Title: HIF2α targeted RNAi therapeutic inhibits clear cell renal cell carcinoma

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Abstract

Targeted therapy against VEGF and mTOR pathways has been established as the standard-of-care for metastatic clear cell renal cell carcinoma (ccRCC); however, these treatments frequently fail and most patients become refractory requiring subsequent alternative therapeutic options. Therefore, development of innovative and effective treatments is imperative. About 80-90% of ccRCC tumors express an inactive mutant form of the von Hippel-Lindau protein (pVHL), an E3 ubiquitin ligase that promotes target protein degradation. Strong genetic and experimental evidence supports the correlate that pVHL functional loss leads to the accumulation of the transcription factor hypoxia-inducible factor 2α (HIF2α) and that an over-abundance of HIF2α functions as a tumorigenic driver of ccRCC. In this report, we describe an RNAi therapeutic for HIF2α that utilizes a targeting ligand that selectively binds to integrins αvβ3 and αvβ5 frequently over-expressed in ccRCC. We demonstrate that functional delivery of a HIF2α specific RNAi trigger resulted in HIF2α gene silencing and subsequent tumor growth inhibition and degeneration in an established orthotopic ccRCC xenograft model.

Introduction

Promising targeted treatment options derived from better understanding of molecular characteristics of cancer biology have resulted in significant improvement in the overall survival of patients with metastatic ccRCC. These treatments include therapies that target molecules regulating angiogenesis and proliferation pathways such as bevacizumab, an antibody against vascular endothelial growth factor (VEGF), and
several multi-kinase-inhibitors that inhibit multiple receptor tyrosine kinases including VEGFR (1-3). These targeted therapies are distinct from small molecule inhibitors that target the mammalian target of rapamycin (mTOR) signaling pathways, such as temsirolimus and everolimus, which disrupt pathways that regulate cell growth, metabolism and survival (4,5). These therapies exhibited significantly improved treatment outcomes over the last decade and have become the standard-of-care in both first- and second-line settings since they were first introduced (1,6,7). However, emergence of resistance to these agents is a frequent limitation for long term success (8-10). More recently, nivolumab (Opdivo®), a monoclonal antibody immunotherapeutic targeting the programmed cell death-1 (PD-1) immune checkpoint pathway (11) was approved as a second-line option for ccRCC. The excitement surrounding this promising new treatment class for oncology in general signals the importance of targeting orthogonal pathological pathways to counter the frequent genomic shifts of heterogeneous cancer cell populations and the complexity of tumor microenvironment cellular interactions (12).

About 80-90% of ccRCC tumors express a mutant form of pVHL, an E3 ubiquitin ligase (13-15). The mutant pVHL is unable to polyubiquitinate the transcription factor hypoxia-inducible factor (HIF) which regulates genes involved in angiogenesis, proliferation, metabolism and invasion/metastasis pathways (16-19). Strong genetic and experimental evidence supports the observation that pVHL functional loss leads to the accumulation of the transcription factor HIF2α, and that an over-abundance of HIF2α functions as a tumorigenic driver of ccRCC (16). In contrast, HIF1α is frequently not expressed due to chromosome 14q deletions commonly occurring in ccRCC.
Evidence suggests that HIF1α functions as a tumor suppressor in renal cancer (16). Accordingly, potential new therapies targeting HIF2α function, including in the current study, are being explored. Recently, preclinical and limited early clinical results using small molecule inhibitors targeting HIF2α were reported (16,20-22). In these studies, the HIF2α antagonists showed antitumor activity in several tumor cell line xenograft mouse models and patient derived xenograft (PDX) models. Early clinical data showed a patient treated with the HIF2α antagonist PT2385 remained free of progression for more than 11 months (20). These encouraging results lend validation towards developing novel therapeutics that target the pVHL/HIF2α pathway. However, these studies also uncovered several preclinical mouse models that are not responsive to these HIF2α antagonists (20,21). In some models, the lack of antitumor activity was attributed to missense mutations that resulted in amino acid substitutions surrounding the binding pocket of HIF2α antagonists (20,21). These observations indicate that an effective alternate therapeutic approach to target HIF2α function is warranted.

The specificity and potency of gene silencing mediated by RNA interference (RNAi) is well established (23,24) and targeted therapeutics utilizing this mechanism to suppress tumor HIF2α expression can potentially overcome conventional drug resistance and improve responsiveness to currently treatments, which includes immune checkpoint inhibitors. To realize the potential of RNAi based therapeutics, efficient delivery of an RNAi trigger (siRNA) has been a major obstacle (25), particularly to target tissues other than liver (26,27). Previously, we described an efficient and selective liver targeting siRNA delivery system termed Dynamic-Polyconjugates (DPC) (28,29). This delivery approach included the use of a membrane active polymer that is reversibly
modified with shielding and targeting moieties to provide highly selective target tissue delivery of siRNA (28,29). For liver targeting, DPC uses N-acetylgalactosamine as the ligand to target asialoglycoprotein receptors (ASGPr) that are abundantly expressed by hepatocytes (30). The incorporation of a targeting ligand, and the reversible masking of a membrane active polymer allowed efficient target cell uptake and subsequent RNAi trigger escape from endosomes (28,29). The modular nature of DPCs allows for exchange of targeting moiety and RNAi trigger for a specific tissue and therapeutic gene of interest. In this report, we describe an RNAi trigger delivery system that preferentially binds to integrin receptors αvβ3 and αvβ5 commonly overexpressed in tumor cells, including ccRCC (31-33). We demonstrate the targeted delivery of RNAi mediated HIF2α gene silencing and the resulting proof-of-concept effect on tumor growth inhibition and degeneration in an established orthotopic ccRCC tumor xenograft model.

Materials and Methods

Cell culture

A498 (2012), 786-O (2014), Caki-1 (2014), Caki-2 (2015), and Hep3B (2009) cells were purchased directly from ATCC, the year when the cell line was acquired is provided in parenthesis. Cells were maintained at 37°C in a humidified atmosphere containing 5% CO₂. Cells were sub-cultured every 3 to 4 days and passaged no more than 20 times before use. Mycoplasma testing was conducted every few months using MycoProbe® Mycoplasma Detection kit (R&D Systems, CUL001B). The A498 and Hep3B cells were cultured in Minimum Essential Media (Cellgro) supplemented with 10% fetal bovine serum (FBS) (Fisher Scientifics) and 1% non-essential amino acids
solution (Gibco). Caki-1 and Caki-2 cells were cultured in McCoy’s 5A medium (Cellgro) supplemented with 10% FBS. 786-O cells were cultured in RPMI medium (Cellgro) supplemented with 10% FBS.

**In vitro RNAi trigger set**

*In silico* analysis of potential specific RNAi triggers cross-reactive to human EPAS1 (accession #NM_001430.4) and NHP transcripts was performed by Axolabs (Kulmbach, Germany). After applying appropriate specificity filters to the sequences, a screen set of 187 RNAi triggers was synthesized using standard phosphoramidite chemistry. The siRNAs contained specific modifications at the 3’ and 5’ ends and 2′-O-methyl/2′flouro-modified nucleotides to protect against nucleases and reduce potential innate immune responses.

**In vitro RNAi trigger screening**

For screening purposes, the human EPAS1 (HIF2α) cDNA sequence (accession #NM_001430.4) was synthesized and cloned (DNA 2.0, Menlo Park, CA) into a commercially-available reporter-based screening plasmid, psiCHECK2 (Promega, C8021) which generated a Renilla/luciferase/EPAS1 fusion mRNA. Hep3B cells were plated at ~10,000 cells per well in 96-well format. Each of the 187 EPAS1 RNAi triggers, was co-transfected at two concentrations, 1 nM and 0.1 nM, with 25 ng of EPAS1-psiCHECK2 plasmid and 0.2 µL LipoFectamine 2000 (Life Technologies) per well. Gene knockdown was determined by measuring Renilla luciferase activities normalized to the
levels of constitutively-expressed firefly luciferase using the Dual Luciferase Reporter Assay (Promega, E1910).

**Candidate RNAi triggers for DPC conjugation**

For the RNA triggers used for *in vivo* testing, the sense strand was synthesized with a 5’ primary amine containing phosphoramidite for attaching click chemistry alkyne functional group for polymer conjugation. Following standard cleavage and deprotection, the sense strand was dissolved in sodium acetate solution (1.2 M) and precipitated with seven volume equivalents of ethanol. The precipitate was then dissolved in sodium bicarbonate solution (0.1M). Six equivalents of the disulfide containing dibezocyclooctyne-methyl-S-S-N-hydroxsuccinimidyl ester (DBCO-Me-S-S-NHS) reagent (see below) dissolved in dimethylformamide (DMF, 10 mg/mL) was added. The reaction progress was monitored by RP-HPLC. Once the reaction was complete, the solution was precipitated with ethanol and purified using RP-HPLC.

**Small molecule synthesis**

Protease masking reagent PEG\(_{12}\)-L-analine-L-citrulline-p-aminobenzyl alcohol p-nitrophenyl carbonate (PEG\(_{12}\)-ACit-PABC-PNP, PEG\(_{12}\) refers to a discrete 12-mer of PEG) was synthesized as previously described (28). RGD or RGE mimetic conjugated-PEG masking reagents, RGD-PEG\(_{20}\)-FCitFP-TFP or RGE-PEG\(_{20}\)-FCitFP-TFP (PEG\(_{20}\) is a discrete 20-mer PEG, FCitFP refers to the amino acid sequence phenylalanine-citrulline-phenylalanine-proline, TFP (tetrafluorophenyl ester), were synthesized using standard amide coupling reagents as previously described (34,35). RGD or RGE
mimetics are arginine-glycine-aspartic acid or glutamic acid mimics, respectively (34). The copper free click chemistry reagent DBCO-Me-S-S NHS was synthesized as previously described (36). See Supplementary Data for structures.

**Competitive solid phase ligand binding assay**

The binding constant of the RGD mimetic was evaluated using an ELISA based competitive assay using recombinant human integrin αvβ3, αvβ5 and αvβ6 (see Supplementary Methods for details).

**RAFT copolymer of N-Boc-ethoxyethylamine acrylate and propyl acrylate (EAP)**

Polymer EAP synthesis is similar as described for N-Boc-ethoxyethylamine acrylate and sec-butyl acrylate (EAB) (28). For polymer EAP, propyl acrylate was used instead of sec-butyl acrylate. Additional details are provided in Supplementary Data.

**Assembly and characterization of RGD-DPC**

RGD-PEG and PEG only masking reagents were added to azide-functionalized EAP polymer in 5 mM pH 8.0 HEPES buffer and incubated at room temp. Masked polymer was then mixed with alkyne-functionalized RNAi trigger at a weight ratio of 1:0.3, respectively. Trigger conjugated RGD-DPC was then purified with tangential flow filtration and characterized. Further details on polymer functionalization, assembly and analytical methods used for characterizations are provided in Supplementary Data.
RGD-Cy3-DPC binding and internalization in cultured cells

Cy3-NHS ester (GE Healthcare) was coupled to polymer following the manufacturer's recommendations. Cells (100,000) plated on cover glass were incubated with 2.5 µg/ml of Cy3-labeled DPC polymer that was modified with PEG12-ACit-PABC and RGD-PEG_{20}-FCitFP-TFP or PEG_{12}-ACit-PABC alone for 24h in complete media. No RNAi triggers were included in these imaging DPCs. Cells were fixed in 10% formalin and counterstained with 20 nM Alexa Fluor® 488-phalloidin (A12379, Invitrogen) and 40 nM TO-PRO®-3 (T3605, Invitrogen) in PBS for 30 min. Cover glass was then mounted onto slides with Vectashield mounting medium (H-1000, Vector Laboratories) and evaluated by confocal microscopy (LSM710, Carl Zeiss).

Orthotopic ccRCC xenografts with A498 cells stably expressing SEAP

Female athymic nude mice 6-8 weeks old were obtained from Envigo Bioproducts Inc. (Madison, WI). All animal studies followed procedures approved by the Institutional Animal Care and Use Committee at Arrowhead Pharmaceuticals. The mice were housed in individually ventilated cages (Super Mouse 1800™, Lab Products) with access to food and water *ad libitum*. For tumor implantation, a 10 µl aliquot of SEAP-expressing A498 cell/Matrigel mixture containing about 400,000 cells (2:1 vol:vol of cell:Matrigel, Corning, cat# 354248), using a 27-gauge needle catheter was injected into the left kidney subcapsular space using a syringe pump. Serum was collected every 7-14 days after implantation to monitor tumor growth using a commercially available SEAP assay kit (Life Technologies, T1016). For most studies, tumor-bearing mice were
used 5-6 weeks after implantation, when tumors were typically 4-8 mm in length and width.

**RGD-DPC binding and internalization in tumor bearing mice**

Cy3-RGD-DPC prepared with Cy3-labeled polymer (100 µg) as described above was injected via the tail vein into tumor bearing mice. Tumor and other organs were harvested 4 h post-injection and frozen tissue sections were prepared as previously described (29). Tissue sections were counterstained and analyzed as described above for *in vitro* binding studies.

**In vivo efficacy studies**

HIF2α-RGD-DPC was injected via the tail vein into A498-SEAP tumor bearing mice. Dosage was calculated based on the amount of polymer before conjugation to masking reagents and siRNA. In multi-dosing studies, serum was collected weekly for SEAP and clinical chemistry evaluations. Clinical chemistry was analyzed using a Cobas® Integra 400 Plus clinical analyzer (Roche Diagnostics). Tumors were excised at the end of the study and total RNA extracted using TriReagent (Molecular Research Center). Caliper measurements of tumor length and width (shorter measurement) were recorded, and in some instances tumor gross morphology was photographed. Tumor volume was calculated using the formula for a hemi-ellipsoid ($\pi/6 \times L \times W \times D$), where tumor width was used as an estimate for depth (D). Tumor weight was determined by subtracting the weight of the contralateral kidney from the kidney weight implanted with tumor.
Tumor gene expression assays

Approximately 500 ng RNA was reverse-transcribed using the High Capacity cDNA Reverse Transcription Kit (Life Technologies). Quantitative PCR was performed by using a 7500 Fast or StepOnePlus Real-Time PCR system (Life Technologies). The ΔΔCT method was used to calculate relative gene expression. Primer sets used are described in Supplementary Data.

Statistical analysis

Significance of relative mRNA expression between treatment and control groups was determined using a Student’s t test. Significance of changes in tumor volume or weight was determined using a Mann-Whitney U test. Data were analyzed using GraphPad Prism.

Additional methods

Flow Cytometry, Immunohistochemistry, RGD-DPC plasma clearance, and creation of SEAP-expressing A498 cells are provided in Supplementary Data.

Results

Functional components and characteristics of RGD-DPC

The DPC delivery system described in the current study utilized protease cleavable masking reagents similar to those previously described (28). The protease cleavable masking reagents were developed to provide the DPC with longer systemic circulation time compared to the pH-labile maleic anhydride (CDM) masking chemistry.
used in first generation DPC (29,37). For targeting tissue outside of the liver, longer circulation times are necessary for targeting receptors that are likely less abundant and potentially less efficient than the Ashwell-ASGPr expressed on hepatocytes (30,38). The exploitation of RGD-binding integrins frequently overexpressed in cancer but more restrictive in normal tissue has been explored by others as a delivery tool, as well as an integrin antagonist in cancer therapy (31,39,40). The current construct, RGD-DPC, targets integrin receptors αvβ3 and αvβ5 using a peptidomimetic RGD molecule (34). This RGD mimetic bound to both αvβ3 and αvβ5 with high affinity and selectivity, with IC$_{50}$ values of 1.3 ± 0.4 nM (n=4) and 4.9 ± 2.3 nM (n=3), respectively, evaluated using an ELISA based competitive binding assay (Supplementary Fig. S1). In contrast, binding to the related integrin αvβ6 had an IC$_{50}$ value of 116 ± 4.9 nM (n=3). Like the NAG-DPC previously described (28), RGD-DPC comprises an ethoxyethylamine acrylate polymer modified with a mixture of protease cleavable PEG masking reagents with or without the ligand conjugated. The optimal number of RGD mimetics per polymer was determined empirically as 10-20 using in vitro cellular uptake, in vivo tumor uptake and gene knockdown as readouts. Upon unmasking, the polymer is expected to perturb endosomal membranes to facilitate cytoplasmic delivery. The trigger was attached to the polymer using a hindered-disulfide linker (41) to confer significant serum and extracellular compartment stability while enabling efficient dissociation from the delivery vehicle in the tumor cell cytoplasm. Using Cy5-labeled RNAi trigger to measure the intact conjugate, the serum half-life of RGD-DPC was 2 hr, while unconjugated RNAi trigger was <5 min. The serum half-life for PEGylated DPC (without RGD) was 12 hr. The shorter serum half-life for RGD-DPC supports an RGD-mediated cellular uptake
mechanism. The nanoparticles are ~20 nm in size and with a near neutral zeta potential, features that are essential for tumor extravasation and to minimize non-specific cellular binding (42,43). A summary of the physical characteristics of the RGD-DPC is summarized (Supplementary Table S1). A schematic of the DPC nanoparticle is shown in Fig 1.

Expression of integrins αvβ3 and αvβ5 and RGD-DPC internalization

To evaluate the utility of integrin receptors as an effective means to deliver RNAi therapeutics, the cell-surface expression profile of integrins αvβ3 and αvβ5 were evaluated on four commercially available RCC cell lines, A498, 786-O, Caki-1 and Caki-2, by flow cytometry. The expression of αvβ3 and αvβ5 tabulated as specific mean fluorescence intensity (sMFI) is summarized in Table 1. The expression of αvβ3 is several-fold higher in A498 and Caki-2 cells compared to 786-O and Caki-1. In contrast, the expression of αvβ5 was similar between the four cell lines. Binding and internalization of Cy3-RGD-DPC was strongest in A498 and Caki-2 cells, consistent with their relatively high expression levels of αvβ3 (Fig. 2). These results suggest that RGD-DPC exhibits a preference for αvβ3 in vitro. However, as the RGD mimetic shows high affinity to both receptors, this may reflect the combined abundance of both receptors rather than preference of αvβ3 versus αvβ5 integrin. Further demonstration of RGD-dependent binding was observed with a DPC without ligand or a DPC with RGE-control ligand showing background fluorescent signals (Supplementary Fig. S2A). Consistent with an endocytic uptake mechanism, after an overnight incubation, approximately 80%
of the internalized RGD-DPC in A498 cells was localized to lysosome marker LAMP-1 positive vesicles (Supplementary Fig. S2B).

To evaluate the potential usage of this delivery approach, the expression of integrin αvβ3 in a commercially available renal cancer tissue microarray was profiled (CT565907, OriGene Technologies). Among the samples showing histological morphology consistent with ccRCC (25/50), approximately 80% (20/25) showed good (medium to strong) β3 membrane staining, and 9 of these (36% of all ccRCC samples) showed strong β3 expression, similar in staining intensity as A498 cells derived tumors (Supplementary Fig. S3). These observations are consistent with those reported in the literature (32,33). The selectivity of the β3 antibody was validated using tumor tissue sections derived from cell lines known to express or lack β3 as determined by flow cytometry. As integrin β3 is known to pair with only αv or αIIb (platelet specific) subunits (44), this should provide a good representation of tumor αvβ3 staining.

**In vivo DPC uptake in established orthotopic xenografts**

While *in vitro* validation of ligand binding provided specificity, efficient *in vivo* delivery to established tumors is an obligatory step for a successful model and ultimately an effective therapeutic. To evaluate *in vivo* delivery to established tumors, 100 µg of Cy3-RGD-polymer was injected intravenously via the tail vein into tumor bearing mice established with either A498, 786-O, Caki-2 or Caki-1 cells in the left kidney sub-capsule. Delivery of Cy3-RGD-DPC to tumor was evaluated by confocal microscopy examination of frozen tissue sections. The efficiency of uptake and internalization to tumor parenchymal cells showed a correlation with the cell lines
expressing higher levels of αvβ3 integrin, A498 (Fig. 2A, 2E) and Caki-2 (Fig. 2C, 2G). A498 tumors exhibited morphology typical of ccRCC whereas Caki-2 showed morphology consistent with papillary renal carcinoma, as reported (45). RGD-polymer with no ligand or RGE control ligand showed no tumor uptake in A498 tumor cells (Supplementary Fig. S2 Panel A, bottom row).

Differences in the tumor architecture of these tumors may also have influenced effective delivery to cancer cells. The parenchymal cancer cells in 786-O and Caki-1 tumors were heavily surrounded by a layer of stromal myofibrocytes (strongly stained by Alexa 488-phalloidin) that potentially form a more restrictive microenvironment that hinders efficiency of RGD-DPC delivery (Fig. 2F and 2H).

**Selection of HIF2α RNAi trigger**

In addition to an efficient delivery vehicle, a potent and specific RNAi trigger is critical to an effective RNAi therapeutic. To identify a lead therapeutic candidate, 187 candidate RNAi trigger sequences highly specific to human EPAS1 (HIF2α), cross-reacting with rhesus and cynomolgus non-human primate sequences, and exhibiting minimal potential off-target hybridization to non-target genes, including HIF1α, and seed regions of microRNAs were selected by *in silico* analysis amongst 5,166 potential human-specific EPAS1 RNAi triggers. Sequences containing known single nucleotide polymorphisms directed against target sites were excluded from the screen set. The candidate sequences were synthesized with 2’-O-methyl/2’-fluoro-modified nucleotides to provide nuclease resistance, and to abrogate potential immune activation (46). The gene inhibition potency of the RNAi trigger screen set was evaluated by *in vitro*
transfection. The relative potency of the siRNAs was ranked using a waterfall plot. Additional sequence selection information is available in our published patent application (35). Five of the most potent sequences were then further evaluated in a gene silencing study in tumor-bearing mice (Supplementary Fig. S4). The most potent trigger (sequence ID T4) was selected for further evaluation and used in subsequent animal studies.

**Ligand dependent dose response and duration of HIF2α gene silencing in A498 tumor bearing mice**

HIF2α-conjugated DPC modified with or without RGD was injected into A498 tumor bearing mice (n = 3 per group) via the tail vein at 5, 10 and 15 mg/kg. Animals were enrolled into the efficacy study 4-5 weeks after tumor establishment (confirmed by serum SEAP levels). Serum SEAP levels were used to randomize animal group assignment. HIF2α mRNA expression relative to mice receiving delivery buffer alone was determined on study day 5 (study day 1 = injection day). Dose-dependent HIF2α gene silencing was observed in the mice receiving DPC with the RGD mimetic targeting ligand, whereas DPC without RGD was ineffective (Fig. 3). At the highest dose administered, an 87 ± 2.5% reduction in relative HIF2α mRNA expression level was observed.

To determine the best dosing regimen for multi-dose studies, the duration of HIF2α gene silencing after a single injection of 15 mg/kg (polymer) of RGD-DPC was evaluated on study days 4, 8, 11, and 15 (day 1 = injection day). Each time point represented a separate group of animals (n = 3). Tumor samples were collected at
each time point and relative gene expression was batch analyzed at the end of the study. The results showed substantial gene silencing on study day 4 (78 ± 7.1%, Fig. 4A), and maximal gene silencing (89 ± 2.1% reduced) on study day 8. Maximum gene silencing was sustained until study day 11. By study day 15, recovery of HIF2α expression began but remained significantly reduced by 79 ± 7.7%. As a tool to monitor tumor growth, serum SEAP levels were evaluated and were lower in the RGD-DPC treated group (Supplementary Fig. S5), suggesting an effect on growth after a single injection. In parallel to HIF2α mRNA expression, recovery of SEAP expression was apparent at the end of the duration study (Supplementary Fig. S5). The effect on tumor growth was further substantiated by the overall smaller, although not statistically significant, tumors observed on day 15 compared to those of the control treated group after excising and weighing (Fig. 4B). Furthermore, histological examination showed evidence of tumor degeneration and the presence of blood-filled cavities surrounding areas of collapsing tumor integrity (Fig. 4C). Maximal anti-tumor effects were observed during the time points corresponding to peak HIF2α gene silencing.

**HIF2α-RGD-DPC multi-dosing study in A498-SEAP tumor bearing mice**

A multi-dosing study was conducted to further assess the effect of HIF-2α gene silencing on tumor growth. Based on the observation that HIF2α gene silencing after a single dose of RGD-DPC injection was sustained for up to 14 days, a multi-dosing study in which 4 bi-weekly doses of 10 mg/kg RGD-DPC was conducted. A submaximal efficacious dose of 10 mg/kg was chosen to allow for better evaluation of potential additive effects of repeat dosing. Four mice per treatment group were randomized
based on pre-treatment SEAP expression. Serum SEAP levels were determined pre-dose and throughout the course of treatment to indirectly evaluate tumor growth. Potential adverse effects of repeat dosing were monitored by daily general physical health check, weekly clinical chemistry evaluations and body weight measurements. One week after the 4th and final RGD-DPC injection, contralateral and tumor-bearing kidneys were excised, photographed, examined for gross morphology and tumor measurements were recorded. Baseline tumor volume was obtained by collecting tumor measurements from a similar cohort of animals on the day treatment was initiated for reference. Tumor tissues were then processed for gene expression and histology evaluations.

Upon excision of kidney tumors on day 49 and study completion, dramatic differences in tumor sizes were apparent by visual examination of gross tumor morphology (Fig. 5A). Animals that were injected with delivery buffer D5W showed significant tumor growth. In contrast, calculated tumor volumes from the RGD-DPC treated group were not significantly different as compared to baseline pre-treatment tumor volume, suggesting highly effective tumor growth inhibition over the course of RGD-DPC treatment (Fig. 5B). Tumor growth inhibition over the course of treatment was also apparent based on relatively unchanged serum SEAP levels (Fig. 5C). Relative HIF2α mRNA expression was 85 ± 7% reduced compared to animals injected with D5W (Fig. 5D). The expression of several known HIF2α regulated genes, VEGFa, Glut1, and EGLN3 were also reduced by 47 ± 10.6%, 42 ± 18.6% and 49 ± 6.4%, respectively. The expression of the tumor suppressor gene p53 was elevated by 28 ± 9% (Fig. 5D). In a separate study, the effects of RGD-DPC containing a control RNAi
trigger (luciferase) were evaluated indicating no significant effects on tumor growth or HIF2α mRNA expression.

Histological examination further revealed widespread tumor structural degeneration in the HIF2α-RGD-DPC treated group. Tumor degeneration was associated with blood-filled cavities where CD31 positive tumor vasculature was significantly reduced, indicative of vascular collapse (Fig. 5E), a phenotype potentially a result of the reduction in tumor VEGFa expression, secondary to HIF2α gene silencing. However, given the histological evidence of general destruction of tumor architecture showing significant tumor degeneration, the overall effects on tumor growth is profound and not fully characterized by evaluating tumor volume alone. The blood-filled cavities are histologically distinct from spontaneous tumor necrosis which is often observed in overgrown untreated tumors. These features likely result from down-regulation of HIF2α-regulated tumor growth promoting genes (Fig. 5D), leading to cell death and disruption of tumor integrity. Importantly, the repeat dosing regimen was well-tolerated, as no significant changes were observed in either body weight or major clinical chemistry parameters including AST, ALT, BUN, creatinine and total bilirubin (Supplementary Table S2). No significant histological findings were observed in the contralateral kidney, liver or spleen.

Discussion

Development of drug resistance, particularly to small molecule drugs, are frequent challenges in cancer therapy. The current standard-of-care treatments for metastatic ccRCC have not escaped from this fate. The high frequency of VHL loss of
function mutation in ccRCC and the subsequent over-expression of HIF2α has long been an attractive therapeutic target (16). In general, transcription factors such as HIF2α have generally been considered undruggable therapeutic targets (47). Recently, several reports described the anti-tumor effects of a small molecule HIF2α antagonist that binds to a hydrophobic binding pocket discovered in HIF2α PAS-B domain (16,20-22). While these studies showed encouraging results that validated HIF2α as a therapeutic target, certain limitations are apparent. These included pre-existing drug resistant mutations, post-treatment mutations discovered surrounding the binding pocket of this small molecule, and the dependence of some tumors on cellular pathways other than VHL-HIF (20-22).

The modular nature of the RGD-DPC delivery system allows each of the individual components to be independently optimized to maximize therapeutic potential. In addition, the receptor/ligand pair or the target gene can be substituted when appropriate. The RGD mimetic used in the current study was designed to target integrins overexpressed in some ccRCC patients (32,33). We showed that the efficiency of RGD-DPC delivery to various tumor cells correlated with overall abundance of RGD-binding integrin expression, particularly αvβ3. The RGD mimetic is necessary for efficient tumor delivery of the DPC. Our results indicate that RGD-binding integrin expression in tumor cells facilitates efficient delivery of RNAi triggers likely through receptor-mediated internalization. Further, expression of αvβ3 was reported to correlate with tumor progression and metastasis (32,48), a patient population where alternate treatment over currently available therapies is needed. The RNAi trigger therapeutic
payload may be specific to one or more potential target genes. Alternate effective RNAi sequences of the same target gene sequence can be readily substituted, if necessary.

The Caki-2 cell line is characterized as a papillary renal cancer with no VHL mutation that expresses both HIF1α and HIF2α (45,49) Although the contribution of HIF in tumor progression in this subtype of renal cancer is unclear, 2 weekly doses of 10 mg/kg of HIF2α-RGD-DPC induced signs of tumor degeneration and apoptosis (unpublished results). HIF2α expression was inhibited ~70%, but no effect was observed on HIF1α expression, validating the specificity of the HIF2α trigger.

The RNAi delivery system described in the current report provided a proof-of-concept approach to an alternative therapeutic modality. Questions such as maximum tolerated dose, biodegradability and toxicity upon repeat dosing, need to be assessed before this technology advances to the clinic. Despite significant efforts to reduce the immunomodulatory potential of the HIF2α-RGD-DPC therapeutic, evaluation of possible cytokine induction, complement activation and other immune reactions warrants attention (46,50). A successful first-in-class RNAi therapeutic for ccRCC will provide a much-needed alternative treatment choice, not only as a potential monotherapy, but more attractively in combination with current treatment options. Tumor targeting by HIF2α inhibition may augment immune-checkpoint control therapies, metabolic regulators or other therapeutics that elicit orthogonal mechanisms of action.
Acknowledgements:

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References


Table 1. Relative expression of integrins αvβ3 and αvβ5 analyzed by flow cytometry. Values of specific mean fluorescence intensity (sMFI) were calculated by dividing MFI values from cells stained with antibody by MFI values from unstained cells alone.

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Figure Legends

**Figure 1.** Schematic illustration of RGD-DPC. Polymer EAP (green curve with amine side chains presented as green bars and alkyl groups depicted as purple bars). The amine side chains of EAP are modified with PEG (gray squiggles) or integrin-targeting ligand RGD (orange spheres) via protease-labile bonds (blue spheres). RNAi trigger is attached to the polymer with a linker containing a hindered-disulfide bond (two magenta spheres) as depicted on the end of the polymer. This illustration is used with permission from Rozema et. al. (28).

**Figure 2.** *In vitro* and *in vivo* cellular uptake of RGD-DPC. **A-D,** RGD-DPC internalization in cultured cells. Indicated renal cancer cell lines cultured on a cover glass were incubated with RGD-DPC formulated using Cy3-labeled polymer (red) in complete media at 2.5 µg/ml for 24 h. Cell were fixed and counterstained with Alexa Fluor® 488-phalloidin (20 nM) for actin (green) and TO-PRO®-3 (40 nM) for cell nuclei (blue). Cover glass mounted slides were analyzed using a LSM710 confocal microscope. Confocal micrographs depicting a single 0.5 µm optical section are shown (magnification 630X). **E-H,** RGD-DCP internalization in tumors implanted in mouse kidneys. Indicated cell lines were implanted into kidney subcapsule. RGD-DPC containing 100 µg of Cy3-labeled polymer (red) was injected via the tail vein. Tumors were excised 4h after injection and frozen tissue sections were prepared as described (29). Tissue sections were counterstained and analyzed as described above. Scale bars for A-D and E-H are 20 µm.
Figure 3. Ligand dependent dose titration in A498-SEAP orthotopic tumor bearing mice. Relative HIF2α mRNA expression on day 5 (day 1 = injection day) compared to control (5% dextrose in water, D5W) measured by qRT-PCR in animals receiving 5, 10 or 15 mg/kg of DPC (base polymer) with or without ligand, accordingly ~1.5, 3 or 5 mg/kg of siRNA, respectively, was dosed. Relative expression shown were normalized geometric means ± SD.

Figure 4. Duration of mRNA knockdown and effects on tumor growth. A, tumor bearing mice injected with a single dose of 15 mg/kg of RGD-DPC (polymer; 5 mg/kg siRNA equivalent) were evaluated for HIF2α mRNA expression over time. Relative expression normalized to mice injected with D5W were used as control. Values shown were normalized geometric means ± SD. Separate groups of mice (n = 3) were used for each time point. D5W, dextrose 5% in water, * P < 0.05. B, effects on tumor weight, mean ± SD. Day 15 tumor weights were not statistically significantly different (NS). C, tumor histology by H&E staining on day 4, 8, 11 and 15 (day 1 = injection day). Scale bars = 100 µm.

Figure 5. Multi-dose tumor growth inhibition study in orthotopic A498-SEAP tumor bearing mice. Mice were injected every 2 weeks with either 10 mg/kg RGD-DPC or D5W. A total of 4 doses were administered to 4 mice per group. A cohort of naïve tumor bearing mice was terminated on day 1 (start of study) as baseline reference. Study was terminated on day 49, 1 week after final injection. A, gross tumor morphologies. From each mouse, the kidney implanted with tumor cells is shown on the left and the contralateral kidney without tumor cell implantation is shown on the
B, tumor volumes calculated with caliper measurements; * P < 0.05. C, SEAP expression during treatment. The SEAP reporter protein expression was used to monitor tumor growth. Serum was collected weekly. Fold-changes relative to pre-dose levels of each animal are shown. D, HIF2α and HIF2α-regulated VEGF-A, GLUT-1, EGLN3 and p53 mRNA expression. Normalized geometric mean ± SD are shown. * P < 0.05. E, tumor histology (H&E) and CD31 staining. Representative images are shown. Scale bar for H&E = 100 µm, CD31 staining = 50 µm.
Figure 1

- **PEG**
- **Disulfide bond**
- **Protease cleavable bond**
- **Polymer**
- **RNAi trigger**
- **Ligand**
Figure 3

![Graph showing relative expression levels for different conditions.](image-url)

- **D5W**
- **5 mg/kg**
- **10 mg/kg**
- **15 mg/kg**

**No ligand**

- **5 mg/kg**
- **10 mg/kg**
- **15 mg/kg**

**With ligand**
Figure 5

A

Day 1 No Rx

Day 49 D5W

Day 49 RGD-DPC

B

Tumor volume (mm³)

Day 1 baseline

Day 49 D5W

Day 49 RGD-DPC

C

Relative change in SEAP expression (fold)

Study Day

D5W-1

D5W-2

D5W-3

D5W-4

DPC-1

DPC-2

DPC-3

DPC-4

D

Relative Expression

D5W

RGD-DPC

E

H&E

CD31

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Molecular Cancer Therapeutics

HIF2α targeted RNAi therapeutic inhibits clear cell renal cell carcinoma

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