Published in final edited form as:

Chem Biol Drug Des. 2015 May ; 85(5): 586–597. doi:10.1111/cbdd.12446.

Targeting Receptor Tyrosine Kinases and Their Downstream Signaling with Cell-Penetrating Peptides in Human Pulmonary Artery Smooth Muscle and Endothelial Cells

Jun Yu¹, Chamila Rupasinghe², Jamie L. Wilson¹, Linda Taylor¹, Nader Rahimi³, Dale Mierke^{2,†}, and Peter Polgar^{1,*†}

¹Department of Biochemistry, Boston University School of Medicine, Boston, MA 02118, USA

²Department of Chemistry, Dartmouth College, Hanover, NH 03755, USA

³Departments of Pathology and Lab Medicine, Boston University School of Medicine, Boston, MA 02118, USA

Abstract

Cell-penetrating peptide (CPP) intracellular delivery of receptor signaling motifs provides an opportunity to regulate specific receptor tyrosine kinase signal transductions. We targeted tyrosine residues Y740 and Y751 of the PDGF receptor β (PDGFR β) and Y1175 of the VEGF receptor 2 (VEGFR2). The Y740 and Y751 motifs activated ERK and Akt, while the Y1175 motif activated ERK. Targeting either Y740 or Y751 of the PDGFR β in human pulmonary artery smooth muscle cells (HPASMC) effectively inhibited PDGF activation of ERK or Akt. Interfering with the Y751 region of the PDGFR β proved more effective than targeting the Y740 region. The phosphorylation of Y751 of the CPP and the length and exact sequence of the mimicking peptide proved crucial. On the other hand, in human pulmonary artery endothelial cell phosphorylation of the VEGFR2 Y1175 CPP was not a determinant in blockage of ERK activation. Likewise, the length of the peptide mimic was not crucial with a very small sequence containing the Y1175 remaining effective. Physiologic proof of concept for the effectiveness of the CPP was confirmed by blockage of HPASMC migration in response to PDGF following culture injury. Thus targeted blockage of tyrosine kinase receptor signaling can be very effective.

Keywords

Akt; cell-penetrating peptide; drug delivery; ERK; human pulmonary artery endothelial cells; human pulmonary artery smooth muscle cells; platelet-derived growth factor receptor β ; receptor tyrosine kinases; vascular endothelial growth factor receptor 2

Cell-penetrating peptides (CPP) are typically short cationic amino acid sequences that mediate intracellular delivery of a range of biological cargos (1). These peptides facilitate the movement of a variety of molecules into various compartments of intact cells (2,3). CPP

coupled to intracellular motifs have been used to regulate G-protein-coupled receptor signaling. Regulations of angiotensin II receptor (4) and endothelin-1 receptor (5) signaling function are examples. Cell-penetrating peptides have a potential for both experimental approaches and therapeutic procedures. As an example, we have used a CPP targeting the second intracellular loop of the ET-1 ETB receptor to ameliorate pulmonary hypertension in hypoxic rats (6). The effect of CPP is similar to that of biased agonists or antagonists, but acts downstream of ligand binding. Thus, CPP can be targeted against distinct signaling motif sites ultimately affecting a much narrower range of, more specific, signal cascades.

Here, we present the use of motif-targeting peptides to regulate the signal transduction of two receptor tyrosine kinases (RTK), platelet-derived growth factor receptor β (PDGFR β), and vascular endothelial growth factor receptor 2 (VEGFR2). Platelet-derived growth factor (PDGF) and its receptor PDGFR β , vascular endothelial growth factor (VEGF), and its receptor VEGFR2 signaling pathways are involved in such vascular smooth muscle and endothelial cell functions as remodeling. Remodeling is associated with diseases such as systematic and pulmonary hypertension and involves cell migration and proliferation (7–11). The blocking of the PDGFR β as well as other RTK has a strong therapeutic potential. Preclinical studies demonstrated that PDGFR β blockage with imatinib, a generalized blocker of PDGFR β signaling, promoted phosphorylation and reduced vascular remodeling. Phase 3 clinical trials showed that imatinib, a generalized blocker of PDGFR β signaling, improved exercise capacity and hemodynamics in patients with advanced pulmonary hypertension (PAH). But treatment was accompanied by serious side-effects (12). Clearly, more specifically targeted PDGFR signal blockers are needed.

We focused on tyrosine 740 and 751 in the PDGFR β C-terminus. Previous reports showed that mutating Y751 to F strongly reduced up-regulation of cell proliferation. It also abolished interaction between PIK3R1 and NCK1. It had no effect on GRB10 binding (13–18). We used peptides mimicking this active domain to inhibit PDGF-induced activation of Akt and ERK.

VEGF and its receptor (VEGFR2) are involved in angiogenesis (19). Binding of VEGF to VEGFR2 leads to endothelial cell proliferation and migration (19). Tyrosine 1175 of this receptor was selected as the target in this study. Phosphorylation of Y1175 creates a binding site for several signaling mediators, particularly phospholipase C γ (PLC γ), which ultimately leads to the activation of ERK (20).

To promote CPP membrane permeability, a critical design feature here is the use of a SynB3 (21)-based CPP. The SynB3 transporter sequence is derived from protegrin, a natural mammalian antimicrobial peptide which possesses a high transmembrane penetrating ability (22). Various motif mimicking sequences with or without modifications were attached to the SynB3 vector. Their effect on PDGF- or VEGF-mediated signal transduction was studied in human pulmonary artery smooth muscle cells (HPASMC) and human pulmonary artery endothelial cells (HPAEC), respectively.

Methods and Materials

Materials

PDGF-BB (the isoform of PDGF that activates PDGFR β), VEGF-165 (the most abundant splice variant of VEGF-A), and epidermal growth factor (EGF) were purchased from R&D Systems (Minneapolis, MN, USA). The MTT assay kit was purchased from ATCC (Manassas, VA, USA). Antibodies for the detection of phospho-ERK1/2(Thr202/Tyr204), phospho-Akt (Ser473), and GAPDH were purchased from Cell Signaling Technologies (Beverly, MA, USA). Protease inhibitor cocktail was from Roche Diagnostics (Indianapolis, IN, USA). Pierce BCA Protein Assay kit and ECL Western Blotting Substrate were purchased from Thermo Fisher Scientific Inc (Rockford, IL, USA).

Cell culture

Primary human pulmonary artery smooth muscle cells (HPASMC) and endothelial cells (HPAEC) isolated from the pulmonary arteries obtained from lung transplants were a generous gift from Dr. Serpil Erzurum (Cleveland Clinic, Cleveland, OH, USA). HPASMC were maintained in 15 mM Hepes buffered DMEM/F12 medium (Life Technologies, Grand Island, NY, USA) supplemented with 10% fetal bovine serum (Lonza, Walkersville, MD, USA) and 250 units/mL penicillin, 250 μ g/mL streptomycin, and 0.625 μ g/mL amphotericin B (Life Technologies) in an atmosphere of 95% air/5% CO₂ at 37 °C. Cell passages of 5–9 were used in experiments. Stain for smooth muscle actin demonstrated that human HPASMC maintain their phenotype in culture. Mass Spec proteomics also attested that they retain their phenotype. For example, myosin heavy chain, myosin light chain, and smooth muscle actins were all expressed in the HPASMC (23). Human pulmonary artery endothelial cells (HPAEC) were grown in EBM-2 basal medium supplemented with EGM-2 (Lonza) on fibronectin-coated plates. Cells were passaged at 70–80% confluency, and primary cultures of passages 6–8 were used in experiments. Extensive immunocytochemistry procedures proved the endothelial phenotype of HPAEC cultures (24).

Peptide synthesis

The various CPP are composed of the targeting sequence and the penetrating sequence (SynB3:RRLSYSRRRF) (22). The structure of the CPP is [targeting sequence]-K (SynB3RKG)-CONH₂, and the N-terminus is not capped. Sketches of the two receptors and the targeted sequences are illustrated in Figure 3A,B. All the compounds were synthesized by Fmoc-based solid-phase peptide synthesis protocols employing microwave heating (CEM Discover S-class microwave synthesizer), employing the appropriately protected amino acids. All compounds were synthesized on Rink-Amide-ChemMatrix resin (N mmol, 0.6 mmol/g, P/N no. 7-600-1310-25), using HBTU for coupling and piperidine for Fmoc deprotection as detailed elsewhere (6). The compounds were then purified by RP-HPLC, and molecular mass confirmed by MALDITOF mass spectroscopy.

Western blot analysis

HPASMC were preincubated with 0, 5, 10, 50 and 100 μ M CPP for 30 min, and then incubated with 1 ng/mL PDGF for 10 min. The cells were then washed twice with ice-cold

PBS. Cell lysates were prepared by addition of ice-cold RIPA buffer, 150 mM NaCl, 1.0% Igepal CA-630, 0.5% sodium deoxycholate, 0.1% SDS, 50 mM Tris, pH 8.0 (Sigma, St Louis, MO, USA), and 1× complete protease inhibitor cocktail (Roche Applied Science, Indianapolis, IN, USA) and centrifuged at 12,000 rpm ($13\,400 \times g$) at 4 °C for 20 min. The protein concentration was measured by BCA assay. The proteins were fractionated on 10% SDS-PAGE gels, and Western blots were carried out using antibodies against phosphorylated ERK1/2, phosphorylated Akt, and GAPDH. Proteins were detected by chemiluminescence and the film scanned with an Epson Perfection 3170 scanner using EPSON SCAN (version 1.22A) software (Epson America, Inc., Long Beach, CA, USA). Analysis of band intensities and normalization was performed using the NIH IMAGEJ image analysis software (25). The IC₅₀ was calculated using GRAPHPAD PRISM 6.0 (GraphPad Software, Inc., La Jolla, CA, USA). The 'log (inhibitor) versus normalized response–Variable slope' equation was used for the analysis.

Measurement of cell viability

The effects of the SynB3-based CPP on cell viability were evaluated with the MTT assay. The assay is based on the detection of the reduction of yellow MTT (3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) by metabolically active cells to purple formazan which is then detected photometrically at 570 nm. HPASMC were seeded in 96-well plates and cultured to 90% confluence, and MTT assay was carried out after 30-min incubation with CPP. Cell viability is measured as the OD value at 570 nm using a BioTek Synergy Multi-Mode microplate reader (Biotek, Winooski, VT, USA).

Migration of HPASMC

HPASMC were grown to confluence on 12-well plates in complete medium. Once confluent, the medium was changed to 0.2% FBS medium (quiescence medium) overnight. The next day, an injury was induced by scratching straight down the middle of the well with a 1000-μL pipette tip. Perpendicular to the scratch, a straight line was drawn with a marker to better identify the same scratch locations under a microscope. After the scratch was made, the medium in each well was replenished with new quiescence medium with either 10 ng/mL PDGF, 10 ng/mL PDGF plus 50 μM CPP, Y751P, or 10 ng/mL PDGF plus 50 μM inactive negative control, SynB3–ETBIC2, and images were taken of each scratch at time 0. Plates were then incubated at 37°C, 5% CO₂ for approximately 24 h. After incubation cells were stained with 20% methanol/PBS crystal violet solution. Each scratch was imaged at 40× magnification. Migration was determined by counting the number of cells which migrated into the wound. NIH IMAGEJ software was used to quantify the results.

Statistical analysis and data analysis

Statistical evaluation of the data was carried out using the Student's *t*-test. Probability values <0.05 were considered significant.

Results

Response of human pulmonary artery smooth cells (HPASMC) and endothelial cells (HPAEC) to PDGF and VEGF

The activation of ERK and Akt in response to PDGF and VEGF was tested in two human lung vascular cell types, pulmonary artery smooth muscle (HPASMC), and endothelial cells (HPAEC). As illustrated in Figure 1, both ERK and Akt were activated by PDGF in HPASMC. VEGF demonstrated no activity in HPASMC. In HPAEC, the actions were opposite, with VEGF showing strong ERK phosphorylation, while PDGF was inactive. However, both VEGF and PDGF failed to activate Akt in HPAEC. Therefore, limiting ERK and Akt activation with CPP in response to PDGF was evaluated in HPASMC, while the effect of VEGFR2 targeting CPP was evaluated in HPAEC. Dose response to either PDGF in HPASMC (Figure 2A) or VEGF in HPAEC (Figure 2B) maximized at 1 ng/mL. All experiments thereafter were conducted at these maximal concentrations.

Targeted motifs

Based on the ERK/Akt activation by PDGFR β and ERK activation by VEGFR2, CPP with various motif sequences were synthesized focusing on PDGFR β Y740 and Y751 and VEGFR2 at Y1175. Schematics of the two receptors and the targeted regions are shown in Figure 3A,B. The SynB3 cell transporter sequence was attached to a variety of motifs in C-terminus of PDGF and VEGF receptors to produce cell-penetrating peptides. The naming code and the targeting sequences are also summarized in Figure 3. As illustrated, the PDGFR β targeting sequences focused on Y740 (GESDGGY⁷⁴⁰MDMSKDES) and Y751 (KDESVDY⁷⁵¹VPMLDMK) regions. Both Y740 and Y751 have been described to interact with Nck1 (non-catalytic region of tyrosine kinase adaptor protein 1) and PI3K directly. Nck1 in turn activates ERK, and PI3K activates Akt (26). The VEGFR2 CPP was targeted against the Y1175 region (QQDGKDY¹¹⁷⁵IVLPISET). This region has been shown to interact with PLC γ (phospholipase C γ), which in turn activates ERK via the PIP2/PKC pathway (20). The sequences of the CPP to be tested are presented in the diagram. With the use of flow cytometry, the SynB3 peptides were demonstrated to penetrate cells effectively (6).

Region Y751 of the PDGFR β —CPP Y751P which contains the fourteen amino acid sequences within the Y751 region and contains a phosphorylated tyrosine was used to block PDGF-stimulated ERK and Akt phosphorylation. The CPP displayed dose-dependent inhibition. At 100 μ M, the inhibition resulted in lowering of pERK close to basal level, while the inhibition of pAkt levels was less effective (Figure 4A).

To determine the role of the phosphate group in the inhibition by this sequence, CPP Y751, composed of the same sequence as CPP Y751P, except that the Y751 is not phosphorylated, was tested in response to PDGF (Figure 4B). A considerably diminished inhibitory effect by this CPP was observed. The peptide had no inhibitory effect at concentration as high as 50 μ M and was only marginally active at 100 μ M. A shorter version of the Y751P, Y751Pshort, was then tested. This peptide while phosphorylated at Y751 is composed of six amino acids as illustrated in Figure 3. The shorter sequence continued to inhibit PDGF-stimulated

phosphorylation of ERK and Akt, but at considerably reduced level compared to the fourteen-amino acid-long CPP Y751P (Figure 4C).

Region of Y740 of the PDGFR β —A sixteen-amino acid sequence containing a phosphorylated Y740 was then synthesized (CPP Y740P) to target the Y740 region. As the peptides targeting Y751, this peptide also inhibited ERK activation but less effectively than the Y751 peptide. Meaningful inhibition was only detected at 100 μ M. Interestingly, this CPP proved very effective against the activation of Akt (Figure 5A). To test the sequence specificity of these peptides, glycine at 739 was replaced with to serine (Y740PGS). This peptide lost all inhibitory action of Y740P as illustrated in Figure 5B.

CPP directly targeting ERK—As confirmation of the regulated ERK activation by the targeted PDGFR β peptides, a CPP containing a peptide directly targeting ERK activation was then tested. This is a cell-permeable SynB3 13-amino acid peptide corresponding to the N-terminus of MEK1 (MAPKK) (MPKKKPTPIQLNP) (27). The peptide clearly inhibited ERK activation in HPASMC in response to PDGF at 50 and 100 μ M. It had no effect on Akt activation (Figure 6).

Targeting the region of Y1175 of the VEGFR2—Human pulmonary artery endothelial cells (HPAEC) were used to study the effect of the CPP targeting VEGFR2-mediated ERK activation at the Y1175 motif. As illustrated in Figure 1, VEGF activates ERK in these cells but not in HPASMC. A CPP (Y1175P) targeting the 15-amino acid region containing Y1175 was synthesized with a phosphorylated tyrosine Figure 2B. Inhibition of VEGF-induced ERK activation by this peptide in HPAEC was very effective reaching basal levels of ERK at 10 μ M (Figure 7A).

An unphosphorylated sequence CPP (Y1175) was synthesized to test the role of the phosphate group. It is the same sequence as Y1175P, except that it does not contain a phosphate group at Y1175. The unphosphorylated CPP proved an effective inhibitor of ERK activation at 50 and 100 μ M (Figure 7B). However, it was somewhat less effective than the full-length phosphorylated peptide at 50 μ M concentration.

A shortened phosphorylated version of the Y1175P was then tested. This CPP (Y1175Pshort), which contained only six residues (KDY(PO₄)IVL), inhibited VEGF ERK activation equally effectively to the full-length phosphorylated peptide (Figure 7C).

The CPP targeted directly against ERK was then tested in HPAEC in conjunction with VEGF stimulation. This CPP also inhibited ERK activation in the HPAEC at 50 and 100 μ M, the same concentration as the VEGF CPP (Figure 8A).

Effect of SynB3–ETBIC2 peptide on VEGFR2—A peptide targeting the second intracellular loop of the endothelin-1 receptor B (ETBIC2) with the sequence IKGIGV attached to the SynB3 vector) (6) was tested as a control CPP. No inhibitory effects were observed (Figure 8B), again illustrating that the effects of the CPP against VEGFR2 Y1175 region are sequence specific.

IC50 values of the CPP

The effective concentrations of the CPP targeted either against the PDGFR β or VEGFR2 sites and representing various motif configurations are illustrated in sum in Tables 1 and 2 in terms of IC50 values. The importance of phosphorylation and sequence length of the PDGFR peptides with respect to their actions is correlated with the IC50 values. While Y751P had an IC50 of approximately 20 μ M with respect to Akt and ERK activation, Y751Pshort only affected ERK but not AKT activation. All three CPP against the VEGFR2 and the ERK–CPP likewise had an IC50 within the 20 μ M range.

Possible cytotoxicity of the CPP

It was important to illustrate that the CPP effect was not due to cytotoxicity. The MTT assay was used to follow cell viability of HPASMC following selected biologically active CPP exposure. Results are shown in Figure 9. Hydrogen peroxide at 0.5 mM was used as positive control. The hydrogen peroxide was the only treatment which showed statistically significant toxicity. The two PDGFR β peptides, Y751P and Y740P, the two VEGFR2 peptides, Y1175P and Y1175Pshort, and the ERK peptide all inhibited ERK or Akt activation, but had no effect on the viability of HPASMC in 100 μ M concentration after 30-min incubation.

Possible non-specific effects of the CPP on ERK and Akt activation

To further confirm the specificity of the CPP, we determined a possible non-specific effect of the CPP on the EGF receptor signaling in HPASMC. As shown in Figure 10, 1 ng/mL EGF induced the phosphorylation of both ERK and Akt. All the CPP tested, either against PDGFR β or VEGFR2, had no inhibitory effect on EGF-induced ERK or Akt activation.

Regulation of HPASMC migration

As proof of concept, we examined the regulation of HPASMC migration by CPP. Cultures of quiescent confluent HPASMC were treated with either 10 ng/mL PDGF, 10 ng/mL PDGF including 50 μ M CPP Y751P or 10 ng/mL PDGF including 50 μ M inactive negative control SynB3–ETBIC2, or no treatment. The cultures were injured by gently scraping off the cells in the middle of the culture plate. Migration of the cells into the empty region was then determined after 24 h. As illustrated in Figure 11A, the active CPP proved very effective in limiting HPASMC migration. An equivalent treatment with the inactive CPP is illustrated in Figure 11B. No activity was observed with the inactive CPP. Microscopy of the cultures treated with CPP for the 24-h migratory test did not show any evidence of cytotoxicity.

Discussion

Flow cytometry experiments have demonstrated the effective entrance of the SynB3-based CPP into pulmonary vascular cells *in vitro* and *in vivo* (6). MTT assay ensured that those CPP at 100 μ M do not reduce cell viability as illustrated in Figure 9. Inspection of the morphology of the cells in the migration assays further supports lack of toxicity of the CPP even after 24-h incubation (Figure 11). The CPP are proving ideal for allowing specific targeting of intracellular components and signaling motifs. This is particularly useful when inhibitors for the targets are not available or are not sufficiently specific. We are thus

inhibiting specific phosphorylation and anchoring actions by the receptor without interfering with the other receptor signaling actions. The advantage of specifically targeting individual receptor activation of downstream kinases, particularly such predominant kinases as ERK or Akt, permits their continued function in other essential physiologic actions. There are a number of other physicochemical advantages of CPP use including ease and relatively low cost of synthesis, versatility of employing an identical backbone/scaffold (only modifying the amino acid side chains), control of serum lifetime (by incorporation of D-amino acids or other non-natural amino acids blocking enzymatic degradation), and low toxicity (1).

To our knowledge, this is the first illustration of signaling control based on the CPP mode of action of either the VEGFR2 or PDGFR β . We selected these two RTKs for study because both PDGF and VEGF play important roles in vascular remodeling in diseases such as pulmonary arterial hypertension. Inhibiting the signaling of these two receptors individually and specifically could prove very effective in the treatment of vascular diseases. Furthermore, PDGFR β is expressed in HPASMC but not HPAEC, while the VEGFR2 is expressed in HPAEC but not HPASMC. Therefore, inhibiting the signaling of these two receptors individually and specifically would also lead to specific treatment based on two vascular cell types.

As pointed out in the introduction, the Y740 and Y751 regions of PDGFR β were targeted because these two motifs are known to interact with NCK1, a docking protein, which recruits MAPK and leads to the activation of ERK and interacts with PI3K which in turn activates Akt (24). We found that CPP targeting the Y751 region is more effective than the Y740 region in shutting down ERK/Akt activation. Also, the size of the targeting region proved important. The Y751P (14 residues) was much more effective than Y751Pshort (six residues) to shut down ERK activation. Nevertheless, the shorter CPP still inhibited Akt signaling effectively. Thus, the CPP Y751Pshort displays some extent of selectivity between the Nck1/ERK and PI3K/Akt activation. We also showed that the phosphorylation of Y751 is crucial for the activity of this CPP. Without the phosphate group, CPP Y751 did not effectively inhibit PDGFR β signaling. The effective sequences are very specific. For example, one amino acid mutation in Y740GS rendered this CPP totally ineffective.

While PDGFR β and VEGFR2 are structurally related, their intracellular signaling is considerably different. PDGFR at Y740 and Y751 binds to the SH2 domain of the p85 subunit of PI3K. It activates the PI3K-Akt and Ras pathways (26). On the other hand, the PI3K pathway is not highly activated by VEGFR (28). Autophosphorylation of Y1175 binds phospholipase C γ (PLC γ) which then regulates the PKC-Raf-MEK-MAPK cascades. Homology alignment showed that there is no motif in the VEGFR2 which corresponds to the Y740/Y751 region of PDGFR β . Conversely, there is no motif in the PDGFR β which corresponds to the Y1175 region of VEGFR2. A Basic Local Alignment Search Tool (BLAST) search showed that these motifs are specific to PDGFR β and VEGFR2, respectively. Phosphorylation of the Y751 CPP was critical for blocking of the PDGF-associated ERK activation. The phosphorylation of Y1175 CPP of the VEGFR2 was not. VEGF-stimulated ERK activation was effectively blocked by the CPP whether Y1175 of the CPP was phosphorylated or not. This illustrates that the effectiveness of a CPP targeting critical tyrosines does not always require phosphorylation and apparently varies with the

receptor and perhaps the given motif. Interestingly, VEGFR2 CPP (Y1175Pshort) with very short sequence (only five residues) appears equally or perhaps more effective than the larger sequence making up the target CPP. Clearly, the optimal length of the targeting region is variable and needs to be experimentally determined.

The specificity of the peptides was established with amino acid exchanges and unrelated peptide sequences. Additionally, EGF-stimulated ERK activation was used in the HPASMC to further illustrate that the CPP targeted against PDGFR β and VEGFR2 do not function in a related but structurally different RTK receptor such as EGFR.

We showed previously that TAT linked peptides which mimic motifs within the intracellular loops of GPCR such as angiotensin II receptor or endothelin-1 B (ETBR) receptor can alter their signal cascades (4,5). A SynB3 CPP containing the second intracellular loop of the ETB receptor was shown to inhibit endothelin-1-stimulated ERK and Akt phosphorylation in rat pulmonary artery smooth muscle, and this CPP ameliorated increases in pulmonary artery pressure, right ventricle hypertrophy, and right ventricle remodeling in a hypoxic rat model of pulmonary arterial hypertension (6). Results herein illustrate that receptor tyrosine kinase signaling and cellular action can also be regulated with peptides duplicating signaling motifs of critical intracellular tyrosines linked to transmembrane carrier molecules. One advantage of this study is that the HPASMC and HPAEC were primary cells at low passage derived from human pulmonary arteries.

At this time, our report deals with the effect of the RTK CPP on signaling and short-term migration in cell culture. These promising *in vitro* results warrant further studies at both the *in vitro* and *in vivo* levels. Careful experimental screening has to be carried out to optimize the peptide design. Also, to obtain optimal effectiveness, the pharmacokinetics of the CPP within the targeted cells and tissues will require careful determination.

Acknowledgments

This work was supported by NIH Grant Number HL025776. We thank Dr. Suzy Comhair and Dr. Serpil Erzurum from the Cleveland Clinic Pathobiology Tissue Sample and Cell Culture Core (supported by NIH PO1 HL081064 and RC37 HL60917) for pulmonary smooth muscle and endothelial cell samples.

Funding Sources

This work was supported by National Institutes of Health (NIH) grant HL025776.

References

1. Heitz F, Morris MC, Divita G. Twenty years of cell-penetrating peptides: from molecular mechanisms to therapeutics. *Br J Pharmacol*. 2009; 157:195–206. [PubMed: 19309362]
2. Schwarze SR, Hruska KA, Dowdy SF. Protein transduction: unrestricted delivery into all cells? *Trends Cell Biol*. 2000; 10:290. [PubMed: 10856932]
3. Murriel CL, Dowdy SF. Influence of protein transduction domains on intracellular delivery of macromolecules. *Expert Opin Drug Deliv*. 2006; 3:739–746. [PubMed: 17076596]
4. Yu J, Taylor L, Mierke D, Berg E, Shia M, Fishman J, Sallum C, Polgar P. Limiting angiotensin II signaling with a cell-penetrating peptide mimicking the second intracellular loop of the angiotensin II type-I receptor. *Chem Biol Drug Des*. 2010; 76:70–76. [PubMed: 20492449]

5. Sallum CO, Wilson JL, Rupasinghe C, Berg E, Yu J, Green DS, Taylor L, Mierke D, Polgar P. Enhancing and limiting endothelin-1 signaling with a cell-penetrating peptide mimicking the third intracellular loop of the ETB receptor. *Chem Biol Drug Des.* 2012; 80:374–381. [PubMed: 22553998]
6. Green DS, Rupasinghe C, Warburton R, Wilson JL, Sallum CO, Taylor L, Yatawara A, Mierke D, Polgar P, Hill N. A cell permeable peptide targeting the intracellular loop 2 of endothelin B receptor reduces pulmonary hypertension in a hypoxic rat model. *PLoS ONE.* 2013; 8:e81309. [PubMed: 24312288]
7. Grimminger F, Schermuly RT. PDGF receptor and its antagonists: role in treatment of PAH. *Adv Exp Med Biol.* 2010; 661:435–446. [PubMed: 20204747]
8. Schermuly RT, Dony E, Ghofrani HA, Pullamsetti S, Savai R, Roth M, Sydykov A, Lai YJ, Weissmann N, Seeger W, Grimminger F. Reversal of experimental pulmonary hypertension by PDGF inhibition. *J Clin Invest.* 2005; 115:2811–2821. [PubMed: 16200212]
9. Nicolls MR, Mizuno S, Taraseviciene-Stewart L, Farkas L, Drake JI, Al Hussein A, Gomez-Arroyo JG, Voelkel NF, Bogaard HJ. New models of pulmonary hypertension based on VEGF receptor blockade-induced endothelial cell apoptosis. *Pulm Circ.* 2012; 2:434–442. [PubMed: 23372927]
10. Becker PM, Verin AD, Booth MA, Liu F, Birukova A, Garcia JG. Differential regulation of diverse physiological responses to VEGF in pulmonary endothelial cells. *Am J Physiol Lung Cell Mol Physiol.* 2001; 281:L1500–L1511. [PubMed: 11704547]
11. Farkas L, Farkas D, Ask K, Möller A, Gaudie J, Margetts P, Inman M, Kolb M. VEGF ameliorates pulmonary hypertension through inhibition of endothelial apoptosis in experimental lung fibrosis in rats. *J Clin Invest.* 2009; 119:1298–1311. [PubMed: 19381013]
12. Hoeper MM, Barst RJ, Bourge RC, Feldman J, Frost AE, Galié N, Gómez-Sánchez MA, et al. Imatinib mesylate as add-on therapy for pulmonary arterial hypertension: results of the randomized IMPRES study. *Circulation.* 2013; 127:1128–1138. [PubMed: 23403476]
13. Kazlauskas A, Durden DL, Cooper JA. Functions of the major tyrosine phosphorylation site of the PDGF receptor beta subunit. *Cell Regul.* 1991; 2:413–425. [PubMed: 1653029]
14. Kashishian A, Kazlauskas A, Cooper JA. Phosphorylation sites in the PDGF receptor with different specificities for binding GAP and PI3 kinase *in vivo*. *EMBO J.* 1992; 11:1373–1382. [PubMed: 1314164]
15. Kazlauskas A, Kashishian A, Cooper JA, Valius M. GTPase-activating protein and phosphatidylinositol 3-kinase bind to distinct regions of the platelet-derived growth factor receptor beta subunit. *Mol Cell Biol.* 1992; 12:2534–2544. [PubMed: 1375321]
16. Nishimura R, Li W, Kashishian A, Mondino A, Zhou M, Cooper J, Schlessinger J. Two signaling molecules share a phosphotyrosine-containing binding site in the platelet-derived growth factor receptor. *Mol Cell Biol.* 1993; 13:6889–6896. [PubMed: 7692233]
17. Wang J, Dai H, Yousaf N, Moussaif M, Deng Y, Boufelliga A, Swamy OR, Leone ME, Riedel H. Grb10, a positive, stimulatory signaling adapter in platelet-derived growth factor BB-, insulin-like growth factor I-, and insulin-mediated mitogenesis. *Mol Cell Biol.* 1999; 19:6217–6228. [PubMed: 10454568]
18. Yokote K, Mori S, Hansen K, McGlade J, Pawson T, Heldin CH, Claesson-Welsh L. Direct interaction between Shc and the platelet-derived growth factor beta-receptor. *J Biol Chem.* 1994; 269:15337–15343. [PubMed: 8195171]
19. Rahimi N. VEGFR-1 and VEGFR-2: two non-identical twins with a unique physiognomy. *Front Biosci.* 2006; 11:818–829. [PubMed: 16146773]
20. Takahashi T, Yamaguchi S, Chida K, Shibuya M. A single autophosphorylation site on KDR/Flk-1 is essential for VEGF-A-dependent activation of PLC-gamma and DNA synthesis in vascular endothelial cells. *EMBO J.* 2001; 20:2768–2778. [PubMed: 11387210]
21. Rousselle C, Smirnova M, Clair P, Lefauconnier JM, Chavanieu A, Calas B, Scherrmann JM, Temsamani J. Enhanced delivery of doxorubicin into the brain via a peptide-vector-mediated strategy: saturation kinetics and specificity. *J Pharmacol Exp Ther.* 2001; 296:124–131. [PubMed: 11123372]
22. Drin G, Cottin S, Blanc E, Rees AR, Temsamani J. Studies on the internalization mechanism of cationic cell-penetrating peptides. *J Biol Chem.* 2003; 278:31192–31201. [PubMed: 12783857]

23. Yao CX, Yu J, Taylor L, Polgar P, McComb M, Costello C. Insight of the role of BMPR2 mutation and endothelin-1 signaling in pulmonary arterial hypertension from the global protein expression profiles by proteomic mass spectrometry. *Int J Mass Spectrom.* 2014 In Press.
24. Comhair SA, Xu W, Mavrakis L, Aldred MA, Asosingh K, Erzurum SC. Human primary lung endothelial cells in culture. *Am J Respir Cell Mol Biol.* 2012; 46:723–730. [PubMed: 22427538]
25. Rasband, WS. ImageJ. U. S. National Institutes of Health; Bethesda, MD, USA: 1997–2014 <http://imagej.nih.gov/ij/>
26. Heldin C-H, Westermark B. Mechanism of action and *in vivo* role of platelet-derived growth factor. *Physiol Rev.* 1999; 79:1283–1316. [PubMed: 10508235]
27. Kelemen BR, Hsiao K, Goueli SA. Selective *in vivo* inhibition of mitogen-activated protein kinase activation using cell-permeable peptides. *J Biol Chem.* 2002; 277:8741–8748. [PubMed: 11756441]
28. Shibuya M, Claesson-Welsh L. Signal transduction by VEGF receptors in regulation of angiogenesis and lymphangiogenesis. *Exp Cell Res.* 2006; 312:549–560. [PubMed: 16336962]

Figure 1.

PDGF- and VEGF-stimulated pERK and pAkt activation in HPASMC and HPAEC. ERK and Akt phosphorylation was measured in response to serum-free medium alone (control), 10 ng/mL PDGF, or 10 ng/mL VEGF. Blot is representative of three separate experiments.

Figure 2.

Dose-response curve of ERK activation upon PDGF stimulation in HPASMC (A) and upon VEGF stimulation in HPAEC (B). HPASMC (A) or HPAEC (B) were stimulated with increasing concentrations of PDGF (A) or VEGF (B) in serum-free medium. The western blot shown is representative of results from three separate experiments.

Figure 3.

Diagrams of the targeting regions of designed cell-penetrating peptides. (A) Illustration of the tyrosine sites in the C-tail of the PDGFR β , their downstream targets, and the amino acid sequences which target the Y740 and Y751 regions in the synthesized SynB3 CPP; (B) illustration of the tyrosine sites in the C-tail of the VEGFR2, their downstream targets, and the amino acid sequences which target the Y1175 region in the synthesized SynB3 CPP. NCK1: non-catalytic region of tyrosine kinase adaptor protein 1; RASA1: RAS p21 protein activator 1; GAP: GTPase-activating protein; GRB10: growth factor receptor-bound protein 10; PLCG1: phospholipase C Gamma 1; PTPN11: tyrosine-protein phosphatase non-receptor type 11; CBL: Cbl proto-oncogene, E3 ubiquitin protein ligase; SH2D2A/TSAD: SH2 domain containing 2A/T cell-specific adapter protein; SHB: SH2 domain-containing adapter protein B; MAPK: mitogen-activated protein kinase; FYN: FYN oncogene related to SRC, FGR, YES.

Figure 4.

Concentration-dependent effect of PDGFR β CPP Y751P (A), Y751 (B), and Y751Pshort (C) on PDGF-induced ERK and Akt activation in HPASMC. HPASMC in 6-well plates were grown to confluence and incubated with increasing concentrations of different CPP in serum-free medium for 0.5 h, followed by stimulation with 1 ng/mL PDGF. Western blot analysis of HPASMC lysates was used to show that ERK and Akt activation in response to PDGF. GAPDH was used as loading control. The results are representative of at least three experiments. The quantification of the Western blot analysis is illustrated as a graph which shows fold change in spot intensity of the bands with basal level as 1. * $p < 0.05$ compared with PDGF-stimulated activation.

Figure 5. Concentration-dependent effect of PDGFR β CPP Y740P (A) and Y740PGS (B) on PDGF-induced ERK and Akt activation in HPASMC. The experiment was performed as described in the legend for Figure 4.

Figure 6.

Concentration-dependent effect of ERK-CPP on PDGF-induced ERK and Akt activation in HPASMC. The experiment was performed as described in the legend for Figure 4 except that the ERK-CPP which inhibits ERK phosphorylation was used.

Figure 7. Concentration-dependent effect of VEGFR2 CPP Y1175P (A), Y1175 (B), and Y1175Pshort (C) on VEGF-A-induced ERK activation in HPAEC. HPAEC in 6-well plates were grown to confluence and incubated with increasing concentrations of VEGFR2 CPP Y1175P in serum-free medium for 0.5 h, followed by stimulation with 1 ng/mL VEGF. Western blot analysis of HPAEC lysates was used to show ERK activation in response to VEGF. GAPDH was used as loading control. The results are representative of at least three experiments. The quantification of the Western blot analysis is illustrated as a graph which shows fold change in spot intensity of the bands with basal level as 1. * $p < 0.05$ compared with VEGF-165-stimulated activation.

Figure 8. Concentration-dependent effect of ERK-CPP (A) and SynB3-ETBIC2 (B) on VEGF-induced ERK activation in HPAEC. The experiment was performed as described in the legend for Figure 7 except that the ERK-CPP which inhibits ERK phosphorylation or the SynB3-ETBIC2 CPP which targets the intracellular loop 2 of endothelin receptor was used.

Figure 9.

Effects of CPP on cell viability. Cells were incubated in quadruplicate with 100 μ M of PDGFR and VEGFR CPP and 0.5 mM H₂O₂ (positive control) for 30 min. MTT assay was then carried out using the manufacturer's standard procedure. The cell viability was presented as the percent of the mean OD value compared with that of the negative control (no CPP treatment). The error bars represent the standard deviation of measurement from four replicates. *denotes statistically significant difference in cell viability compared with control (no treatment) ($p < 0.05$).

Figure 10.

Effects of CPP on EGF-induced ERK and Akt activation in HPASMC. The experiment was performed as described in the legend for Figure 5 except that the 100 μ M of the various CPP were used.

Figure 11.

Effect of PDGFR β CPP Y751P (A) and ETRB CPP SynB3-ETBIC2 (B) on PDGF-induced HPASMC migration. Wound assays were performed on confluent HPASMC. The number of cells that migrated into the wound after 24 h was measured in control, 10 ng/mL PDGF and 10 ng/mL PDGF + 50 μ M PDGFR β CPP Y751P (A) or control, 10 ng/mL PDGF and 10 ng/mL PDGF + 50 μ M SynB3-ETBIC2 (B). Representative images 24 h after the wound from three separate experiments are shown. The black lines represent the cell borders at time 0 of the wound. Heights of the bar graph represent the average number of cells that migrated into the wound, and the standard deviation is represented by the error bars. HPASMC under those conditions were compared for their rates of migration into a wound area. * $p < 0.05$ compared with control. ** $p < 0.05$ compared with PDGF-induced migration.

Table 1IC50 values for ERK and Akt activation of the CPP targeting PDGFR β

CPP	IC50 (μ M)	
	pERK	pAkt
Y751P	37.05	22.64
Y751	N/A	N/A
Y751 Pshort	~49.32	N/A
Y740P	24.37	~51.62
Y740PGS	N/A	N/A
ERK–CPP	18.18	N/A

Table 2

IC50 values for ERK activation of the CPP targeting VEGFR2

CPP	IC50 (μ M)
Y1175P	24.72
Y1175	25.22
Y1175Pshort	16.14
ERK–CPP	15.87
SynB3–ETBIC2	N/A