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Fusogenic-oligoarginine peptide-mediated silencing of the CIP2A oncogene suppresses oral cancer tumor growth *in vivo*

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Abstract

Intracellular delivery and endosomal escape of functional small interfering RNAs (siRNAs) remain major barriers limiting the clinical translation of RNA interference (RNAi)-based therapeutics. Recently, we demonstrated that a cell-penetrating endosome-disruptive peptide we synthesized, termed 599, enhanced the intracellular delivery and bioavailability of siRNAs designed to target the CIP2A oncoprotein (siCIP2A) into oral cancer cells and consequently inhibited oral cancer cell invasiveness and anchorage-independent growth *in vitro*. Thus, to further assess the therapeutic potential of the 599 peptide in mediating RNAi-based therapeutics for oral cancer and its prospective applicability in clinical settings, the objective of the current study was to determine whether intratumoral dosing of the 599 peptide-siCIP2A complex could induce silencing of CIP2A and consequently impair tumor growth using a xenograft oral cancer mouse model. Our results demonstrate that the 599 peptide is able to protect siRNAs from degradation by serum and ribonucleases *in vitro* and upon intratumoral injection *in vivo*, confirming the stability of the 599 peptide-siRNA complex and its potential for therapeutic utility. Moreover, 599 peptide-mediated delivery of siCIP2A to tumor tissue induces CIP2A silencing without any associated toxicity, consequently resulting in reduction of the mitotic index and significant inhibition of tumor growth. Together, these data suggest that the 599 peptide carrier is a clinically effective mediator of RNAi-based cancer therapeutics.

Graphical abstract

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Keywords

Fusogenic peptide; Cell-penetrating peptide; Oral cancer; CIP2A; siRNA delivery; RNAi

Introduction

The discovery that exogenous siRNAs induce sequence-specific inhibition of gene expression has resulted in the investigation of the use of RNAi-based approaches to treat many diseases, including cancer. Though the therapeutic applications of siRNAs are promising, intracellular delivery and endosomal escape remain major barriers limiting the clinical translation of RNAi therapeutics. Intracellular trafficking of siRNAs is prevented by their hydrophilicity, large molecular weight, negative charge, and instability in serum [1,2]. Further, upon entering the cell, siRNAs must escape the endosome to reach their site of action in the cytosol and avoid lysosomal degradation, which diminishes their therapeutic activity [2]. Therefore, new strategies in improving the delivery of siRNAs are necessary to further advance RNAi therapeutics towards clinical translation.

Peptide carriers have been explored to overcome the barriers of nucleic acid transport and cell-penetrating peptides (CPPs) have proven to be a promising approach for efficient delivery. CPPs are a class of short peptide sequences that are cationic in nature, allowing for efficient translocation through the cell membrane independent of receptors, with the ability to transport protein or oligonucleotide cargo [3,4]. CPPs typically contain arginine rich regions, including many positively charged amino acids, which are able to form complexes with oligonucleotides and allow effective delivery across the cell membrane through electrostatic interactions [4]. Several CPPs have been developed and proven to promote transfection of oligonucleotides [5] or siRNAs [6-8] into cells. Although CPPs promote cellular uptake of siRNAs, the gene silencing effect and consequently, the therapeutic outcome, are often limited due to retention of CPP-associated cargo within the endosome, resulting in degradation by lysosomes [9,10]. Therefore, peptides with endosomolytic properties are necessary for effective delivery of bioactive siRNAs. Endosomal escape of CPPs and their associated cargo can be enhanced using a class of peptides referred to as pH-dependent membrane active peptides [11]. A prototypical example is the HA2 fusogenic peptide, a synthetic peptide based on the endosome-disruptive domain of the hemagglutinin HA2 subunit of the influenza virus [11-13]. The HA2 subunit comprises N-terminal residues that form an α -helix structure under acidic conditions of the endosome, which inserts into the endosomal membrane resulting in fusion and endosomal escape [14,15]. The co-incubation of synthetic HA2 fusogenic peptides with CPPs has been used for delivery of various therapeutic macromolecules [3]. However, co-incubation of CPPs with fusogenic peptides appears to only slightly improve endosomal escape [16]. Consequently, endosomal co-localization of both the CPP and fusogenic peptide must occur in order to achieve

maximum therapeutic activity, which is not guaranteed using co-incubation strategies. It has been shown that single hybrid peptides containing both cell-penetrating and endosome-disruptive properties can allow cellular uptake and significantly enhance endosomal escape of various cargo, including, proteins, DNA, and siRNAs [8,10,17,18]. Moreover, chemical modifications of CPPs with endosomolytic moieties have also been demonstrated to facilitate the endosomal escape of anti-microRNA oligonucleotides and siRNAs [19,20]. However, despite these promising findings, no studies have demonstrated the therapeutic utility of these hybrid peptides for RNAi-based therapies *in vivo*, in particular, in treating diseases, such as cancer.

Recently, we designed a novel chimeric peptide, termed 599, combining cationic cell-penetrating nona(D-arginine) residues and an influenza virus-derived fusogenic peptide sequence, INF-7, with endosome-disruptive properties to mediate both cellular entry and endosomal escape of siRNAs [21]. More specifically, our studies found that at a 50:1 peptide-to-siRNA molar ratio, the 599 peptide completely bound siRNAs, and mediated intracellular delivery of siRNAs into oral cancer cells with minimal cytotoxicity [21]. Furthermore, the 599 peptide complexed to siRNAs designed to target CIP2A (siCIP2A), an oncoprotein overexpressed in human oral squamous cell carcinomas (OSCCs) [22,23], promoted significant silencing of CIP2A mRNA and protein expression, resulting in decreased anchorage-independent growth and cancer cell invasiveness *in vitro* [21]. Thus, to further assess the therapeutic potential of the 599 peptide in mediating RNAi-based therapeutics for oral cancer and its prospective applicability in clinical settings, this study examined whether intratumoral dosing of the 599 peptide complexed to siCIP2A (599+siCIP2A) could induce silencing of the CIP2A oncoprotein using a xenograft oral cancer mouse model. Our results demonstrate the ability of the 599 peptide to protect siRNAs from degradation by serum and ribonucleases (RNases) *in vitro* and upon intratumoral injection *in vivo*, confirming the stability of the 599+siRNA complex and potential utility for therapeutic applications. Moreover, we also demonstrate that 599 peptide-mediated delivery of siCIP2A to tumor tissues induces CIP2A silencing, resulting in the significant inhibition of tumor growth.

Methods and Materials

Peptide synthesis

Peptide 599 (GLFEAIEGFIENGWEGMIDGWYGGGRRRRRRRRRK) was synthesized and purified (> 95% purity) by high-performance liquid chromatography at GenScript USA Inc. (Piscataway, NJ). The 599 peptide was also biotinylated on the lysine residue and the nine arginine residues were D-arginine.

siRNAs

The siRNA targeting CIP2A (siCIP2A) was previously described [21,24] and synthesized by GE Healthcare Dharmacon (Lafayette, CO). Additionally, siCIP2As with either a Cy5.5 or DY547 label at the 3'-end of the anti-sense strand (Cy5.5/D-siCIP2A) were also synthesized and used for some experiments. The control siGENOME Non-Targeting siRNA #5 (siNT) was purchased from GE Healthcare Dharmacon.

Cell culture

The human tongue squamous cell carcinoma (SCC) cell line CAL 27 was purchased from American Type Culture Collection (ATCC, Manassas, VA) and cultured in ATCC-specified complete growth media in a 37°C incubator with 5% CO₂.

RNase and serum protection assays

For the RNase A protection assay, the 599 peptide was complexed with 30 pmol siCIP2A at a 50:1 (599:siRNA) molar ratio for 20 minutes at room temperature (RT) and then incubated in the absence or presence of RNase A (0.18 µg) for 60 minutes at 37°C. For the serum protection assays, the 599 peptide was complexed with 25 pmol siCIP2A at a 50:1 (599:siRNA) molar ratio for 20 minutes at RT and then incubated in the absence or presence of either 10% or 50% fetal bovine serum (FBS) or human serum (HS) for 1 hour at 37°C. As a control, naked siCIP2A was also analyzed under these same conditions. After the incubations, a 4% SDS solution was added and the samples were examined by electrophoresis on a 2% agarose gel stained with ethidium bromide.

Generation of tumor xenografts

Based on protocols approved by the Institutional Animal Care and Use Committee of MUSC, CAL 27 cells, grown to 50-60% confluence, were suspended in Dulbecco's Modified Eagle's Medium containing 10% FBS / Matrigel (1:1 ratio; BD Biosciences, Bedford, MA) at 750,000 cells per 50 µl and injected submucosally in the floor-of-mouth of anesthetized eight-week-old nude mice (NCI Athymic Ncr-nu/nu strain; *n*=3/group for *in vivo* and *ex vivo* imaging studies; Charles River, Bar Harbor, ME) or NOD-SCID mice (NOD.CB17-*Prkdc*^{scid} strain; *n*=2/group or *n*=3-4/group for single or three-dose bioactivity studies, respectively; Jackson Laboratory, Bar Harbor, ME). Oral tumors were allowed to grow for 14 days before treatment was administered. Of note, tumor cell dose was optimized for the CAL 27 cell line to produce tumors corresponding to an average volume of ~70-mm³, within this time period.

Formulation and in vivo treatment with the 599+siCIP2A complex

Upon tumor formation in nude or NOD-SCID mice, the 599+siRNA complexes were formed in nuclease-free water at a 50:1 (599:siRNA) molar ratio in half of the injection volume (i.e. 25 µl) by mixing 10 µg/µl 599 and 100 pmol/µl siRNA (siNT or Cy5.5-/D-/siCIP2A) stock solutions. After 30 minutes incubation, 25 µl of 1.8% saline solution was added to the complex. Subsequently, 50 µl of solution containing 5 µg of siRNA complexed with 599 peptide was delivered in either a single dose or three doses every 72 hours (of note, 2.5 µg of siRNA was delivered in the third dose) via intratumoral injection into tumors formed within the oral cavity of the mice. As controls, mice were also either left untreated or treated with Cy5.5-/D-/siCIP2A alone or with 599 peptide complexed to siNT (599+siNT). Following the specified treatments, the mice were either subjected to *in vivo* / *ex vivo* imaging or sacrificed at the specified time points, after which tumor tissues were collected for Western blot, fluorescence microscopy, real-time PCR, and/or histopathological analyses.

In vivo and ex vivo imaging

Mice intratumorally injected with Cy5.5-siCIP2A alone or 599 peptide complexed to Cy5.5-siCIP2A were imaged using the PerkinElmer Maestro 2 *In Vivo* Imaging System (Caliper Life Sciences, Hopkinton, MA) 1, 3, 6, 24, 48, and 72 hours post-injection. After 72 hours, mice were euthanized and the tumors and internal organs were harvested for *ex vivo* imaging. Mono-images (gray scale images) were acquired using 1×1 binning at 250 ms and cube images (fluorescent images) were acquired using 2×2 binning at 500, 1000, 2000, and 4000 ms at each time point. Unmixed images depicting Cy5.5-siCIP2A fluorescence were obtained via spectral analysis using the Maestro software and heat map images of fluorescence intensity were obtained using the ImageJ [25] HeatMap Histogram plugin (National Institutes of Health; freely available at <http://rsb.info.nih.gov/ij/>). Fluorescence within the tumor tissue was also quantified at each imaging time point by identifying the area of the tumor as the region of interest and measuring the average fluorescence intensity using ImageJ [25].

Processing of tumor tissues

48 hours post-treatment for the one-dose bioactivity experiment or two days after the last treatment for the three-dose bioactivity study, the mice were euthanized by CO₂ asphyxiation followed by cervical dislocation, and the tumors were surgically excised. For the single-dose bioactivity experiment, the tumor tissues were snap-frozen in OCT compound. For the three-dose bioactivity study, prior to processing the tumors, the tumors were weighed and measured in two dimensions using digital calipers to determine the tumor volume calculated based on the formula: $a \times a \times b/2$, where a is the smaller dimension [26]. Afterwards, the tumor tissues were dissected into thirds, where one-third of the tumor was either preserved in RNeasy[®] at -80°C (Life Technologies, Grand Island, NY), snap-frozen in OCT compound, or fixed in 10% formalin solution and paraffin-embedded.

Fluorescence microscopy

Frozen OCT-embedded tumor tissue sections cut at 5-μm using a TBS Minotome PLUS Cryostat (Durham, NC) were placed on slides and allowed to thaw at RT for 10 minutes prior to being either mounted immediately with a glass coverslip using VECTASHIELD Mounting Medium with 4',6-diamidino-2-phenylindole (DAPI, VECTOR Laboratories, Burlingame, CA) or fixed and permeabilized with ice cold acetone for 10 minutes. For tissues that were fixed and permeabilized, the slides were subsequently blocked in 10% normal goat serum (Sigma, Saint Louis, MO) for 20 minutes and then incubated with a rabbit polyclonal anti-CIP2A antibody (1:100, Bethyl Laboratories, Montgomery, TX) diluted in 1× PBS containing 1.5% normal goat serum at RT for 1 hour. Next, the tissues were washed with 1× PBS and incubated with the corresponding secondary antibody, Alexa Fluor[®] 488-conjugated goat anti-rabbit IgG (1:200, Life Technologies) diluted in 1× PBS containing 1.5% normal goat serum, at RT for 1 hour, after which the slides were mounted with coverslips, as described above. Fluorescence images were obtained using a Zeiss (Thornwood, NY) Axio Observer.D1 microscope equipped with a LD A-Plan ×20/0.3 Ph1 objective.

Western blot analysis

For the single-dose bioactivity experiment, proteins were extracted based on a protocol described by Scicchitano *et al.* [27], where four 5- μ m-thick OCT-embedded tumor tissue sections preserved at -80°C were placed into a tube containing ice cold RIPA buffer (50 mM Tris-HCl, pH 7.4, 150 mM NaCl, 0.5% sodium deoxycholate, 0.1% SDS, and 1% NP-40) with protease inhibitor cocktail (Thermo Fisher Scientific, Rockford, IL), vortexed, homogenized, subjected to four freeze/thaw cycles in dry ice, centrifuged and supernatant collected, and stored at -80°C . Alternatively, for the three-dose bioactivity study, proteins were extracted from tumor tissues preserved in RNeasy Lysis Buffer (Life Technologies) using the AllPrep DNA/RNA/Protein Mini kit (Qiagen, Valencia, CA), according to the manufacturer's instructions. Between 5-20 μ g of the protein lysates were then resolved in Mini-PROTEAN[®] TGX[™] Precast 4-15% gels (BIO-RAD, Hercules, CA) and transferred to PVDF membranes (Millipore, Bedford, MA). The PVDF membranes were blocked in 5% non-fat dried milk in Tris-HCl-buffered-saline supplemented with 0.1% Tween-20 (TBS-T) for one hour at RT, followed by overnight incubation at 4°C with either of the following primary antibodies: mouse monoclonal anti-CIP2A (1:200, clone 2G10-3B5, Santa Cruz Biotechnology, Santa Cruz, CA), rabbit polyclonal anti-CIP2A (1:2,000, Bethyl Laboratories), rabbit monoclonal anti-GAPDH (1:10,000, Cell Signaling, Danvers, MA) or mouse monoclonal anti- β -actin (1:10,000, Sigma). The membranes were then washed four times with TBS-T and incubated with the corresponding secondary antibodies, either horseradish peroxidase-conjugated goat anti-mouse (1:2,000 or 1:30,000) or anti-rabbit IgG (1:2,000 or 1:10,000, SouthernBiotech, Birmingham, AL), for 1 hour at RT. Immunoreactive bands were detected by the SuperSignal Chemiluminescent system (Thermo Fisher Scientific, Waltham, MA), according to the manufacturer's instructions.

Real-time PCR

For CIP2A, OAS1, and STAT1 mRNA and 18S rRNA quantitation, total RNA was harvested from tumor tissues preserved in RNeasy Lysis Buffer (Life Technologies) using the AllPrep DNA/RNA/Protein Mini kit (Qiagen) and reverse-transcribed using the High-Capacity cDNA Reverse Transcription kit (Life Technologies). Afterwards, quantitative real-time PCR was performed in an Applied Biosystems[®] StepOnePlus[™] Real-Time PCR machine (Life Technologies) using predesigned TaqMan[®] Gene Expression Assays (Life Technologies) for CIP2A (Hs00405413_m1), OAS1 (Hs00973637_m1), STAT1 (Hs01013996_m1), and 18S (4310893E) combined with TaqMan[®] Fast Universal PCR Master Mix (Life Technologies), according to the manufacturer's instructions.

Histochemistry

5- μ m-thick paraffin-embedded tumor tissue sections were cut using a Thermo Fisher Scientific HM 340E Electronic Rotary Microtome and then stained with hematoxylin and eosin (H&E). The histopathology of the H&E-stained tumor tissue sections was evaluated by an experienced pathologist, who was blinded to the treatment groups. Histological images were taken by an Olympus (Center Valley, PA) BX43 microscope equipped with an Olympus SC30 digital camera. For mitotic rate analyses in tumor specimens, the number of mitoses in twenty consecutive 400 $\times$ magnification fields was quantified for each tumor, after

which the average number of mitoses per field was calculated to determine the mitotic index for each tumor sample.

Statistical analysis

The results were presented as mean $\pm$ SEM. Comparisons between groups were performed using either Student's *t* test or one-way ANOVA (Bonferroni's Multiple Comparison Test). A value of $P < 0.05$ was considered statistically significant. Statistical analyses were performed using GraphPad Prism 6 software (La Jolla, CA).

Results

The 599 peptide protects siRNAs from RNase and serum-mediated degradation in vitro

siRNAs delivered *in vivo* encounter RNases in the physiological environment (e.g. serum) that degrade RNAs before they reach their therapeutic target. Therefore, delivery systems for siRNAs must protect them from degradation in order to maintain their bioactivity. To examine the resistance of the 599 peptide-siRNA complex against RNases, the 599 peptide was complexed to siCIP2A at a 50:1 (599:siRNA) molar ratio and incubated either in the presence of RNase A or sera for 1 hour (**Fig. 1**). Of note, the 50:1 molar ratio was used for these and subsequent experiments because it was previously demonstrated by our laboratory to be optimal in delivering bioactive siRNAs into oral cancer cells *in vitro* [21]. As shown in **Fig. 1A**, no detectable degradation of siRNAs was observed when complexed with 599 peptide in the presence of 10 $\mu\text{g/ml}$ RNase A after a one hour incubation, while naked siRNAs were completely degraded under these conditions. Moreover, examination of the stability of the complex in fetal bovine and human sera (**Fig. 1B**) demonstrated that siRNAs complexed with 599 peptide exhibited either no degradation or very minor levels of degradation in the presence of 10% and 50% serum, respectively. Conversely, naked siRNAs were either largely or completely degraded by sera at both concentrations. Thus, these results indicate that the 599 peptide protects siRNAs from degradation by serum ribonucleases and RNases directly. These data, in combination with our previous *in vitro* findings that demonstrated that the 599+siCIP2A complex preserves its silencing activity in serum [21], made evident the possible utility of this complex for *in vivo* applications.

The 599 peptide protects siRNAs from degradation in vivo

After demonstrating the ability of the 599 peptide to protect siRNAs from degradation in the presence of RNase A and sera *in vitro*, we assessed the ability of the peptide to protect siRNAs *in vivo* after intratumoral delivery. To determine siRNA stability *in vivo*, 599 peptide complexed to Cy5.5-labeled siCIP2A (Cy5.5-siCIP2A) or Cy5.5-siCIP2A alone were intratumorally injected in nude mice bearing xenograft floor-of-mouth oral tumors and the mice were imaged 1, 3, 6, 24, 48, and 72 hours post-injection. Fluorescence image analyses revealed stable and strong fluorescence signals at all time points in tumors injected with 599 peptide complexed to Cy5.5-siCIP2A (599+Cy5.5-siCIP2A), in comparison to tumors injected with siRNA alone, which exhibited low to negligible fluorescence during this same time period (**Fig. 2A**). Furthermore, *in vivo* imaging between 1-6 hours revealed fluorescence in the midsection of the mice intratumorally injected with siRNA alone, with no detectable signal in any tissues beyond 6 hours, indicating the potential degradation

and/or elimination by other organs of the naked siRNAs. In contrast, very low levels of siRNA were detected in the midsection of the animals upon complexation with 599 peptide, signifying that very little siRNA was eliminated from the site of injection and that most of the 599 peptide-complexed siRNA was retained within the tumor tissues. To further confirm these results, we dissected the internal organs 72 hours post-injection and performed *ex vivo* imaging, which revealed high levels of fluorescence in tumor tissues treated with 599+Cy5.5-siCIP2A, but no detectable fluorescence in tumor tissues treated with naked siRNA. Of note, negligible fluorescence was detected in the other dissected organs for both treatment groups. Interestingly, the heat map derived from the siRNA fluorescence signals within the tumors treated with 599+Cy5.5-siCIP2A displayed a pattern of bands of decreasing signal intensity in the tumor tissue, which indicated that the siRNA signal was heterogeneous in the tumor tissue, with highest signal intensity likely near the sight of injection and decreasing intensity further from the sight of injection. Quantitation of the siRNA fluorescence within the tumors further corroborated the above findings demonstrating significant differences at all time points between the fluorescence signals of tumors injected with 599+Cy5.5-siCIP2A, in comparison to tumors injected with siRNA alone, with average fluorescence signals of 96 ± 5 A.U. (arbitrary units) compared to 10 ± 3 A.U., respectively (**Fig. 2B**). Taken together, these results demonstrate the ability of the 599 peptide to protect siRNAs from degradation *in vivo* and mediate its delivery into tumor tissues, further substantiating the potential therapeutic applicability of the 599 peptide.

The 599 peptide mediates delivery of bioactive siRNAs into xenograft mouse tumors following a single-dose treatment

In addition to providing protection for siRNAs, the 599 peptide must effectively deliver bioactive siRNAs to tumor tissues *in vivo* in order to be considered a viable delivery vehicle for RNAi-based cancer therapeutics. As a result, the *in vivo* delivery of bioactive siRNAs was assessed using a xenograft floor-of-mouth tumor mouse model, where following tumor generation, mice were intratumorally injected with 599 peptide complexed with either a DY547-fluorescent-labeled siCIP2A (599+D-siCIP2A) or a control non-targeting siRNA (599+siNT). As controls, in addition to the 599+siNT treatment, mice were also either left untreated or treated with D-siCIP2A alone. 48 hours post-treatment, tumors were excised and examined for siRNA uptake and silencing of the targeted CIP2A by fluorescence microscopy and western blot analyses (**Fig. 3**). In line with our *in vivo* and *ex vivo* imaging data (see **Fig. 2**), fluorescent imaging of tumor tissue sections displayed no uptake of naked D-siCIP2As, in contrast to tumors treated with the 599+D-siCIP2A complex, which were found to have effective uptake of siRNAs (**Fig. 3A**), thus, confirming the ability of the 599 peptide to protect siRNAs from degradation and to mediate their delivery into cancer cells/tissues *in vivo*. More importantly, Western blot analysis of tumors treated with the 599+D-siCIP2A complex depicted very effective silencing of CIP2A protein expression, in comparison to untreated tissues or tissues treated with D-siCIP2A alone or 599+siNT (**Fig. 3B**). Furthermore, indirect-immunofluorescence microscopy analyses of the tumor tissues confirmed CIP2A protein knockdown in tissues treated with the 599+D-siCIP2A complex compared to untreated tissues or tissues treated with D-siCIP2A alone or 599+siNT (**Fig. 3C**). Notably, in tissues treated with 599+D-siCIP2A, CIP2A knockdown occurred in areas where D-siCIP2A was also detected. Interestingly, analyses of the 599+D-siCIP2A-treated

tumor tissue sections also revealed differences in siRNA fluorescence intensity throughout the tumor (compare **Figs. 3A** and **3C**), corroborating our *in vivo* and *ex vivo* imaging results (see **Fig. 2**), which similarly found the siRNA signal to be heterogeneous within the tumor tissue. However, it remains to be determined whether these differences in siRNA fluorescence signal intensities correlate with the degree of gene silencing. Regardless, these results demonstrate that the 599 peptide delivers bioactive siRNAs *in vivo*, and more importantly that silencing of CIP2A protein expression can be achieved after a single-dose intratumoral injection of the 599+D-siCIP2A complex.

599 peptide-complexed siCIP2A mediates tumor growth inhibition following a three-dose schedule

We previously established that silencing of CIP2A mediated by the 599+siCIP2A complex decreased oral cancer cell invasiveness and anchorage-independent growth *in vitro* [21]. Therefore, upon demonstrating *in vivo* silencing of CIP2A after a single-dose treatment of the 599+siCIP2A complex, we evaluated the ability of this complex to inhibit tumor growth after multiple dosing treatments. Intratumoral injection of the 599+siCIP2A complex in three doses every 72 hours resulted in a significant decrease in both weight and volume of the excised tumors 8 days post-initial treatment compared to untreated tissues or tissues treated with siCIP2A alone or 599+siNT (**Fig. 4**). Tumor weights for the 599+siCIP2A treated mice decreased approximately two-fold in comparison to the control groups, with an average tumor weight of 234 ± 15 mg for the 599+siCIP2A treated mice, compared to 466 ± 16 mg in the control groups. Additionally, tumor volumes decreased by approximately three-fold in the 599+siCIP2A treated mice in comparison to the control groups, with an average tumor volume of 235 ± 28 mm³ in the 599+siCIP2A treated mice, compared to 619 ± 46 mm³ in the control groups. Together, these data demonstrate the ability of the 599+siCIP2A complex to inhibit tumor growth and its potential utility as a cancer therapeutic.

The 599+siCIP2A complex mediates silencing of CIP2A in tumor tissues exhibiting impaired growth

Though delivery systems combining endosome disruptive and cell penetrating components have been shown to deliver siRNAs and silence targeted genes *in vitro* [9,10], little is known about the *in vivo* utility of these systems to silence oncogenes and function as cancer therapeutics. In order to confirm CIP2A gene silencing in tumor tissues in which growth was inhibited by the 599+siCIP2A complex, real-time PCR and Western blot analyses were performed to evaluate CIP2A mRNA and protein expression levels, respectively (**Fig. 5**). Quantitation of mRNA levels by real-time PCR demonstrated a significant ~40% reduction in CIP2A mRNA expression levels on average in 599+siCIP2A-treated tumors compared to untreated tumors or tumors treated with siCIP2A alone or 599+siNT (**Fig. 5A**). Furthermore, Western blot analyses followed by densitometric quantification of the Western blot bands confirmed the silencing of CIP2A protein expression levels with a significant ~8.5-fold and ~6-fold reduction in CIP2A protein expression (normalized to untreated tissues) in tumor tissues treated with the 599+siCIP2A complex compared to tissues treated with siCIP2A alone or 599+siNT, respectively (**Figs. 5B and 5C**). Thus, these results substantiate that the inhibition of tumor growth mediated by the 599+siCIP2A complex was the result of CIP2A

oncogene silencing. Moreover, these results confirm the *in vivo* functionality of the 599+siCIP2A complex and demonstrate its potential utility for RNAi-based cancer therapies.

The 599+siCIP2A complex does not trigger an interferon response or induce acute toxicities in xenograft mouse tumor tissues

Under certain circumstances, apparent therapeutic effects of siRNAs may be confounded by induction of the interferon (IFN) pathway [28-31]. More specifically, exogenous siRNAs have been demonstrated to induce the IFN pathway and mediate sequence-independent gene suppression by signaling through the Toll-like receptor 3 (Tlr3) [31], a receptor commonly found to be expressed in various cancers [32], including OSCC tissues and cell lines [33]. Because the CAL 27 cell line we used to generate the xenograft tumors for this study express Tlr3 [33], we examined the 599+siCIP2A-treated tumor tissues for induction of the principal IFN-responsive genes, *Oas1* and *Stat1* [34-36], by real-time PCR and found that treatment of tumor tissues with this complex did not significantly induce the expression of either gene compared to untreated tumors or tumors treated with siCIP2A alone or 599+siNT (**Fig. 6A**). Furthermore, to confirm that the therapeutic effects observed for the 599+siCIP2A-treated tumors were a consequence of RNAi-based therapeutics and not due to any associated toxicities with the complex, histopathological examinations of the 599+siCIP2A-treated tumor tissues in relation to the untreated or control-treated tumor tissues revealed no visible signs of acute toxicities (**Fig. 6B**). Thus, based on these data, the therapeutic effects observed for the 599+siCIP2A-treated tumors appear to be a consequence of the RNAi mechanism.

Tumors treated with the 599+siCIP2A complex exhibit a significant decrease in mitotic index

Depletion of CIP2A has been previously reported to inhibit cell proliferation and delay mitotic progression in human cancer cells [24,37]. As a result, to investigate differences in proliferation between the 599+siCIP2A-treated tumors and untreated tumors or tumors treated with siCIP2A alone or 599+siNT, tumor sections from each group were stained with hematoxylin and eosin and examined in a blinded fashion for the number of mitotic figures per high-power field (mitotic index; **Fig. 7**). Histological examination of the 599+siCIP2A-treated tumors and the control groups demonstrated a disparity in mitoses (**Fig. 7A**; arrows), where 599+siCIP2A-treated tumors exhibited reduced mitoses compared to the untreated tumors or tumors treated with siCIP2A alone or 599+siNT. More specifically, quantitation of the mitotic indices demonstrated a statistically significant ~56% reduction in mitoses on average in 599+siCIP2A-treated tumors compared to untreated tumors or tumors treated with siCIP2A alone or 599+siNT (**Fig. 7B**). Taken together, these data imply that 599 peptide-mediated silencing of CIP2A impairs tumor growth due to its ability to reduce mitoses, and consequently, cancer cell proliferation.

Discussion

Intracellular delivery and endosomal escape of siRNAs are barriers that must be overcome to achieve clinical translation of RNAi therapeutics. Hybrid peptides containing cell-

penetrating and endosome-disruptive properties have shown promise for addressing these barriers to RNAi therapeutics [10,17-19]; however, few studies have demonstrated the utility of these peptides *in vivo*. Furthermore, among the studies that have demonstrated *in vivo* utility [19], none have established the therapeutic utility of these hybrid peptides using a disease model. As a result, the current study sought to assess the therapeutic potential of a chimeric peptide, we previously designed and characterized *in vitro*, termed 599 [21], in mediating RNAi-based cancer therapy *in vivo*. Results from our study found that intratumoral dosing of the 599 peptide complexed to siCIP2A effectively silenced the CIP2A oncoprotein and consequently impaired tumor growth using a xenograft oral cancer mouse model. The significance being that these data demonstrate the successful *in vivo* utility of a cell-penetrating fusogenic chimeric peptide for RNAi-based therapy in a disease model.

Due to the susceptibility of siRNAs to degradation by RNases in the physiological environment, it is necessary to use delivery vehicles that protect siRNAs from degradation *in vivo*. Both the *in vitro* serum and RNase protection assays in our study confirmed the ability of the 599 peptide to protect siRNAs from degradation when compared to siRNAs alone. These results may be attributable to the ability of the 599 peptide to completely complex siRNAs at a 50:1 molar ratio [21], as well as the inherent proteolytic resistance of D-amino acids [38,39], which compose the cell-penetrating portion of the 599 peptide. The stability of the 599+siCIP2A complex against degradation, combined with its ability to preserve the bioactivity of siRNAs in serum *in vitro* [21], verified the potential of the 599 peptide to protect siRNAs in the physiologic environment and warranted the evaluation of the complex for *in vivo* use.

Assessment of whether the chimeric 599 peptide effectively protected siRNAs from degradation *in vivo* and delivered bioactive siRNAs to tumor tissue following a single-dose treatment was determined using a xenograft floor-of-mouth tumor mouse model. The results of the *in vivo* / *ex vivo* bioimaging and single-dose bioactivity studies revealed that the 599 peptide mediated the internalization of fluorescently-labeled siCIP2As into tumor tissues, with siRNA fluorescence signal remaining for at least 72 hours post-treatment in comparison to tumors treated with fluorescently-labeled siCIP2A alone, which exhibited low to negligible fluorescence, thus, confirming the stability of the complex and the ability of the 599 peptide to protect siRNAs from degradation *in vivo*. The lack of internalization of naked siRNAs was likely due to several factors: (1) RNase-mediated degradation in the physiological environment, (2) charged repulsion between the negatively-charged siRNAs and the negatively-charged cell membranes, and/or (3) rapid elimination from the animal. Upon establishing internalization of the 599+siCIP2A complex into tumor tissue, we subsequently evaluated the functionality of the complex *in vivo* to determine the ability of the 599 peptide to mediate delivery of bioactive siCIP2As. Western blot and immunofluorescence microscopy analyses both revealed that a single-dose treatment of the 599+siCIP2A complex administered intratumorally effectively mediated CIP2A oncoprotein silencing, thus, demonstrating the potential utility of the 599 peptide for RNAi-based cancer therapeutics. Interestingly, in a study by Ye *et al.* [40], it was reported that intratumoral administration of nanoparticles modified with both cell-penetrating peptides and fusogenic

peptides could synergistically enhance intracellular delivery and gene transduction of a reporter plasmid construct. Our study further demonstrates that fusogenic and cell-penetrating peptides can be combined in a single hybrid peptide and directly complexed with siRNAs to silence gene expression in tumor tissues.

To evaluate the ability of the 599+siCIP2A complex to mediate tumor growth inhibition, we performed a multiple-dosing study, where three doses of the 599+siCIP2A complex were administered intratumorally to xenograft floor-of-mouth tumors in mice over a period of 8 days. Measurement of both final tumor weights and volumes revealed that treatment with the 599+siCIP2A complex significantly decreased both tumor growth parameters in comparison to untreated tumors or tumors treated with siCIP2A alone or 599+siNT. Moreover, analyses of the tumor tissues exhibiting tumor growth inhibition confirmed significant silencing of CIP2A at both the mRNA and protein levels with no apparent toxicities associated with the treatment or induction of the IFN pathway, thus confirming that the therapeutic effect was a consequence of the RNAi mechanism. Although a few studies have examined the ability of peptides exhibiting combined cell-penetrating and endosome-disruptive properties to deliver bioactive nucleic acid cargo *in vivo* [19,40], none of these studies have examined their therapeutic effects on tumor growth. Therefore, our study is significant because it demonstrates the *in vivo* utility of a cell-penetrating endosome-disruptive chimeric peptide for RNAi-based cancer therapy.

Lastly, tumor growth inhibition mediated by the 599+siCIP2A complex appeared to be due to inhibition of cell proliferation, as evidenced by the significant reduction in the mitotic index for these treated tissues. In fact, depletion of the CIP2A oncogene has been reported to inhibit cell proliferation and delay mitotic progression in human cancer cells [24,37]. More specifically, the inhibition of *in vivo* tumor growth could possibly have been due to several factors related to the silencing of the CIP2A oncogene. First, CIP2A downregulation is known to activate PP2A tumor suppressor function, resulting in destabilization of the proto-oncogenic transcription factor c-Myc, a known regulator of cell proliferation [24,41]. Second, CIP2A depletion has also been reported to significantly reduce the stability of the Plk1 protein, a kinase that plays a crucial role in mitosis, causing a delay in mitotic progression, and possibly, tumor development [37]. Despite these possibilities, further studies will be needed to clarify the mechanisms that are perturbed by CIP2A depletion which impair the oncogenic properties of cancer cells. Regardless of the mechanisms involved, our results are significant because they demonstrate that 599 peptide-mediated silencing of CIP2A impairs tumor growth due to its ability to suppress cancer cell proliferation.

Conclusion

Our present study found that the 599 peptide, comprised of endosome-disruptive and cationic cell-penetrating nona(D-arginine) residues, protected siRNAs from degradation by serum and ribonucleases *in vitro* and upon intratumoral injection *in vivo*, confirming the stability of the 599+siRNA complex and its potential for therapeutic utility. Moreover, 599 peptide-mediated delivery of siCIP2A to tumor tissue induced CIP2A silencing, consequently resulting in reduction of the mitotic index and significant inhibition of tumor

growth in a xenograft floor-of-mouth tumor mouse model. Together, these data demonstrate the therapeutic potential of the 599 peptide and warrant its further preclinical evaluation and development as a delivery vehicle for RNAi-based human oral cancer therapeutics.

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Figure 1.

The 599 peptide protects siRNAs from RNase A and serum ribonuclease-mediated degradation *in vitro*. Naked and 599 peptide-complexed siRNAs targeting the CIP2A oncogene (siCIP2A) at a 50:1 (599:siCIP2A) molar ratio were incubated in the absence or presence of either (**A**) RNase A or (**B**) sera for 1 hour at 37°C. The position of the intact siRNA is indicated. Fetal bovine serum (FBS); human serum (HS).

Figure 2.

The 599 peptide protects siRNAs from degradation *in vivo*. **(A)** Representative *in vivo* images of mice 1 to 72 hours post-treatment with Cy5.5-fluorescent-labeled siCIP2A (Cy5.5-siCIP2A) alone or in complex with the 599 peptide (599+Cy5.5-siCIP2A). *Ex vivo* images of the tumors and internal organs dissected from the respective mice 72 hours post-treatment are also shown. **(B)** Quantitative analysis of siRNA fluorescence intensity over time in tumor tissues treated with either naked Cy5.5-siCIP2A or 599+Cy5.5-siCIP2A. Data are mean $\pm$ SEM of three independent samples, where **P<0.01 and ***P<0.001 compared to 599+Cy5.5-siCIP2A treated tumors (Student's *t* test). Black and white image (B/W); site of tumor (arrow); heart (H); brain (B); lung (Lu); kidney (K); liver (Li), spleen (S); tumor (T); arbitrary units (A.U.).

Figure 3.

The 599 peptide mediates delivery of bioactive siRNAs into xenograft mouse tumors following a single-dose treatment. **(A)** Fluorescence microscopy analysis of DY547-conjugated siCIP2A (D-siCIP2A; red) uptake into tumor tissues 48 hours post-treatment with or without complexation to the 599 peptide. Nuclei (blue) were counterstained with DAPI. Scale bar: 50 μ m. **(B)** Western blot analysis of CIP2A protein expression levels in excised xenograft tumor tissues 48 hours post-treatment with 599 peptide complexed to D-siCIP2A (599+D-siCIP2A), compared to tissues treated with D-siCIP2A alone or 599 peptide complexed to a control non-targeting siRNA (599+siNT). The CIP2A protein levels in untreated tumor tissues were also analyzed for comparison. Tissue samples from two mice (M1 and M2) per treatment group were analyzed. GAPDH protein levels were monitored to ensure equal loading of samples. **(C)** Indirect immunofluorescence analysis of CIP2A protein expression levels (green) and D-siCIP2A (red) uptake in excised xenograft tumor tissues 48 hours post-treatment with 599+D-siCIP2A compared to untreated tissues or tissues treated with D-siCIP2A alone or 599+siNT. Nuclei (blue) were counterstained with DAPI. Representative images of xenograft tumors from mice in the untreated group and treatment groups are shown. Scale bar: 100 μ m.

Figure 4.

The 599+siCIP2A complex significantly impairs tumor growth in mice following a three-dose schedule. The (A) weights and (B) volumes of excised xenograft tumors 8 days post-initial treatment with 599+siCIP2A, compared to tissues treated with siCIP2A alone or 599+siNT. The weights and volumes of untreated tumor tissues were also analyzed for comparison. Data are mean $\pm$ SEM of four independent samples (3 samples for untreated group), where *P<0.05, **P<0.01, and ***P<0.001 compared to 599+siCIP2A treated tumors (one-way ANOVA).

Figure 5.

The 599+siCIP2A complex mediates silencing of CIP2A in tumor tissues following a three-dose schedule. **(A)** Real-time PCR analysis of CIP2A mRNA levels in excised xenograft tumor tissues 8 days post-initial treatment with 599+siCIP2A, compared to untreated tissues or tissues treated with siCIP2A alone or 599+siNT. The CIP2A mRNA levels were normalized to 18S rRNA. Data are mean $\pm$ SEM of four independent samples (3 samples for untreated group) performed in duplicate, where $**P < 0.01$ compared to 599+siCIP2A treated tumors (one-way ANOVA). **(B)** Western blot analysis of CIP2A protein expression levels in excised xenograft tumor tissues 8 days post-initial treatment with 599+siCIP2A, compared to untreated (Unt) tissues or tissues treated with siCIP2A alone or 599+siNT. Tissue samples from four mice (M1-4) per treatment group were analyzed, except for the untreated group which included samples from only two mice. β -actin protein levels were monitored to ensure equal loading of samples. **(C)** Densitometric quantification of CIP2A protein expression levels (from Western blot shown in panel B) in treated tissues normalized to untreated tissues. CIP2A protein levels were normalized to β -actin. Data are mean $\pm$ SEM of four independent samples, where $**P < 0.01$ and $****P < 0.0001$ compared to 599+siCIP2A treated tumors (one-way ANOVA).

Figure 6.

The 599+siCIP2A complex does not trigger an interferon response or induce acute toxicities in xenograft mouse tumor tissues. (A) Real-time PCR analysis of mRNA levels of the interferon-responsive genes, *OAS1* and *STAT1*, in excised xenograft tumor tissues 2 days post-final treatment with 599+siCIP2A, compared to untreated tissues or tissues treated with siCIP2A alone or 599+siNT. The *OAS1* and *STAT1* mRNA levels were normalized to 18S rRNA. Data are mean $\pm$ SEM of four independent samples (3 samples for untreated group) performed in duplicate, where $P > 0.05$, not significant (ns), compared to 599+siCIP2A treated tumors (one-way ANOVA). (B) Histopathological examinations of hematoxylin and eosin-stained tumor tissues treated with 599+siCIP2A, compared to untreated tissues or tissues treated with siCIP2A alone or 599+siNT. Representative images of xenograft tumors from mice in the untreated group and treatment groups are shown. Scale bar: 50 μ m.

Figure 7.

Treatment of tumors with 599 peptide complexed to siCIP2A causes a significant decrease in the number of mitotic figures. (A) Representative high-power fields of hematoxylin and eosin-stained tumor tissues treated with 599+siCIP2A, compared to untreated tissues or tissues treated with siCIP2A alone or 599+siNT. Arrows indicate mitotic figures. Scale bar: 20 μ m. (B) Graphic representation of mitotic index in twenty high-power fields (HPF) from excised xenograft tumor tissues 8 days post-initial treatment with 599 peptide+siCIP2A, compared to untreated tissues or tissues treated with siCIP2A alone or 599+siNT. Data are mean $\pm$ SEM of four independent samples (3 samples for untreated and siRNA alone-treated groups), where ** P <0.01 and *** P <0.001 compared to 599+siCIP2A treated tumors (one-way ANOVA).