ratios when grown in co-culture with low EGFR-expressing SCC-15 cells (Fig. 1A). In fact, very little siRNA was detected in SCC-15 cells, thus, indicating that GE11R9 had high specificity for cells overexpressing EGFR. Of note, the protein expression level of EGFR in each cell line was confirmed by Western blot analysis (Fig. 1B). Subsequent assessment of the functionality of siRNAs delivered by GE11R9, however, revealed no detectable difference in CIP2A protein expression in cells treated with GE11R9-complexed siCIP2A at either GE11R9:siCIP2A molar ratio in comparison to cells treated with GE11R9-complexed siNT or untreated cells (Fig. 1C). Conversely, near complete knockdown of CIP2A protein expression was detected in cells treated with 599-complexed siCIP2A, which was previously demonstrated to be very effective at mediating gene silencing [25]. Interestingly, further analysis demonstrated endosomal entrapment of the internalized DY547-siCIP2A cargo in complex with GE11R9, 4 hours post-treatment of CAL 27 cells, as opposed to treatments with the endosome-disruptive 599 in complex with DY547-siCIP2A, which displayed minimal co-localization with endosomal markers (Fig. 1D). Thus, based on the above findings, we reasoned that complexing siRNAs with a homing peptide alone was not sufficient to instill a competent cancer cell-targeting strategy. Instead, the vector would require the use of a dual peptide delivery strategy, combining both the homing and endosome-disruptive properties of the GE11R9 and 599 peptides, respectively, within a single complex, to enable targeted delivery of bioactive siRNAs into EGFR-overexpressing cancer cells.

The dual peptide-siRNA complex retains the ability to target cells with high EGFR expression and delivers bioactive siRNAs with no significant cytotoxic effects in vitro

To determine whether a dual peptide complex consisting of both GE11R9 and 599 could specifically target siRNAs to cells exhibiting high EGFR expression, we treated co-cultured CAL 27 and SCC-15 cells with various molar ratios of the dual peptide complexed with DY547-siCIP2A, including 60:5:1, 60:10:1, 60:20:1, 60:30:1, 60:40:1, and 60:50:1 (GE11R9:599:DY547-siCIP2A). As controls, we also treated cells with GE11R9-complexed DY547-siCIP2A and 599-complexed DY547-siCIP2A at 60:0:1 and 0:50:1 molar ratios, respectively. Fluorescence microscopy images showed that the dual peptide-complexed siRNAs preferentially accumulated in high EGFR-expressing CAL 27 cells at molar ratios from 60:5:1 through 60:30:1, with specificity comparable to cells treated with GE11R9-complexed DY547-siCIP2A (Fig. 2). Slight nonspecific uptake was detected at higher molar ratios, including 60:40:1 and 60:50:1 and even greater nonspecific uptake into low EGFR-expressing SCC-15 cells was observed for cells treated with 599-complexed DY547-siCIP2A, verifying that its uptake mechanism was nonspecific. Thus, based on these findings, our results demonstrated that although uptake of the 599 peptide-siRNA complex was nonspecific, its complexation with the GE11R9 peptide in a dual peptide strategy, at particular molar ratios, did not perturb the ability of the complex to specifically target cancer cells with high EGFR expression.

To examine whether the dual peptide-siRNA complex delivered functional siRNAs, we next analyzed CIP2A protein and mRNA levels in CAL 27 cells 48 hours post-treatment with dual peptide-complexed siCIP2A at various GE11R9:599:siRNA molar ratios. More specifically, Western blot analyses revealed effective CIP2A silencing by the dual peptide-siCIP2A complex at molar ratios of 60:20:1 and greater compared to cells treated with dual peptide-complexed siNT at the same molar ratios (Fig. 3A). More importantly, the CIP2A silencing was comparable to knockdown observed for cells treated with 599-complexed siCIP2A. Subsequent quantitative assessment by real-time PCR further demonstrated that cells treated with dual peptide-complexed siCIP2A had the greatest and optimal silencing effect at 60:30:1 and 60:40:1 molar ratios, both exhibiting a significant ~60% reduction in CIP2A mRNA expression (Fig. 3B). Taken together, these results illustrate that complexation of the nonfunctional GE11R9 peptide-siRNA carrier with the 599 peptide in a dual peptide strategy, at specific molar ratios, can restore the bioactivity of siRNAs delivered into oral cancer cells.

Finally, the long-term cell viability of the dual peptide-siRNA complex was also assessed to further optimize the dual peptide molar ratio for siRNA delivery. In particular, treatment of CAL 27 cells with dual peptide-complexed siNT (using molar ratios that were previously found to effectively induce gene silencing) for 48 hours revealed no significant cytotoxic effects at both the 60:20:1 and 60:30:1 molar ratios (Fig. 4). Consequently, based on the cumulative analyses of this assay and that of the in vitro targeting specificity and gene silencing assays, the dual peptide-siRNA molar ratio of 60:30:1 was determined to be the optimal molar ratio for siRNA delivery and used in subsequent experiments.

The dual peptide carrier enhances delivery of bioactive siRNAs to xenograft oral cancer tumors following systemic administration

Analyses of both the particle size and surface charge of the dual peptide-siRNA carrier at its optimized molar ratio demonstrated that it forms uniform nanocomplexes with an average diameter of 156 ± 4 nm and a zeta potential of 45 ± 2 mV (Fig. 5A); the significance being that this size falls within the particle size range (20–200 nm) necessary for effective retention in tumor tissues [32]. Moreover, the dual peptide complex at its optimal molar ratio was found to completely bind free siRNAs, as evidenced by its ability to completely retard the migration of siRNAs in the gel-shift assay (Fig. 5B). Of note, the strong siRNA-retardation effect exhibited by the dual peptide carrier at the optimal molar ratio (as well as the other peptide:siRNA molar ratio combinations) was most likely due to the complete encapsulation of the negatively-charged siRNAs and the resulting overall cationic-surface charge of the complex combined with its larger size (see Fig. 5A), in comparison to the average size of a single siRNA molecule, which is typically <10 nm [33]. Furthermore, the dual peptide carrier at its optimized molar ratio was also found to protect siRNAs from serum and RNase-mediated degradation (Fig. 5C), thus, making evident its ability to encapsulate and safeguard the RNA for potential use in intravenous-administered forms of therapy.

Because prospective siRNA delivery systems should enable targeted delivery and accumulation of the therapeutic in the tumor tissue, while clearing the organs and other tissues, we next proceeded to examine the ability of the dual peptide-siRNA complex to target tumor tissue using a xenograft oral cancer mouse model. In particular, we examined the biodistribution of Cy5.5-labeled siCIP2A (Cy5.5-siCIP2A) in complex with the dual peptide carrier at the optimal molar ratio (Dual peptide+Cy5.5-siCIP2A) upon intravenous administration into mice bearing orthotopic tumors derived from the high-EGFR expressing CAL 27 oral cancer cell line. Of note, mice treated with Cy5.5-siCIP2A complexed with the 599 peptide at a 0:30:1 molar ratio (599+Cy5.5-siCIP2A) were used as the non-targeting control group. Analysis of heat map images captured 1, 6, 24, and 48 hours post-injection revealed that the Cy5.5 fluorescent signal concentrated in the tumor region one-hour post-injection for both treatment groups (Fig. 6A), with average fluorescence intensities of 38 and 12 arbitrary units in mice treated with Dual peptide+Cy5.5-siCIP2A and 599+Cy5.5-siCIP2A, respectively (Fig. 6B). In fact, the Cy5.5 fluorescence intensity in the tumor region was greater in mice treated with Dual peptide+Cy5.5-siCIP2A at all time points in comparison to mice treated with 599+Cy5.5-siCIP2A, with a significant difference observed at the 48 hour time point. Additionally, Cy5.5 fluorescence was also observed in the midsection of the mice with dissipating intensity over time (the signal appeared to be largely cleared from circulation within 24–48 hours after administration), possibly indicative of clearance of the complex by the organs of the reticuloendothelial system, including the liver and spleen. Regardless, subsequent analysis of the functionality of the systemically administered siRNAs by real-time PCR analysis demonstrated a significant ~49% reduction in CIP2A mRNA expression (normalized to untreated tumor tissues) in Dual peptide+Cy5.5-siCIP2A-treated tumors compared to tissues treated with dual peptide carrier complexed to siNT (Dual peptide+siNT) (Fig. 6C). Conversely, although a slight ~23% reduction in CIP2A mRNA expression was detected in 599+Cy5.5-siCIP2A-treated tumors, this

difference was not significant. Accordingly, the above results demonstrate that the dual peptide carrier, following systemic administration, mediates enhanced delivery and accumulation of bioactive siRNAs into oral cancer tumors expressing high levels of EGFR. Moreover, these results demonstrate the clinical potential of the dual peptide complex for targeted delivery of siRNA therapeutics.

Discussion

Peptides are promising versatile vectors for siRNA therapeutics, including several classes that address the barriers to siRNA delivery, such as CPPs, endosome-disruptive peptides, and homing/targeting peptides. Although these peptides have been used individually to deliver siRNAs [34,35], few studies have combined different classes of peptides to overcome multiple barriers [36,37]. Consequently, in this study, we developed a dual peptide strategy in which we co-complexed the previously designed endosome-disruptive peptide, 599 [25,26], and the EGFR-targeting peptide, GE11R9, with siRNAs. Our results demonstrated that the dual peptide carrier mediated specific delivery of siRNAs to cells overexpressing EGFR, while also mediating significant gene silencing both in vitro and in vivo. Together, these data suggest that the dual peptide strategy could be a viable vector design for targeted delivery of RNAi-based therapeutics.

Because EGFR is commonly overexpressed in many cancers, including oral cancer [28], we designed the GE11R9 peptide to enable specific targeting of siRNAs to oral cancer cells. Our results showed that GE11R9-complexed siRNAs preferentially accumulated in OSCC cells overexpressing EGFR; however, GE11R9 was unable to deliver bioactive siRNAs, evidenced by the lack of gene silencing and the apparent endosomal entrapment of siRNAs. Due to the fact that GE11R9 acts as a targeting ligand, it allows siRNAs to enter the cell through receptor-mediated endocytosis. However, endocytosed nanocomplexes are trafficked to endosomal compartments and later degraded in lysosomes if they lack a mechanism for endosomal escape [38]. Consequently, we developed the dual peptide approach for delivering siRNAs by combining the endosome-disruptive peptide, 599, with the EGFR-targeting peptide, GE11R9. Our results demonstrated that the dual peptide carrier maintained the ability to preferentially target cells that overexpressed EGFR, suggesting that receptor-mediated endocytosis was the preferred method of cellular entry for the dual peptide carrier. Furthermore, gene silencing results demonstrated that the dual peptide carrier restored the functionality of delivered siRNAs. Based on these findings, we suggest that the GE11R9 and 599 peptides work synergistically to deliver bioactive siRNAs to targeted cells. The proposed model is that initially, GE11R9 binds to EGFR to induce receptor-mediated endocytosis. Once inside the endosome, under acidic conditions, 599 fuses with the endosomal membrane, resulting in its disruption, and consequently, the release of siRNAs into the cytosol, where they are incorporated into the RNA-induced silencing complex (RISC) to mediate gene silencing (Fig. 7). Interestingly, a recent study by Jun et al. used a similar approach, combining a targeting peptide with an endosome-disruptive peptide for delivery of siRNAs, but showed no knockdown of the targeted gene [36]. The investigators attributed the lack of gene silencing to the inability to effectively liberate the siRNA cargo from the blended nanocomplex [36]. Only when they replaced selected arginines with alanine residues within the stretch of nine D-arginine residues (that

were similarly built into the design of their targeting peptide) did they observe more efficient liberation of the siRNAs, with one particular peptide variant being able to significantly restore gene silencing [36]. Intriguingly, the more efficient release of bioactive siRNAs from this peptide variant appeared to be dependent on the apparent modification of its predicted 3D structure [36], thus implying that the structure of our dual peptide nanocomplex was conducive to promoting the dissociation of the siRNA cargo.

Examination of the clinical potential of the dual peptide carrier for RNAi therapeutics using a mouse model of human oral cancer demonstrated that the dual peptide carrier, upon intravenous administration, could mediate enhanced accumulation of siRNAs into high-EGFR expressing oral tumor tissues compared to siRNAs complexed to the non-targeting 599 peptide alone. The significance of this finding was that it demonstrated the ability of the dual peptide technology to mediate targeted delivery in vivo. However, despite this promising ability, one drawback we observed was the rapid clearance of the complex from systemic circulation, thus potentially limiting the therapeutic effectiveness of the administered siRNA cargo. A potential cause for this rapid clearance was the cationic property of the dual peptide-siRNA complex, as it is well documented that cationic nanoparticles attract negatively-charged serum proteins, which lead to aggregation and rapid plasma clearance [39,40]. Regardless, real-time PCR analyses showed that dual peptide-complexed siCIP2A could induce significant CIP2A silencing; whereas 599-complexed siCIP2A could not, thus, further demonstrating the ability of the dual peptide technology to deliver bioactive siRNAs systemically.

Finally, it is interesting to note that the Jun et al. study [36], which used a similar dual peptide-mediated siRNA delivery approach to ours, did not evaluate the in vivo tumor-targeting capability of their multicomponent peptide-woven nanocomplex. Furthermore, although they demonstrated siRNA bioactivity in vivo, the route of administration of the nanocomplex was intratumoral, and the gene silencing effect was not reported to be statistically significant compared to the control treatments [36]. Thus, to our knowledge, our study is the first to demonstrate the enhanced accumulation of siRNAs into tumor tissues following intravenous administration using a dual peptide carrier approach. This strategy is advantageous due to the potential versatility of the dual peptide carrier to be tailored to target other receptors/markers overexpressed in various cancers and/or specific cancer cell-types by modifying the sequence of the targeting peptide, while keeping the 599 peptide constant to enable functionality of the complex. In fact, this versatility in the design of the dual peptide carrier could prove critical for the overall successful treatment of oral cancer. Considering the cellular heterogeneity of tumors, there is a need to not only target cancer cells of epithelial origins, such as OSCC cells that exhibit EGFR overexpression in >75% of tumor cases [29], but also subpopulations of cells, such as cancer stem cells and cancer cells of mesenchymal phenotype, which have low EGFR expression levels [29,41–43]. Consequently, future studies will expand the cancer cell-type targeting repertoire of the dual peptide carrier design, examine biochemical strategies for enhancing the systemic circulation of the dual peptide-siRNA complex, and determine its efficacy in reducing tumor burden. Together, these advancements will ultimately help establish the therapeutic potential of this technology for RNAi-based therapy in human oral cancer, as well as its translational applicability towards the treatment of other diseases.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

We thank Anca Dumitriu for technical and experimental insights and discussions. We also thank Tilman Heise and Gunhild Sommer in the Department of Biochemistry and Molecular Biology at MUSC for the use of their Zeiss Axio Observer microscope. This work was supported in part by the NIGMS grant P30GM103331 (A.J. subproject investigator) and NIDCR grants R00DE018191 (A.J.), T32DE017551 (A.A.A.), and R25DE022677 (H.Z. and R.M.S.). Further support was provided in part by the Cell & Molecular Imaging Shared Resource, Hollings Cancer Center, MUSC (NCI P30CA138313).

Role of the Funding Source

The funding sources had no involvement in the study design, the collection, analysis and interpretation of the data, the writing of the report or in the decision to submit it for publication.

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Highlights

- Developed a dual peptide carrier approach for siRNA delivery to human oral cancer.
- Targets siRNAs to EGFR-overexpressing oral cancer cells.
- Enhances accumulation of siRNAs in oral tumors upon intravenous administration.
- Mediates significant silencing of the target oncogene in vivo.
- Has clinical potential as a targeted RNAi-based therapy for human oral cancer.

Fig. 1.

The GE11R9 peptide targets delivery of siRNAs to cells with high expression of EGFR, but is not capable of delivering bioactive siRNAs. **(A)** Fluorescence microscopy analysis of targeted siRNA delivery following co-culture of high EGFR-expressing CAL 27 and low EGFR-expressing SCC-15 oral cancer cells incubated for 4 hours with DY547-conjugated siCIP2A (DY547-siCIP2A, red) in complex with GE11R9 peptide at 100:1 or 60:1 (GE11R9: DY547-siCIP2A) molar ratios. Of note, to distinguish between the two cell lines in co-culture, cells were stained for vimentin (green, top panels), which was found to be

differentially expressed, with CAL 27 cells being vimentin negative and SCC-15 cells being vimentin positive. Nuclei (blue) were counterstained with DAPI. The bottom panels are identical to the top panels, except that the vimentin fluorescent signal was removed in the bottom panels to better visualize the degree of fluorescent siRNA signal in SCC-15 cells, which are indicated by arrowheads. Scale bar: 100 μ m. **(B)** Western blot analysis of EGFR and vimentin protein expression levels in CAL 27 and SCC-15 cells. GAPDH protein levels were monitored to ensure equal loading of samples. **(C)** Western blot analysis of CIP2A protein expression levels in CAL 27 cells performed 48 hours post-treatment with GE11R9 peptide complexed to either a control non-targeting siRNA (siNT) or siCIP2A at a 60:1 or 100:1 (GE11R9:siRNA) molar ratio compared to 599 peptide at a 50:1 (599:siRNA) molar ratio or untreated cells. β -Actin protein levels were monitored to ensure equal loading of samples. **(D)** Indirect immunofluorescence microscopy analysis of endosomal co-localization following transfection of CAL 27 cells with DY547-siCIP2A (red) complexed with GE11R9 at 100:1 or 60:1 (GE11R9:DY547-siCIP2A) molar ratios or 599 peptide at a 50:1 (599:DY547-siCIP2A) molar ratio for 4 hours. In the top panels, cells were stained for endosomes (green) and nuclei (blue) were counterstained with DAPI. The co-localization of DY547-siCIP2A with endosomes (white; bottom panels) was determined using the ImageJ [44] Colocalization plug-in (National Institutes of Health, Bethesda, MD; freely available at imagej.nih.gov/ij/). Scale bar: 50 μ m.

Fig. 2.

The dual peptide complex retains the ability to specifically target siRNAs to cells overexpressing EGFR. Fluorescence microscopy analysis of the co-culture of CAL 27 (high EGFR, vimentin negative) and SCC-15 (low EGFR, vimentin positive) oral cancer cells incubated for 4 hours with DY547-siCIP2A (red) in complex with GE11R9 and/or 599 peptides at various molar ratios (GE11R9:599:DY547-siCIP2A). Cells were stained for vimentin (green) and nuclei (blue) were counterstained with DAPI. Scale bar: 100 μ m.

Fig. 3.

The dual peptide complex mediates delivery of bioactive siRNAs into oral cancer cells. **(A)** Western blot analyses of CIP2A protein expression levels in CAL 27 cells 48 hours post-treatment with siNT or siCIP2A in complex with GE11R9 and/or 599 peptides at various GE11R9:599:siRNA molar ratios. β-Actin protein levels were monitored to ensure equal loading of samples. **(B)** Real-time PCR analysis of CIP2A mRNA levels in CAL 27 cells 48 hours post-treatment with siNT or siCIP2A in complex with either the dual peptide at various GE11R9:599:siRNA molar ratios or the 599 peptide alone. The CIP2A mRNA levels

were normalized to 18S rRNA. Data are mean $\pm$ SEM of three independent experiments performed in triplicate, where *P<0.05, ***P<0.001, ****P<0.0001, and P 0.05 is not significant (ns) compared to siNT treated cells (Student's t test).

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Fig. 4.

The dual peptide-siRNA complex displays no significant cytotoxicities at GE11R9:599:siRNA molar ratios up to 60:30:1. Assessment of long-term viability of CAL 27 cells 48 hours post-treatment with siNT in complex with the dual peptide at various GE11R9:599:siNT molar ratios. Cells were treated with siNT alone or left untreated as negative controls and untreated cells were defined as 100% viable. Data are mean ± SEM of three independent experiments performed in triplicate, where ** $P < 0.01$, *** $P < 0.001$, and $P \geq 0.05$ is not significant (ns) compared to untreated cells (one-way ANOVA).

Fig. 5.

Biochemical characterization of the dual peptide carrier at the optimized GE11R9:599:siRNA molar ratio of 60:30:1. **(A)** Size distribution and zeta potential of the dual peptide complexed with siCIP2A at the 60:30:1 molar ratio 20 minutes after formulation in water. **(B)** Agarose gel shift assay examining the ability of the dual peptide to complex free siCIP2As at the 60:30:1 (GE11R9:599:siCIP2A) molar ratio in comparison to GE11R9 or 599 at a 60:0:1 or 0:30:1 molar ratio, respectively. Non-complexed siCIP2As (0:0:1 molar ratio) and the molecular weight marker (MWM) are also shown. **(C)** Stability of siRNAs in complex with the dual peptide carrier was examined in the presence and absence of serum or RNase A. In particular, naked and dual peptide-complexed siCIP2As at the 60:30:1 molar ratio were incubated in the absence or presence of either 50% fetal bovine serum (FBS) or 5 μ g/ml RNase A for one hour at 37°C. The position of intact siCIP2A is indicated.

Fig. 6.

The dual peptide carrier enhances delivery of bioactive siRNAs to xenograft oral cancer tumors following systemic administration. **(A)** Representative in vivo images of siRNA biodistribution in mice and **(B)** quantitative analysis of tumor fluorescence 1 to 48 hours post-treatment with Cy5.5-labeled siCIP2A (Cy5.5-siCIP2A) in complex with the dual peptide at a 60:30:1 (GE11R9:599:siRNA) molar ratio (Dual peptide+Cy5.5-siCIP2A) or the 599 peptide alone at a 0:30:1 molar ratio (599+Cy5.5-siCIP2A). Black and white image (B/W); site of tumor (arrow); Arbitrary units (A.U.). Data are mean $\pm$ SEM of four independent samples, where $*P < 0.05$ compared to 599+Cy5.5-siCIP2A complex-treated tumors (Student's *t* test). **(C)** Real-time PCR analysis of relative CIP2A mRNA levels in excised xenograft tumor tissues, 48 hours post-treatment with Dual peptide+Cy5.5-siCIP2A, 599+Cy5.5-siCIP2A, or dual peptide complexed to siNT (Dual peptide+siNT), normalized to untreated tissues. The CIP2A mRNA levels were normalized to 18S rRNA. Data are mean $\pm$ SEM of four independent samples performed in triplicate, where $*P < 0.05$ and $P = 0.05$ is not significant (ns) compared to Dual peptide+siNT (one-way ANOVA).

Fig. 7.

Model of targeted delivery and gene silencing using the dual peptide-siRNA nanocomplex. Both the GE11R9 and 599 peptides complex with siRNA using a stretch of densely packed cationic nona(D-arginine) amino acid residues, which enable complexing via electrostatic interactions. The GE11R9 peptide mediates cellular entry through receptor-mediated endocytosis initiated by binding of GE11R9 to the epidermal growth factor receptor (EGFR). Upon entering the cell, 599 mediates endosomal escape of siRNA under acidic conditions and siRNA in the cytoplasm enter the RNA-induced silencing complex (RISC), which together enable sequence-specific inhibition of gene expression.