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Therapeutic efficacy of a synthetic epsin mimetic peptide in glioma tumor model: uncovering multiple mechanisms beyond the VEGF-associated tumor angiogenesis

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Abstract

Binding of epsin ubiquitin-interacting motif (UIM) with ubiquitylated VEGFR2 is a critical mechanism for epsin-dependent VEGFR2 endocytosis and physiological angiogenesis. Deletion of epsins in vessel endothelium produces uncontrolled tumor angiogenesis and retards tumor growth in animal models. The aim of this study is to test the therapeutic efficacy and targeting specificity of a chemically-synthesized peptide, UPI, which compete for epsin binding sites in VEGFR2 and potentially inhibits Epsin-VEGFR2 interaction *in vivo*, in an attempt to reproduce an epsin-deficient phenotype in tumor angiogenesis. Our data show that UPI treatment significantly inhibits and shrinks tumor growth in GL261 glioma tumor model. UPI peptide specifically targets VEGFR2 signaling pathway revealed by genetic and biochemical approaches. Furthermore, we demonstrated that UPI peptide treatment caused serious thrombosis in tumor vessels and damages tumor cells after a long-term UPI peptide administration. Besides, we revealed that UPI peptides were unexpectedly targeted cancer cells and induced apoptosis. We conclude that UPI peptide is a potent inhibitor to glioma tumor growth through specific targeting of VEGFR2 signaling in the tumor vasculature and cancer cells, which may offer a potentially novel treatment for cancer patients who are resistant to current anti-VEGF therapies.

Keywords

Epsin; Tumor angiogenesis; VEGFR2 signaling; Glioma tumor therapy; Mimetic peptide; UPI

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DISCLOSURE

The authors declare that they have no conflict of interests to disclose.

INTRODUCTION

Tumor angiogenesis is the process by which new blood vessels form from pre-existing vessels and a vital step in changing benign tumors into malignant ones through endothelial cells (ECs) [1–5]. Angiogenesis, under normal circumstances, is an adaptive quality of mammalian bodies to be able to self-repair through wound healing and growth of cellular tissues. However, tumorigenesis hijacks this process into routing nutrients to the growing tumor [1, 6]. Although there are numerous growth factors (EGF, PDGF, VEGF, TGF- β , etc.) and subsequent pathways which, when stimulated, cause angiogenesis to occur, we chose to focus on Vascular Endothelial Growth Factor (VEGF) and its subsequent receptor site Vascular Endothelial Growth Factor Receptor 2 (VEGFR2) because VEGF has been demonstrated, in previous literature, to be the most important receptor pathway in vascular injuries, as well as the vasculature itself [7, 8]. In 1971, Dr. Judah Folkman proposed an idea to block tumor growth by inhibiting tumor angiogenesis [1]. Since then, targeting tumor angiogenesis has becoming a powerful approach in developing cancer therapeutics [4, 9–21].

Mechanism-wise, VEGF begins the angiogenic process by first causing a signaling cascade through binding and interactions with VEGFR2. This interaction initiates a tyrosine signaling cascade, leading to vessel permeability, proliferation, migration, and differentiation into mature blood vessels [22]. In essence, tumor metastasis is able to be stimulated through the blood vessels themselves into creating more functional vessels. The tyrosine kinase receptor is dependent on epsin, a family of endocytotic proteins which emerges in two forms in mammalian vertebrates: epsin1 and epsin2 [23, 24]. Epsin is one of the key players in clathrin-mediated endocytosis [24]. Ubiquitin interacting motifs (UIMs) located at the downstream of ENTH domain in the N-terminal of the protein help to promote the interaction between epsin and VEGFR2, thus promoting endocytosis [25]. UIM is a 18-sequence motif that is able to specifically detect and bind to ubiquitylated VEGFR2 through specific residues on UIM and VEGFR2 respectively [26, 27]. After binding to the ubiquitylated VEGFR2, it acts as a type of cargo carrier, which promotes epsin binding [26, 27]. Epsin binds to the activated VEGFR2 and degrades it, which eventually causes VEGFR2 to break down into endosomes and lysosomes, leaving only parts of VEGFR2 remaining. This process significantly decreases VEGFR2 and VEGF signaling and produces functional tumor vessels through normal angiogenesis.

Previous literature have suggested that if epsin1 and epsin2 are disrupted or inhibited, then non-functional vessels are created, i.e. nonproductive angiogenesis, which cannot sustain further tumor growth and proliferation [24]. In our study, we use UPI (containing UIM, Plasma Membrane Anchoring Peptide, and iRGD) a chemically synthesized peptide, which competes for binding locations on VEGFR2 [26]. Due to the competitive nature of UPI from its high affinity of specific targeting and binding to VEGFR2, this process leaves excess epsin in the cytosol, which mimics an induced double knockout (iDKO), or the epsin-deficient condition [26]. With an inhibited epsin-VEGFR2 interaction *in vivo*, we hypothesized that tumorigenesis would significantly decrease because the upregulated, but dysfunctional and tangled vessels, could no longer maintain tumor growth. These processes and interactions could potentially present an alternative and novel treatment option for patients who are resistant to anti-VEGF therapies.

MATERIALS AND METHODS

Antibodies, reagents and chemicals

Epsin1 antibody (goat, cat# sc-8673) was purchased from Santa Cruz Biotechnology Inc. (Santa Cruz, CA), Epsin2 antibody was home-made and approved specific. Anti-VEGFR2 (cat# 2479), and anti-PDGFR- β (cat# 3175) antibodies were purchased from Cell Signaling Technology (Danvers, MA). Anti-FGFR1 (cat# ab823) antibody was purchased from Abcam (San Francisco, CA). Anti-EGFR antibody (cat# 600-401-905) was purchased from Rockland Immunochemicals (Pottstown, PA). VEGF-A, FGF and PDGF were purchased from R&D systems (Minneapolis, MN). 4-hydroxytamoxifen was purchased from Sigma (St. Louis, MO). Anti-CD31 (cat# 550274) antibody and matrigel were purchased from BD Biosciences (San Jose, CA). Hoechst stain was bought from Sigma (Cat# B-2883). All other chemicals were purchased from Sigma or elsewhere. Anti-VEGF antibody (Clone B20-4.1.1) was a kind gift from Genentech (South San Francisco, California).

Animals

Mice were raised in the Live Animal Research Center (LARC) in the Oklahoma Medical Research Foundation (OMRF) and were either C57BL/6 wild types or genetically-modified with either one copy of VEGFR2 and induced knocked out epsin using tamoxifen (denoted EC-iDKO-Flk), or two copies of VEGFR2 and induced knocked out epsin (denoted EC-iDKO). Wild type (WT) mice had both copies of VEGFR2, and also had both epsins. All animal protocols were approved by the IACUC committee in OMRF.

Peptide synthesis

Peptides were synthesized by Fmoc solid phase. Briefly, 2-Chlorotrityl Chloride resin (1.05 mMole/g substitutions) was allowed to swell in DCM for 30 min, then the amino acid coupling was performed. During this process, the generation and consumption of free primary amine was monitored through ninhydrin test. The deprotection and coupling sequence was repeated until the desired length of peptide was maintained, then the conjugate was cleaved using a cleavage mixture (95% TFA in the presence of TIS and EDT) for 2 hours at room temperature. The cleaved product was precipitated using cold ether. The crude product was then dissolved in a certain proportion of H₂O and ACN. The crude sequence was qualitatively analyzed by 220nm on HPLC system using a Waters C-18 reverse phase column. The crude sequence was in a H₂O:ACN gradient with ACN starting at a reasonable gradient. The purified fractions were collected and masses were confirmed using an API150-ESI mass spectrometry system. The products were further HPLC purified and lyophilized. Peptides were dissolved in purified H₂O. Endotoxin of synthetic peptides (EU/mg) was monitored using LAL kit (Pierce) according to manufacturer's instructions.

Establishment of glioma tumor model, peptide administration and MRI scan

10,000 Glioma tumor cells were implanted into the right forebrain of each mouse. The tumors were allowed to grow for 14 days. On day 14 post-implantation, we began to treat the mice with either control (scrambled) or UPI peptide (20 mg/kg, in 100 μ l each time by i.v) or Anti-VEGF antibody (2.5 mg/kg per injection by i.v). Genetically modified mice

(EC-iDKO, EC-iDKO-Flk) were treated with either UPI or control peptides. Treatments were administered intravenously every-other-day. The mice were anesthetized with isoflurane in the terminal. Treatments were administered every other day post-implantation. MRI scans were also taken of each mouse starting on day 14 post-implantation and continued about twice every week. Tumor volumes were collected and analyzed using ParaVision™ software from Bruker Corporation™.

Immunofluorescent staining

Tumors were fixed in 4% PFA (Paraformaldehyde) overnight (O/N), and washed with PBS four times by 15 minutes each wash. Samples were then put into 20% sucrose O/N and embedded in OCT (optimal cutting temperature compound) solution. Cryostat sections were cut in 8~10 microns. Rehydrate tissue sections with two washes of PBS, detergent permeabilization can be achieved by 10 minutes with PBS + 0.1% Triton X-100, followed by blocking with PBS+2% BSA for 45 minutes from the species in which the secondary antibodies are made (e.g. if secondary is made in donkey, use 20% donkey serum). Wash sections with PBS+0.5% BSA for 5 times. Dilute primary antibody to the desired concentration in PBS+0.5% BSA (usually starting with 1:50 up to 1:400). Gently drop ~100 µl antibody solution over the sections (make sure fully covered). Incubate for 1 hr at room temperature (RT) or leave it O/N in cold room (4°C). Initial antibody optimization should be accomplished in a range up to 5 µg/ml antibody in PBS+0.5% BSA. Primary antibodies can be added together, but the host must be from two different species. Wash sections 5 times with PBS+0.5% BSA and then add secondary antibody to the section for 60 minutes at RT. Secondaries can be added together if using more than one, but they must be against two separate species. Wash sections with PBS+0.5% BSA 5 times, followed by 5 times of wash with PBS to remove the BSA that would bind Hoechst indiscriminately. Add Hoechst stain 30 seconds (up to 5 minutes in some cases) to stain the nuclei (Hoechst stain=1 mg/100 ml dH₂O, Sigma CatNo.B-2883). Wash sections 3 times with PBS. Adhere cover glass over the samples with mounting solution, place slides horizontal in slide box, and allow cover glass to adhere to slide O/N at 4°C in the dark. Images were captured using Olympus fluorescent microscopy with a digital camera.

Hypoxia assay

Mice bearing GL261 tumors were intraperitoneal injected with the hypoxia probe, pimonidazole hydrochloride (50 mg/ml stock solution; Hypoxyprobe, Inc, Burlington, MA), at 60 mg/kg body weight for 1 hour. Mice were then sacrificed and tumors were fixed in 4% PFA and sectioned in 10 microns. Hypoxia was measured by immunofluorescence staining of tumor samples. Areas of hypoxia were quantified using Image J software.

UIM pull-down assay for UPI peptide targeting by using *ex vivo* tumors

GL261 tumor lysate was made in lysis buffer containing 50 mM Tris-HCl pH7.5, 150mM NaCl, 0.05% NP-40, 10% Glycerol, 1xcocktail (Roche) and 20mM NEM (N-ethylmaleimide). Lysate protein concentration was determined using BCA kit (Pierce). 2mg of total lysate was co-incubated with 50 µg of either biotinylated UIM peptide or control peptide, necessary for UIM to bind to ubiquitinated (activated) VEGFR2 or other receptors in the lysate. 30–40 µl Neutri-Avidin beads (Invitrogen) were then added in order to bind

onto the biotinylated UIM or control peptide. These beads were subjected to boiling and then two washes with lysis buffer and two washes with ½ lysis solutions. After each wash, the samples were centrifuged at 6000 rpm for 4 minutes each time. Afterwards, the beads were suspended for 3 minutes before the next wash cycle. The supernatant was extracted in 2x loading buffer at 95°C for 5 minutes and used for western blot for VEGFR2, PDGFR-β, EGFR and FGFR analysis.

In situ VEGFR2 monitoring in the orthotopic GL261 glioma model

Control or UPI-peptide treated GL261 glioma tumor-bearing mice were anesthetized with isoflurane, set up with a tail-vein catheter, put on a cradle, and inserted in a 7.0 Tesla small animal MRI system (Bruker Biospin). VEGFR2-targeted MRI probe (anti-VEGFR2-Gd-albumin-biotin) was administered as previously described [28]. Pre- and 90 min post-contrast MRI images were taken following administration. Intensity of VEGFR2 was quantified using Image J software.

TUNEL assay

Tumor samples were fixed in 4% PFA and sectioned to 10 microns. TUNEL assay was performed using “In Situ Cell Death Detection Kit” (Roche) according to the manufacturer’s instructions. Images were taken using Olympus fluorescent microscopy with digital camera.

Western blot

Western blot was conducted as previously described [29]. In brief, cells were washed once with cold PBS and immediately lysed in lysis buffer containing 50mM Tris-HCl, pH 6.8, 6M urea, 5% β-mercaptoethanol, 2% SDS, 0.2mM NaF, 0.1mM Na₂VO₃ and protease inhibitors (Roche). Protein concentration was determined by BCA kit (Fisher Scientific) for equal amount of total protein loading. Proteins were separated in Tris-glycine SDS-PAGE gel and transferred to 0.45 μm Nitrocellulose Blotting Membrane (GE Healthcare Life Sciences), followed by blotting with different antibodies and visualized by SuperSignal West Pico Chemiluminescent Substrate from Thermo Scientific (Cat# 34080).

Transmission electron microscopy (TEM)

Tumor processing and TEM analysis refer to our previous publication (22). In brief, tumor tissues were fixed with 3% PFA and 2% glutaraldehyde in 0.1 M cacodylate buffer, pH7.4, post-fixed in 1% osmium tetroxide and mordanted in 1% tannic acid, both in cacodylate buffer. Post-fixed tissues were dehydrated, treated with propylene oxide and embedded in epoxy resin (EMS Inc., Hatfield, PA). Ultra-thin sections (80 nm), counterstained with 1 % lead citrate and 0.5 % uranyl acetate, were examined on a Hitachi H7650 electron microscope (Hitachi High Technologies America, Inc.). Morphometry of EM was done based on at least 30 to 40 micrographs taken from random fields in each sample.

Data analysis

Data was presented by mean value with standard deviation. A student *t* test was performed to test difference between groups. *P* values less than 0.05 were considered significant. Figures were made using Prism™ software.

RESULTS

UPI peptide can be efficiently delivered to GL261 glioma tumor vasculature and produces leaky vessels

Due to the natural blood–brain barrier (BBB) in the brain, a major obstacle to drug delivery, the glioblastoma therapy remains challengeable. To test if UPI peptide can be delivered to brain tumor, in 15-day established GL261 tumors, we intravenously injected FITC-conjugated UPI peptide (FITC-UPI) at 20 mg/kg dosage to GL261 tumor mice. Four-hour post-injection, mice were sacrificed and tumors were fixed and processed for CD31 immunofluorescent staining. Our data show that FITC-conjugated UPI can be co-localized with CD31 stained vessels (Figure 1A), suggesting that UPI peptide can be efficiently delivered to brain tumors. A possible mechanism for UPI peptide to pass the BBB could be through the tumor vessels. To further confirm if UPI peptide can be taken up by tumor endothelial cells (TEC), we isolated TEC from GL261 tumors and co-incubated TEC with FITC-UPI peptide overnight (17 hours) at 50 μ M. Our data demonstrate that FITC-UPI can be highly taken up by TEC (Figure 1B), since TEC highly express integrins which facilitates the iRGD binding [30]. For the design of UPI peptide, a plasma membrane (PM) targeting peptide from Lyn kinase is conjugated for PM enrichment [26]. We want to know if our peptide can be enriched on the inside of the PM. To test this, we used confocal to detect the FITC-UPI peptide distribution inside cells. As shown in Figure 1C, the FITC-UPI peptide is highly enriched in the PM. In the next, we tested the permeability of tumor vessels. After 20-day continuing injection, we found that tumor vessel leakage is more profound as evidenced by more leakage of FITC-conjugated UPI peptide (Figure 1D and 1E).

UPI treatment significantly inhibits and shrinks tumor growth in GL261 glioma tumor model

To explore the therapeutic efficacy in glioblastoma, the GL261 glioma tumor model was established as previously reported [28]. As shown in Figure 2A & 2B, GL261 tumors grew quickly in the control group, roughly doubling every two days. However, UPI peptide injection can significantly inhibit tumor growth (Figure 2), a therapeutic efficacy comparable to the current anti-VEGF antibody, which is the best drug used in human glioma and colon cancers. Interestingly, after cease of drug administration, anti-VEGF antibody treated mice were relapsed quickly (about a week, 100% relapse, as shown in Figure 2C), however, in the UPI treated group, approximate 40% mice (2 out of 5) remains healthy, and tumors gradually disappear after 6 months (Figure 2D), while other three mice gradually died but with relative longer survival days compared to anti-VEGF antibody administration (not shown). This data suggest that UPI peptide is a potent inhibitor to tumor growth and may trigger irreversible apoptotic program of tumor cells through an unknown mechanism. It is therefore possible that UPI peptide administration can inhibit glioma tumor and recovery from glioblastoma.

UPI peptide treatment upregulates VEGFR2 and makes aberrant tumor vessels in tumor

As we have reported before, loss of epsins in the endothelium produces non-productive tumor angiogenesis [24]. After 20-day treatment, mice were sacrificed and tumors were fixed and processed for immunofluorescent staining. In terminal mice, we have identified

that UPI peptide treatment significantly increases the vessel density, accompanying with upregulates VEGFR2 expression (Figure 3C). Specifically, UPI peptide treatment makes aberrant, large diameter vessels (Figure 3A, b to e). In order to visualize the distribution of VEGFR2, we performed *in situ* VEGFR2 expression analysis by MRI image tracking approach for VEGFR2 using probe as previously reported. As illustrated in Figure 3D, UPI peptide treatment drastically elevated the expression of VEGFR2, either in tumor periphery (TP) or tumor interior (TI) compared to control peptide treated group.

UPI specifically targets to VEGFR2 in genetic modified animal models

To test whether UPI specifically targets to VEGFR2, but not other receptors such as PDGFR, EGFR, and FGFR, genetically modified mice deficient in endothelial-epsins (EC-iDKO) or deficient endothelial-epsins with one copy of VEGFR2 (EC-iDKO-Flk) were employed for UPI treatment. Genetically, if UPI only targets to VEGFR2 alone, we would expect a result shown in Supple Figure 1A. Indeed, we obtained the expected results as displayed in Supple Figure 1B & 4C. In WT mice, UPI treatment can remarkably retard tumor growth, because UPI can block VEGFR2 endocytosis and excess VEGFR2 remains in the plasma membrane (PM) made by two copies of VEGFR2, transmitting abnormal tumor angiogenesis. In EC-iDKO mice, regular endocytosis is inhibited, and excessive VEGFR2 is attained on the PM for abnormal vessel growth, leading to smaller tumors. In UPI treated EC-iDKO mice, the tumor size is similar to EC-iDKO saline controls, suggesting that UPI did not target to other vessel receptors. Otherwise, the tumor sizes could be variable in sizes from off-site receptor targeting. In EC-iDKO-Flk mice, there is only one copy of VEGFR2 but lacks epsins, in which the amount of VEGFR2 is reduced to a regular level compared to EC-iDKO mice, and the tumor volume is rescued to be a WT-like phenotype. After UPI was injected to EC-iDKO-Flk mice, a similar tumor size was observed in EC-iDKO-Flk control group, implying that UPI did not further impact other receptors; otherwise, the tumor volume in these mice could be smaller. However, this has not occurred (Supple Figure 1). Taken together, UPI peptide treatment did not further affect tumor growth in EC-iDKO or EC-iDKO-Flk mice. The therapeutic efficacy of UPI is strictly dependent on the amount of epsins and VEGFR2, suggesting that UPI targets and affects VEGFR2 signaling specifically.

UPI specifically targets to VEGFR2 in biochemical pull-down assay

To verify the results obtained from the genetic mutant mice, we take a biochemical approach to test if UPI peptide binds VEGFR2 in *ex vivo* glioma tumors. In theory, if UPI peptide is biotinylated and UPI targets to a specific receptor, this receptor should be able to be pulled down via Neutr-Avidin beads (see cartoon in Supple Figure 2A). Results from the pull-down assay and western blot show that biotinylated UIM peptide (and UPI) specifically binds to VEGFR2 only, as evidenced in Supple Figure 2, while biotinylated UPI peptide did not bind to PDGFR- β , EGFR, and FGFR. These results also answer two critical questions: 1.) Although UIM could, in theory, interact with other ubiquitinated growth factor receptors, our results make it clear that epsin UIM specifically interacts with VEGFR2, but not other growth receptors involved in tumor angiogenesis. 2.) Epsin is important in maintaining epsin-VEGFR2 endocytosis. Therefore, if epsin-VEGFR2 interaction is limited or artificially interrupted by UPI treatment, then VEGF-induced, VEGFR2 endocytosis is greatly reduced. The excessive VEGFR2 could be attained on the PM, transmitting abnormal

VEGFR2 signaling and create disorganized vessels that are nonfunctional. We used this mechanism to design the UPI peptide to block tumor growth.

UPI peptide treatment increases thrombi in the late stage of treatment and causes mitochondrial vacuolization

We analyzed the tumor structure by transmission electron microscopy (TEM) for tumors treated over 25 days. As shown in Figure 4, in control group, vessels are intact, red blood cells are inside the vessels, and cancer cells are well nourished and grow vigorously (big nuclei as indicated by red arrows) (Figure 4A). UPI peptide treatment caused significant leakage as plenty of red blood cells (RBC) were leaked out from the blood vessels (Figure 4B). Due to the hyper leakage features of the UPI-induced vessels in early stage of treatment, profound thrombi was formed later (Figure 4B & 4C), and completely blocked the blood stream, which may, at least in part, which can be interpreted as to why UPI peptide treatment also blocks metastasis beyond dysregulated tumor angiogenesis in our previous report [26]. Importantly, the nuclei of cancer cells are much smaller in the UPI peptide treated group compared to the control group, suggesting that all cancer cells are dying (Figure 4B, as indicated by red arrows). Furthermore, we characterized that there are numerous mitochondrial vacuolization in UPI treated tumors, suggesting that UPI peptide treatment induce cell abnormality and apoptosis (Figure 4C & 4D).

UPI peptides target tumor endothelium and cancer cells to trigger apoptosis in tumor

UPI peptide treatment remarkably increases hypoxic in tumors (Figure 5A), causing necrotic or apoptosis. To test this, we measured the apoptotic signaling in control or UPI peptide treated tumors. As presented in Figure 5, a significant cleaved caspase 3 and cleaved PARP were detected in UPI treated tumors, while anti-apoptotic molecule AKT is dramatically downregulated (Figure 5C & 5D). To further confirm this result, we performed TUNEL assay. As shown in Figure 5E & 5F, TUNEL staining is significantly magnificant in UPI treated tumors.

To test if UPI peptide from the leakage vessels can directly target tumor cells, we treated glioma U87 cells with varied concentration of UPI peptide *in vitro*. After 24 hours, FITC conjugated UPI peptides were taken up by all U87 cancer cells (Supple Figure 3A). We then conducted follow-up validations to determine if UPI peptide could induce apoptosis. Our results demonstrated that UPI peptide treatment induced substantial apoptosis in a concentration dependent fashion, as detected in cell culture and caspase activation in western blot analysis in U87 cells (Supple Figure 3B to 3D). Anti-apoptotic molecule AKT expression was obviously reduced (Supple Figure 3D). Furthermore, the expression of metastatic molecule snail and vimentin was significantly reduced by UPI peptide treatment (Supple 3E & 3F). Taken together, UPI peptide can directly target cancer cells from the leaking tumor vessels and induce apoptosis (Supple Figure 4), suggesting that, additional mechanisms exist for the inhibition of tumor growth and metastasis by UPI peptide beyond the dysfunctional tumor angiogenesis.

DISCUSSION AND CONCLUSION

The data that was extrapolated confirms our hypothesis that UPI is an effective, alternative treatment for certain types of cancers. Glioma tumors were largely inhibited in GL261 mouse model, and long-term administration of UPI peptide shrink glioma tumor growth. Compared to our previous research on UPI peptide, we identified multiple, novel mechanisms with regards to tumor inhibition and metastasis. The present study is more imperative to pre-clinics than our previous report [26]. First, we demonstrated that UPI administration can shrink glioma tumor growth, even after withdrawal of the UPI peptide administration in the long term (after 6 months), although the ratio (~40%) can be further improved. This observation was not seen in the VEGF antibody treated mice (Figure 2). Secondly, UPI peptide can directly induce tumor cell apoptosis (Supple Figure 3) in addition to dysfunctional tumor angiogenesis-induced apoptosis and necrosis. This can occur from the leaking tumor vessels (remodeling of tumor vessels is usually incomplete and approx. 30–40% tumor vessels are leaking). Third, UPI peptide can significantly increase tumor vessel thrombosis after long-term administration of the peptide (Figure 4). Additionally, UPI peptide can inhibit metastatic snail and vimentin expression in tumor cells (Supple Figure 3). Factors, such as tumor vessel thrombosis in later stages of peptide administration, cancer cell apoptosis, and the downregulated snail and vimentin, taken together, may better explain the mechanisms as to why the UPI peptide can effectively block metastasis, rather than solely regulate dysfunctional tumor angiogenesis as we previously reported [26].

Inhibition of epsins demonstrates our understanding that epsin is essential in the mechanisms of cellular angiogenesis because of its role in the degradation of VEGFR2 and production of functional tumor vessels [24, 26]. The leading peptide, iRGD, also specifically targets tumor vasculature by targeting $\alpha v \beta -3$ and $\alpha v \beta -5$ integrin, which is found in higher concentrations in a growing tumor. The plasma membrane anchoring peptide allowed the UPI to remain in the inner PM, instead of scattering into the cytosol, attempting to deliver maximal candidate drug efficacy. This assertion is further supported by previous research comparing the efficacy of UI (without PM-anchoring peptide) to UPI. UI compound efficacy was relatively decreased because it was highly concentrated in the cytosol compared to UPI [26]. Previous research further supports this because UPI was found enriched in organs that contained tumor cells, but not in other organs (unpublished data). Collectively, UPI peptide is a potent inhibitor for glioma tumors and the survival rate in UPI treated mice is significantly increased. Consequently, these results also show that UPI is comparable to current Anti-VEGF antibody treatments on the market today but with sustainable power to shrink tumor growth (Figure 2). Needless to say, future refining of UPI treatments could be potential, highly effective first-line of treatments for cancer patients or a promising option for cancer patients who are resistant to anti-VEGF antibody therapy.

As we reported before [24], deletion of epsins in ECs exhibits an anti-tumor phenotype in several tested cancer models through upregulation of VEGFR2 protein and signaling [24]. Epsin UIM is a critical determinant for epsin-VEGFR2 interaction [24]. This unique molecular mechanism underlying tumor-suppressing action of epsin-deficiency provides us with a strong rationale to ectopically disrupt epsin-VEGFR2 interaction, mimicking therapeutic treatment in cancer patients. In our proof of concept study, the UPI peptide

faithfully recapitulates a tumor-inhibition epsin knockout phenotype [24] by substantially increasing disorganized, dilated and dysfunctional leaky tumor vessels and VEGFR2 proteins in tumor ECs, resulting in smaller tumors that are hypoxic and necrotic (Figure 3 & Figure 5). The hyper-leaky feature generated by UPI peptide treatment may have great potential for combinational therapy using cancer-killing agents such as Doxorubicin [31]. Intuitively, these hyper-permeable tumor vessels could be exploited by cancer cells for speedy dissemination. However, our data demonstrates that the UPI peptide actually attenuates metastasis. A possible reason could be, at least in part, a non-productive vessel leakage-triggered thrombosis in the UPI peptide treated tumors (Figure 4), which may adversely affect cancer cell intravasation and circulation.

A previous report has demonstrated that iRGD can be taken up by tumor cells largely because tumor cells also express high levels of integrins and neuropilin-1 [30]. It is possible that, in UPI peptide treated tumors, the vessels are hyper-leaky, such as a sum of UPI peptides was leaked out from tumor vessels and taken-up by the vessel-surrounding cancer cells. Accordingly, we could not exclude the possibility that the UPI peptide also has an impact on cancer cells as the secondary effect owing to UPI-generated leakage vessels (Supple Figure 3). Whether UPI peptides directly influence cancer cells warrants further investigation.

We demonstrate that UPI therapy increases abnormal, leaky vessels. Clinically, leaky vessels could be devastating to patients with glioblastoma. However, clinically, leaky vessels also cause buildup of fluid, which exacerbates tissue damage due to increased intracranial pressure. Unlike other places in the body, the intracranial space is a closed chamber and swelling from leaky vessels can cause herniation of brain tissue. In our experiments, we did not observe any increased edema in T2-weighted images nor did the mice appear sick any sooner than the control group. Mice treated with UPI had a significantly longer survival and decreased tumor volumes compared to untreated mice. If there was increased intracranial pressure, this would result in mice appearing sick earlier than untreated mice, and we would have had to terminate them, i.e. decreased survival, not increased survival. However, this side-effect has not occurred in our mice treated by the UPI peptide. Clinically, if intracranial pressure (detected as edema in T2-weighted images) is present, shunts, the relievers for such cases, could be used. In addition, we have demonstrated that UPI peptide cannot be targeted to normal brain and non-tumor vessel, suggesting that the side effect from the peptide is minimal, whereas a thorough evaluation of peptide off-target warrants further investigation.

In conclusion, an anti-angiogenic approach is still considered a major combating strategy for several types of cancers in the colon, lung and brain [5, 32–34]. However, many tumors are refractory to this treatment, either at an initial stage or during the course of treatment. Our chimeric UPI peptide adopted a multiple mechanisms by targeting the angiogenic process and cancer cells, required for both tumor growth and metastasis, by a novel molecular mechanism of exacerbating leaky tumor angiogenesis, which may potentially represent a more potent alternative to current anti-angiogenic therapies.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Figure 1.

UPI peptide can be efficiently targeted to GL261 glioma tumor vasculature. (A) FITC-conjugated UPI peptide (FITC-UPI) was injected intravenously to established GL261 glioma tumor models. Mice were sacrificed 4 hours post-injection. Tumors were collected and fixed for immunofluorescent staining using antibody CD31, a specific marker for vessel endothelium. FITC-UPI was recorded in “green” channel. $n=5$ mice. Scale bar: 200 μm . (B) Tumor endothelial cells (TEC) were isolated as indicated in “Material and Methods”. TECs were cultured in regular endothelial medium EBM-2 basal with all supplements and amplified 1–2 passages. TECs were co-incubated with 50 μM FITC-UPI overnight (16–18 hours) in a 24-well plate. Cells were washed with 1xPBS for 4 times and recorded by fluorescent microscopy. $n=3$ repeats. Scale bar: 100 μm . (C) TECS were cultured in coverslip and co-incubated with 50 μM FITC-UPI as stated in (B). Cells were imaged using confocal microscopy. Scale bar: 10 μm . (D) In established GL261 glioma tumor models, UPI peptide administration was conducted by i.v-injection at 10 mg/kg every-other-day for 20 days. In terminal mice, FITC dextran perfusion was performed to test vessel permeability. Note the leakage feature of the UPI-treated tumor vessels and numerous FITC (green) were leaked to surrounding cancer cells. Red: CD31 staining. $n=5$ mice. Scale bar: 100 μm . (E) Quantification of leakage FITC area (mm^2/field). $n=5$ fields for each tested mice. $P<0.01$.

Figure 2.

UPI peptide administration inhibits tumor growth and prevented tumor relapse after cease of peptide treatment. (A) GL261 mouse models were i.v.-injected with control or UPI peptide at 20 mg/kg as indicated, and anti-VEGF antibody as a positive control at a dosage of 2.5 mg/kg. At day 27 post-tumor cell implantation, withdraw all treatments. Mice tumor volume was continuing to record using MRI methodology. $n=5$ in each group. * $P < 0.001$, run by t test. (B) Representative images of different treatments. (C) A representative image after stopping treatment for 15 days from anti-VEGF antibody group, showing tumor relapse. (D)

A representative image after stopping treatment for 6 months from UPI peptide group, showing tumor shrink. Note a visible scar remnant in the right forebrain.

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Figure 3.

UPI peptide induces hyper-dilated and dysfunctional vessels with upregulated VEGFR2 expression in G261 glioma tumors. (A) Representative images for enlarged vessels in UPI peptide treated (i.v-injection, 20 mg/kg, every-other-day) G261 glioma tumors. (B) Fold change of vessel CD31 staining in CTR and UPI treated G261 glioma tumors, n=5; *P<0.001. (C) Western blot analysis of VEGFR2 expression in CTR and UPI treated G261 glioma tumors, n=5; *P<0.001. (D) VEGFR2 is upregulated in GL261 brain tumors following UPI peptide treatment revealed by VEGFR2-targeted molecular MRI technology. The % MRI SI of VEGFR2 for the UPI group is significantly higher than the control group (*P<0.0001). Abbreviations: TP: tumor periphery; TI: tumor interior; SI, signal intensity.

Figure 4.

UPI peptide treatment caused serious thrombosis in tumor vessels and damages in tumor cells after longer administration of UPI peptide using electronic microscopy. Tumors were from mice treated by control or UPI peptide for 15 days. (A) Control peptide tumor vasculature. V: vessel; RBC: red blood cell; CC: cancer cell (note: normal cancer cells with big nuclei as indicated by red arrows). Scale bar: 200μm. (B) A magnificent thrombi was formed in UPI peptide treatment. Note that the RBCs (blue arrows) were leaked out from the vessel and scattered around the surrounding cancer cells, which are dying (red arrows; note: dying cancer cells with much smaller nuclei). Scale bar: 200μm. (C) An enlarged image showing thrombi and abnormal mitochondria (black arrows) caused by UPI peptide treatment. Scale bar: 500nm. (D) Mitochondrial vacuolization (black arrows). Scale bar: 10nm.

Figure 5.

UPI peptide treatment increases tumor hypoxia and induces apoptosis in glioma GL261 tumor models. (A) UPI treatment significantly increased hypoxia in tumors in terminal mice. Tumors were collected and processed for hypoxia probe staining. This is a representative of five tumors in each group. Scale bar: 100 μ m. (B) Statistical analysis of tumor hypoxia for control or UPI peptide treated tumors, n=5 in each group. *P<0.001. (C) Tumor cells were lysed and subjected to SDS-PAGE gel and blotted with apoptotic cascade antibodies cleaved-casp3, cleaved-PARP and AKT. (D) Quantification of apoptotic signaling molecules as in Figure (C), *, ** or # all P<0.001, control vs. UPI. (E) TUNEL staining for tumors of

control or UPI treated GL261 samples. Five tumors were used to do this assay in each group, and six fields of each sample were selected for analysis. Scale bar: 100 μ m. (F)
Quantification for TUNEL assays. n=5 tumors in each group; *P<0.001.

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