

First-Generation Radiolabeled Cyclic Peptides for Molecular Imaging of Platelet-Derived Growth Factor Receptor α

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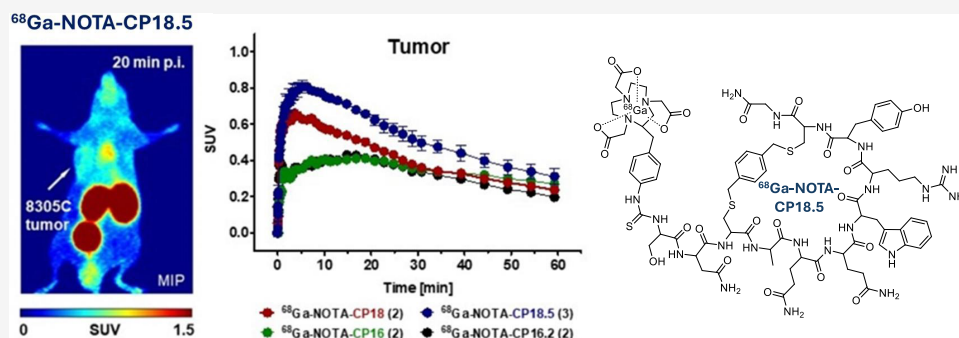
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ABSTRACT: Occult nodal spread and metastatic disease require longstanding imaging and biochemical assessments for thyroid cancer, a disease that has a propensity for diffuse, small-volume disease. We have developed a ^{64}Cu -labeled platelet-derived growth factor receptor α (PDGFRA) antibody for immuno-PET of PDGFRA in metastatic papillary thyroid cancer (PTC). The present work describes the discovery of small cyclic PDGFRA-targeting peptides, their binding features, and radiolabeling with positron emitter gallium-68 (^{68}Ga) for *in vitro* and *in vivo* characterization in thyroid cancer models. Phage-display technology with two separate libraries and seven different cell lines was used through three rounds of biopanning as well as flow cytometry and comparative analysis with recombinant protein to select specific peptide sequences. Phenotypic binding analysis was completed by using phosphorylation and cell migration assays. *In vitro* protein binding was analyzed with thermophoresis and flow cytometry using the fluorescent-labeled PDGFRA peptide. Peptide candidates were modified with the NOTA chelator for radiolabeling with ^{68}Ga . *In vitro* cell uptake was studied in various thyroid cancer cell lines. *In vivo* studies of ^{68}Ga -labeled peptides included metabolic stability and PET imaging. From the original library (10^{13} compounds), five different peptide groups were identified based on biopanning experiments with and without the α subunit of PDGFR, leading to ~ 50 peptides. Subsequent phenotypic screening revealed two core peptide sequences (CP16 and CP18) that demonstrated significant changes in the level of PDGFRA phosphorylation and cell migration. Alanine scan sublibraries were created from these two lead peptide sequences, and peptides were radiolabeled using ^{68}Ga - GaCl_3 at pH 4.5, resulting in RCP > 95% within 34–40 min, including SPE purification. Cyclic peptide CP18.5 showed the strongest effects on cell migration, flow cytometry, and binding by visual interference color assay. ^{68}Ga -labeled PDGFRA-targeting peptides showed elevated cell and tumor uptake in models of thyroid cancer, with ^{68}Ga -NOTA-CP18.5 being the lead candidate. However, metabolic stability *in vivo* was compromised for ^{68}Ga -NOTA-CP18.5 vs ^{68}Ga -NOTA-CP18 but without impacting tumor uptake or clearance profiles. First-generation radiolabeled cyclic peptides have been developed as novel radiotracers, particularly ^{68}Ga -NOTA-CP18.5, for the molecular imaging of PDGFRA in thyroid cancer.

KEYWORDS: PDGFRA, cyclic peptides, PET, thyroid cancer

INTRODUCTION

The increasing incidence of thyroid carcinoma worldwide, in many cases, small-volume disease, has sparked broad interest in better monitoring and prognostic tools to avoid overtreatment.^{1,2} While the majority of these cases are ultimately amenable to surgical or radioactive iodine therapy, staging and surveillance remain a challenge given the high propensity for small-volume lymphatic and distant spread that can occur many years after the original diagnosis.^{3,4} Lymphatic

metastases are the most common sites of recurrence, and previously, we identified that a significant driver of lymphatic

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spread and disease recurrence is platelet-derived growth factor receptor α (PDGFRA). PDGFRA overexpression is associated with poor survival in patients with papillary thyroid cancer (PTC) and is a driver of epithelial-to-mesenchymal transition (EMT), creating a cell phenotype with increased cell migration and invasive potential.^{5,6} The PDGFRA protein is typically minimally expressed in most normal tissues, but elevated PDGFRA protein expression has been described in human cancers, including ovarian, melanoma, gastrointestinal, stromal tumors, glioblastoma, prostate, breast, lung, renal cell, and sarcoma.^{7,8} These findings make PDGFRA a promising target and a potential theranostic for the management of carcinoma, especially for those patients at risk of or presenting with metastatic disease such as papillary thyroid cancer.⁹

Molecular imaging with radiolabeled probes using positron-emission tomography (PET) is a highly sensitive and noninvasive approach to monitor and characterize PDGFRA and associated diseases. Radiopharmaceuticals such as ¹⁸F-FDG¹⁰ and ^{99m}Tc-MIBI¹¹ have been described for use in molecular imaging of papillary thyroid cancer. Limitations of ¹⁸F-FDG in PTC are low sensitivity due to the slow-growing nature of PTC and potentially less glycolytic activity, as well as the lack of specificity due to uptake in inflammatory disease.¹² ^{99m}Tc-MIBI has a high negative predictive value (NPV) but suffers from low specificity and positive predictive value (PPV).¹¹ Those limitations in PTC can be addressed by the development of a ⁶⁸Ga-labeled PDGFRA-targeting peptide as an imaging probe with improved image quality and diagnostic accuracy.

Recently, we created a ⁶⁴Cu-radiolabeled antibody that targets PDGFRA in PTC in a mouse xenograft model.¹³ However, while demonstrating selectivity, the disadvantages of antibodies include high molecular weight, prolonged clearance times, and immunogenicity.^{14,15} Conversely, peptide-radioisotope conjugates represent favorable alternatives due to easier synthesis, higher tissue penetration, faster clearance from blood, and lower immunogenic potential.^{16,17} Cyclic peptides are an emerging trend in drug discovery and design of radiopharmaceuticals and radiotheranostics to improve metabolic stability and binding affinity.^{18,19} To date, only PDGFRB subtype targeting peptides have been reported in the literature.^{20–23} Askoxylakis et al. identified PDGFR-**P1** peptide (IPLPPSRPFKY-NH₂), a radiolabeled ¹²⁵I/¹³¹I conjugate that displayed promising *in vitro* PDGFR β targeting but lacking metabolic stability and *in vivo* binding enhancement.²² Effendi et al. used the same peptide to create a ⁶⁷Ga-radiopeptide, evaluating different linkers to improve metabolic stability and binding affinity.²³ These ⁶⁷Ga-peptides with an ethylene glycol linker exhibited high *in vivo* metabolic stability but without sufficient tumor accumulation, possibly due to the fact that the β subunit of PDGFR exhibits relatively high levels of expression in many normal tissues. Unlike PDGFRB, few studies have successfully defined PDGFRA ligand or drug-binding pocket structures, and directed efforts to target PDGFRA have been limited.²⁴ The goal of the present study was to describe the discovery of novel cyclic PDGFRA-targeting peptides and their *in vitro* and *in vivo* binding characteristics by using a combination of phenotypic and molecular screening techniques. We explore the radiolabeling with ⁶⁸Ga ($t_{1/2}$ = 68 min), *in vivo* metabolic stability, and *in vivo* characterization with PET imaging in thyroid cancer mouse models. We believe that targeting the α subunit of PDGFR not only has potential use in thyroid carcinoma but

also in other carcinomas as well including GIST, glioblastoma, and lymphomas, where a large body of literature demonstrates the efficacy of tyrosine kinase inhibition of PDGFRA signaling.

MATERIALS AND METHODS

Materials. All chemicals were obtained from Millipore-Sigma (ON, Canada). PDGFRA peptides were synthesized via automated solid-phase peptide synthesis (SPPS) using the Syro I peptide synthesizer (MultiSynTech/Biotage, Charlotte, North Carolina). A 50 mCi (1850 MBq) iThemba Laboratories ⁶⁸Ge/⁶⁸Ga generator from IsoSolutions Inc. (Vancouver, BC, Canada) was used as the ⁶⁸Ga source. Mass spectra were recorded on an Agilent Technologies 1260 HPLC with G6130B MSD (LCMS ESI, Santa Clara, California) or a Bruker 9.4T Apex-Qe FTICR (Bruker Daltonics, Billerica, Massachusetts) for HR-MALDI. Analytical HPLC was performed on a Shimadzu system (Mandel Scientific, Guelph, ON, Canada) equipped with a DGU-20AS degasser, a SIL-20A HT autosampler, a LC-20AT pump, a SPD-M20A photodiode array detector, and a Ramona Raytest radiodetector using a Phenomenex Luna 10u C18(2) 100A, 250 mm $\times$ 4.6 mm column. Semipreparative HPLC was performed on a Gilson system (Mandel Scientific, Guelph, ON, Canada) with a 321 pump and a 155 dual wavelength detector installed with a Phenomenex Jupiter 10u Proteo 90A, 250 mm $\times$ 10 mm, 4.5 μ m C18 column. UV absorbance was monitored at wavelengths of 210 and 254 nm. The mobile phase consisted of water/0.2%TFA as solvent A and acetonitrile as solvent B. Human thyroid cancer cell line BCPAP expressing PDGFRA (BCPAP(+)) per transfection or not (BCPAP(–)) from mock transfection and human thyroid anaplastic carcinoma cell line 8305C (American Type Tissue Culture Center, Manassas, Virginia) were cultivated in High Glucose Dulbecco's modified Eagle's medium (DMEM) supplemented with Ham's F-12 and 10% heat-inactivated fetal bovine serum (FBS) and 1% penicillin/streptomycin from Invitrogen (Life Technologies Inc., Grand Island, New York). All animal studies were carried out according to the guidelines of the Canadian Council on Animal Care (CCAC) and approved by the Cross Cancer Institute Animal-Care Committee. *In vivo* studies were done using control BALB/c and male BCPAP(+) and BCPAP(–) tumor-bearing NSG nude mice (body weight: 20–24 g, Charles River Laboratories, Saint-Constant, QC, Canada). BCPAP(+) tumor-bearing mice also received a doxycycline slow-releasing pellet (0.5 mg/21 days; Innovative Research of America, Sarasota, Florida) that was implanted into the right flank at the same time. For tumor xenografts, about 3×10^6 BCPAP(+) or BCPAP(–) or 8305C cells in 200 μ L of PBS/Matrigel (50/50) were injected subcutaneously into the upper left flank of male nude NSG mice. After 3–5 weeks, BCPAP(+), BCPAP(–), and 8305C tumors reached sizes of ~ 500 mm³ and were used for the experiments as described.

Phage-Display Libraries. The phage-display library Cy5-MBX-SXC6C of macrocyclic glycopeptides (10^{13} unique cyclic peptide sequences) was generated in one rapid, biocompatible step by reacting phage-displayed libraries of peptides with dichloro-oxime derivatives as previously described.²⁵ After incubating the 2×10^6 cells with 10^{11} pfu phage library for 1 h at 4 $^{\circ}$ C, Cy5 cells were sorted and submitted to deep sequence analysis using Illumina platform and MatLab software analysis.²⁹ Similarly, phage library MBX-SXC6C was panned for binding to recombinant PDGFR α -His protein; after 90 min incubation at room temperature, the

bound phage was eluted and amplified to create a subset library (10^6 clones).

Peptide Synthesis. All linear PDGFRA-targeting peptides were synthesized by Fmoc-based solid-phase peptide synthesis (SPPS) using a fully automated peptide synthesizer (Syro I, MultiSynTech/Biotage). Fifty milligrams of Rink amide 4-methylbenzhydrylamine (MBHA) resin (50 mg, 100–200 mesh, loading: 0.78 mmol/g) was used as the solid support. Rink amide MBHA resin was allowed to swell in 2 mL of dimethylformamide (DMF) for 15 min. Fluorenylmethyloxycarbonyl (Fmoc) group deprotection was achieved by treatment with 40% piperidine/DMF for 5 min, followed by treatment with 20% piperidine/DMF for 15 min. Fmoc-protected amino acids (5 equiv) were activated and coupled using 5 equiv of *O*-benzotriazole-*N,N,N',N'*-tetramethyluronium-hexafluoro-phosphate (HBTU), 5 equiv of ethyl-2-cyano-2-(hydroxyimino) acetate (Oxyma), and 10 equiv of *N,N*-diisopropylethylamine (DIPEA) over a 60 min time period followed by washing steps with DMF. Treatment with an acidic solution containing 87.5% TFA, 5% water, 5% thioanisole, and 2.5% 1,2-ethylenedioxy-diethanedithiol for 3 h 50 min induced cleavage of the assembled peptides from the resin with simultaneous deprotection of amino acid side chains. The resin was removed from the peptide solution through a syringe filter, and peptides were precipitated by the addition of ice-cold diethyl ether. Residual ether was removed by a syringe filter, and the precipitated crude peptides were dried under vacuum. HPLC purification using a Phenomenex Jupiter 10u Proteo 90A, 250 mm $\times$ 10 mm, a 4.5 μ m C18 column at a flow rate of 2 mL/min and a gradient of water/0.2% TFA as solvent A and acetonitrile as solvent B: 0–5 min 20% B, 10 min 35% B, 20 min 50% B, 30–40 min 70% B, and subsequent lyophilization gave linear peptides as white solids.

Linear CP18.5. 23.8 mg (18.1 μ mol, 47% isolated yield). MW $C_{54}H_{79}N_{19}O_{16}S_2$ 1313.54, measured ESI-MS (positive) m/z 1314.3 $[M + H]^+$, 657.8 $[M + 2H]^{2+}$. HPLC: t_R = 14.5 min; purity: 99.8%.

Linear CP18. 19.0 mg (14.2 μ mol, 37% isolated yield). MW $C_{56}H_{81}N_{19}O_{16}S_2$ 1339.56, measured ESI-MS (positive) m/z 1340.5 $[M + H]^+$, 670.9 $[M + 2H]^{2+}$. HPLC: t_R = 14.3 min; purity: 94.0%.

Linear CP16. 21.6 mg (18.1 μ mol, 48% isolated yield). MW $C_{52}H_{73}N_{15}O_{14}S_2$ 1195.49, measured ESI-MS (positive) m/z 1196.3 $[M + H]^+$, 598.8 $[M + 2H]^{2+}$. HPLC: t_R = 16.4 min, purity: >85%.

Linear CP16.2. 22.0 mg (19.3 μ mol, 50% isolated yield). MW $C_{50}H_{70}N_{14}O_{13}S_2$ 1138.47, measured ESI-MS (positive) m/z 1139.3 $[M + H]^+$, 570.3 $[M + 2H]^{2+}$. HPLC: t_R = 16.8 min, purity: >92%.

PDGFR-P1 (PDGFR β Peptide). 21.7 mg (13.9 μ mol, 36% isolated yield). MW $C_{79}H_{116}N_{18}O_{15}$ 1556.88, measured ESI-MS (positive) m/z 779.2 $[M + 2H]^{2+}$, 520.2 $[M + 3H]^{3+}$. HR-MALDI (positive): 1557.89178 $[M + H]^+$. HPLC: t_R = 16.2 min, purity: 95.3%.

Cyclization of Linear PDGFRA-Targeting Peptides. The cyclization reaction was performed according to Timmerman et al. 2005²⁶ using the linchpin α,α -dibromo-*m*-xylene. Briefly, a 0.5 mM peptide solution (7.0 mg linear PDGFRA-targeting peptide (1 equiv, 5.2–6.1 μ mol)) in MeCN/20 mM NH_4HCO_3 buffer (pH 7.8) 1:7 was stirred with 1.6–2.0 mg (1.05 equiv, 5.5–7.5 μ mol) α,α -dibromo-*m*-xylene at room temperature for 20 min to yield 80–96% cyclized PDGFRA-targeting peptide. The reaction mixture was purified with

semipreparative HPLC purification using a Phenomenex Jupiter 10u Proteo 90A, 250 mm $\times$ 10 mm, 4.5 μ m C18 column at a flow rate of 2 mL/min and a gradient of water/0.2% TFA as solvent A and acetonitrile as solvent B: 0–10 min 10% B, 25 min 50% B, 30–40 min 80% B, 40–45 min 90% B (t_R (cyclic CP18.5) = 27.5 min, t_R (cyclic CP18) = 27.7 min, t_R (cyclic CP16) = 29.9 min, t_R (cyclic CP16.2) = 30.3 min). HPLC solvent was reduced under a vacuum using a rotary evaporator. Subsequent lyophilization yielded cyclic PDGFRA-targeting peptides as a white powder. Quality control was performed on an analytical Shimadzu HPLC system using a Phenomenex Luna 10u C18(2) 100A, 250 mm $\times$ 4.6 mm column at a constant flow rate of 1 mL/min, and the following gradient with water/0.2% TFA as solvent A and acetonitrile as solvent B: 0–3 min 10% B, 10 min 30% B, 17 min 50% B, 23 min 70% B, 27–30 min 90% B.

Cyclic CP18.5. 6.1 mg (4.3 μ mol, 81% isolated yield). MW $C_{62}H_{85}N_{19}O_{16}S_2$ 1415.59, measured ESI-MS (positive) m/z 1416.4 $[M + H]^+$, 708.9 $[M + 2H]^{2+}$. HR-MALDI (positive): 1416.59223 $[M + H]^+$. HPLC: t_R = 15.5 min, purity: 98.2%.

Cyclic CP18. 4.3 mg (3.0 μ mol, 60% isolated yield). MW $C_{64}H_{87}N_{19}O_{16}S_2$ 1441.60, measured ESI-MS (positive) m/z 1442.4 $[M + H]^+$, 721.9 $[M + 2H]^{2+}$. HPLC: t_R = 15.6 min, purity: 98.4%.

Cyclic CP16. 4.6 mg (3.5 μ mol, 61% isolated yield). MW $C_{60}H_{79}N_{15}O_{14}S_2$ 1297.54, measured ESI-MS (positive) m/z 1298.3 $[M + H]^+$, 649.9 $[M + 2H]^{2+}$. HR-MALDI (positive): 1298.54409 $[M + H]^+$. HPLC: t_R = 17.5 min, purity: 99.0%.

Cyclic CP16.2. 4.6 mg (3.7 μ mol, 60% isolated yield). MW $C_{58}H_{76}N_{14}O_{13}S_2$ 1240.52, measured ESI-MS (positive) m/z 1241.3 $[M + H]^+$, 621.4 $[M + 2H]^{2+}$. HR-MALDI (positive): 1241.52246 $[M + H]^+$. HPLC: t_R = 18.1 min; purity: 96.1%.

Synthesis of Optical Probe FITC-CP18.5. 2.9 mg portion (1 equiv, 2.0 μ mol) of cyclic CP18.5 peptide was incubated with 1.5 mg (1.9 equiv, 3.8 μ mol) of FITC isomer I and 6.9 μ L of triethylamine (25 equiv, 49.7 μ mol) in 500 μ L of DMF at rt for 16 h in the dark. The pH was 9.0. HPLC purification of the reaction mixture was performed using a Phenomenex Jupiter 10u Proteo 90A, 250 mm $\times$ 10 mm, 4.5 μ m C18 column at a flow rate of 2 mL/min and a gradient of water/0.2% TFA as solvent A and acetonitrile as solvent B: 0–10 min 10% B, 25 min 50% B, 30–40 min 80% B, 40–45 min 90% B (t_R = 30.0 min). HPLC solvent was reduced under a vacuum using a rotary evaporator, and lyophilization gave the FITC-modified peptide FITC-CP18.5 as a bright yellow powder (2.5 mg, 1.4 μ mol, 70% isolated yield). MW $C_{83}H_{96}N_{20}O_{21}S_3$ 1804.62, measured ESI-MS (positive) m/z 903.4 $[M + 2H]^{2+}$, 602.9 $[M + 3H]^{3+}$. HR-MALDI (positive): 1805.62646 $[M + H]^+$. Quality control was performed on an analytical Shimadzu HPLC system using a Phenomenex Luna 10u C18(2) 100A, 250 mm $\times$ 4.6 mm column at a constant flow rate of 1 mL/min and the following gradient with water/0.2% TFA as solvent A and acetonitrile as solvent B: 0–3 min 10% B, 10 min 30% B, 17 min 50% B, 23 min 70% B, 27–30 min 90% B (t_R = 18.0 min, purity: 99.8%).

Synthesis of Labeling Precursor NOTA-Modified PDGFRA-Targeting Peptide. Three milligram (1 equiv, 2.1–2.4 μ mol) portion of cyclic PDGFRA-targeting peptide (2.5 mg (1 equiv, 1.7 μ mol for cyclic CP18.5)) was dissolved in 75 μ L of DMF in a LoBind Eppendorf tube before 1.3–1.8 mg (2.3–3.1 μ mol) of 1.3 equiv pSCN-Bn-NOTA in 75 μ L of DMF was added. The pH was adjusted to 9 by the addition of 14–24 μ L (104–176 μ mol, 50 equiv) triethylamine (TEA).

The reaction mixture was incubated at 37 °C for 2 h, then at 25 °C overnight before it was subjected to semipreparative HPLC purification. HPLC purification was performed using a Phenomenex Jupiter 10u Proteo 90A, 250 mm × 10 mm, 4.5 μm C18 column at a flow rate of 2 mL/min and a gradient 10 mM NH₄OAc_{aq} (pH 6.6) as solvent A and acetonitrile as solvent B: 0–10 min 10% B, 25 min 50% B, 30–40 min 80% B, 40–45 min 90% B (*t_R* (NOTA-CP18.5) = 27.0 min, *t_R* (NOTA-CP18) = 26.9 min, *t_R* (NOTA-CP16) = 28.8 min, *t_R* (NOTA-CP16.2) = 28.5 min). HPLC solvent was reduced under vacuum using a rotary evaporator, and lyophilization gave the chelator-modified peptide NOTA-peptides as acetate salts (white powder). Quality control was performed on an analytical Shimadzu HPLC system using a Phenomenex Luna 10u C18(2) 100A, 250 mm × 4.6 mm column at a constant flow rate of 1 mL/min and the following gradient with water/0.2% TFA as solvent A and acetonitrile as solvent B: 0–3 min 10% B, 10 min 30% B, 17 min 50% B, 23 min 70% B, 27–30 min 90% B.

NOTA-CP18.5. 1.3 mg (0.7 μmol, 47% isolated yield). MW C₈₂H₁₁₁N₂₃O₂₂S₃ 1865.74, measured ESI-MS (positive) *m/z* 934.0 [M + 2H]²⁺, 623.2 [M + 3H]³⁺. HR-MALDI (positive): 1866.75107 [M + H]⁺. HPLC: *t_R* = 16.4 min, purity: >95%.

NOTA-CP18. 1.9 mg (1.0 μmol, 49% isolated yield). MW C₈₄H₁₁₃N₂₃O₂₂S₃ 1891.76, measured ESI-MS (positive) *m/z* 946.9 [M + 2H]²⁺, 631.9 [M + 3H]³⁺. HR-MALDI (positive): 1892.76759 [M + H]⁺. HPLC: *t_R* = 16.2 min, purity: 94.0%.

NOTA-CP16. 2.2 mg (1.3 μmol, 55% isolated yield). MW C₈₀H₁₀₅N₁₉O₂₀S₃ 1747.69, measured ESI-MS (positive) *m/z* 874.9 [M + 2H]²⁺, 583.9 [M + 3H]³⁺. HR-MALDI (positive): 1748.70203 [M + H]⁺. HPLC: *t_R* = 18.7 min, purity: 95.0%.

NOTA-CP16.2. 2.2 mg (1.3 μmol, 54% isolated yield). MW C₇₈H₁₀₂N₁₈O₁₉S₃ 1690.67, measured ESI-MS (positive) *m/z* 846.7 [M + 2H]²⁺, 564.8 [M + 3H]³⁺. HR-MALDI (positive): 1692.66453 [M + H]⁺. HPLC: *t_R* = 19.2 min; purity: 97.0%.

Synthesis of Ga-NOTA-Modified PDGFRA-Targeting Peptides. 1.2 mg (1 equiv, 0.6 μmol) NOTA-CP18.5 and 2.0 mg (12 equiv, 7.8 μmol) ^{nat}Ga(NO₃)₃ were dissolved in 220 μL of 10% MeCN/1 M NaOAc buffer (pH 4.5, trace-metal grade) in a LoBind Eppendorf tube. The pH of the reaction mixture was 4.5. After incubation at 37 °C for 2.5 h, semipreparative HPLC purification was performed using a Phenomenex Jupiter 10u Proteo 90A, 250 mm × 10 mm, 4.5 μm C18 column at a flow rate of 2 mL/min and a gradient of 10 mM NH₄OAc_{aq} (pH 6.6) as solvent A and acetonitrile as solvent B: 0–10 min 10% B, 25 min 50% B, 30–40 min 80% B, 40–45 min 90% B (*t_R* (^{nat}Ga-NOTA-CP18.5) = 28.2 min). HPLC solvent was reduced under a vacuum using a rotary evaporator, and lyophilization gave ^{nat}Ga-NOTA-CP18.5 peptide as a white powder. Quality control was performed on an analytical Shimadzu HPLC system using a Phenomenex Luna 10u C18(2) 100A, 250 mm × 4.6 mm column at a constant flow rate of 1 mL/min and the following gradient with water/0.2% TFA as solvent A and acetonitrile as solvent B: 0–3 min 10% B, 10 min 30% B, 17 min 50% B, 23 min 70% B, 27–30 min 90% B.

^{nat}Ga-NOTA-CP18.5. 0.5 mg (0.3 μmol, 42% isolated yield). MW C₈₂H₁₀₈GaN₂₃O₂₂S₃ 1931.65, measured ESI-MS (positive) *m/z* 967.3 [M + 2H]²⁺, 645.5 [M + 3H]³⁺. HPLC: *t_R* = 15.9 min, purity: >99.2%.

Western Blot and Flow Cytometry for PDGFRA Expression in BCPAP and BON1 Cell Lines. Stable gene transfer and expression of PDGFRA in BCPAP cells have been

described elsewhere.²⁷ To express human PDGFRA complementary DNA in Bon1 cells, we used a doxycycline-inducible retrovirus system (Lenti-X Lentiviral Expression Systems; Clontech Laboratories, Inc., Mountain View, California). Briefly, cells were first transduced with the LVX-Tet-On advanced lentivirus (Neo+), followed by selection in G418 (1.0 mg/mL). Resistant cells were then transduced with the LVX-Tight-Puro (Puro+) vector or sequence-verified derivatives expressing wild-type human PDGFRA complementary DNA, followed by selection in puromycin (2.5 μg/mL). Complementary DNA expression was induced by the addition of doxycycline (2 μg/mL) (Supporting Figure S3).

Visual Interference Color Assay (VICA).²⁸ Typically, 18 μL drops of protein–peptide mixtures and their corresponding controls were deposited onto 7 mm diameter circles on the anodized aluminum oxide surface and incubated for 20 min at room temperature under 100% relative humidity. The slides were rinsed thoroughly with deionized water and subsequently washed in 1 × PBS solution for 5 min. The aluminum oxide slides were photographed with the use of a polarizing film to eliminate p-polarized light off the device surface and viewed at an angle of incidence of 75° to generate the strongest color contrast by matching s-polarized light reflection intensities off the alumina and underlying surfaces. The red, green, and blue (RGB) coordinate system was used to quantitatively define the visible colors. The color difference or distance (ΔC) was calculated according to the following formula

$$\text{distance2} = (R2 - R1)^2 + (G2 - G1)^2 + (B2 - B1)^2$$

Flow Cytometry. BCPAP(–) and BCPAP(+) cells were seeded into a 6-well plate with a concentration of 100 000 cells per well one night before. Cells were pretreated with 2 mM cyclic CP18.5, 5 mM PDGFRA-P1 peptide, or 100 μg of PDGFRA monoclonal antibody (Lartruvo, Eli Lilly) (Supporting Figure S3) illustrates the specific binding of Lartruvo to PDGFRA expressing cells) in 200 μL PBS (Phosphate Buffer Saline) for 30 min. The PBS was then aspirated, and cells were washed with 5 mL of PBS per well. Cells were then incubated with 20 μM FITC–CP18.5 (1% DMSO/PBS solution) in 200 μL of PBS for 30 min, with gentle rocking every 5 min. All procedures containing FITC-labeled compounds were performed in the dark. PBS was aspirated, and cells were washed again with 5 mL of PBS. Two hundred microliters of trypsin was added to each well, and the plates were placed in the sterile 37 °C incubator for 2 min. A total of 1 mL of DMEM/F-12 (Thermo Fisher Scientific) with 10% fetal bovine serum (Thermo Fisher Scientific) and 1% penicillin–streptomycin medium was added to inactivate trypsinization. The cell solution was then transferred into 15 mL centrifuge tubes and was centrifuged at 1600 rpm for 5 min. The supernatant was aspirated, and the pellet was resuspended in 10 mL of PBS and centrifuged again at 1600 rpm for 5 min. PBS was aspirated, and cells were fixed in 5 mL of ethanol/PBS (1:1) solution for 1 h, with occasional mixing. After fixation, the tubes were centrifuged at 1600 rpm for 5 min. The supernatant was aspirated, and the cell pellet was washed with 10 mL of PBS. The pellet was resuspended in 1 mL of PBS and was transferred to a labeled FACS tube (352054; Corning), wrapped in foil, and then placed on ice until they were analyzed. Quantification was done using a BD FACSCanto II (BD Biosciences, San Jose, California) Flow Cytometer using FACSDiva 8.0 Software. Analysis was performed using FlowJo

8.0. A Student's *t*-test was performed to determine statistical significance.

Thermophoresis. Twelve microliter aliquot of 6× His-tag-PDGFR protein (1 $\mu\text{g}/\mu\text{L}$) was diluted in 930 μL of phosphate-buffered saline (pH 7.4) with 0.05% Tween-20 (PBS-T). Twenty microliters of 100 nM of Monolith His-Tag Labeling Kit RED-tris-NTA second generation (MO-L018) (NanoTemper Technologies) was added to the His-tag-PDGFR dilution, and the mixture was incubated on a thermoshaker at 600 rpm for 60 min at room temperature in the dark. The final concentration of the fluorescently labeled 6× His-tag-PDGFR protein was 100 nM. All of the compounds were first diluted to a concentration of 6 mM in DMSO and then to 3 mM with PBS-T to prevent precipitation. Low-binding tips and tubes were used for the entire process of MST. Serial dilutions of each compound were prepared by adding 1 μL of stock solution to 9 μL of PBS-T (Tube 1, final concentration of 300 μM) and transferring 5 μL of tube 1 solution to tube 2 that already had 5 μL of PBS-T inside (Tube 2, final concentration of 150 μM). Five microliter tube 2 solution was then transferred to tube 3, which already had 5 μL of PBS-T (tube 3, final concentration of 75 μM). This process stopped until tube 16 reached a final concentration of 0.0046 μM . Five microliter portion of the labeled 6× His-tag-PDGFR protein (100 nM) was added to each serially diluted tube, and then the mixed solutions were loaded into capillaries. MicroScale thermophoresis experiments were performed on a NanoTemper Monolith NT.115 (NanoTemper Technologies).

Phosphorylation and Transwell Invasion Assays. To document the effect of the cyclic peptides on PDGFRA activation by PDGF-AA, BCPAP cells expressing PDGFRA and controls were pretreated with 10 mM–10 μM of cyclic peptide for 30 min, followed by activation with PDGF-AA (50 nM) for 10 or 20 min. Cells were lysed in RIPA buffer (150 mM NaCl, 100 mM Tris (pH 8.0), 1% Triton X-100, 1% deoxycholic acid, 0.1% SDS, 5 mM EDTA, and 10 mM NaF) supplemented with 1 mM sodium vanadate, 2 mM leupeptin, 2 mM aprotinin, 1 mM phenylmethylsulfonyl fluoride (PMSF), 1 mM DTT, 2 mM pepstatin, and 1:100 protease inhibitor cocktail set III on ice. After centrifugation at 4 °C at 18 000 rpf for 15 min, the supernatant was harvested as the total cellular protein extract, aliquoted, and stored at –80 °C. Protein concentration was determined using Pierce BCA Protein Assay Kit (Pierce Biotechnology, Rockford, Illinois). Aliquots (usually 50 μg) of protein extract samples were resolved by SDS-PAGE and transferred to a nitrocellulose membrane, blocked in 5% nonfat milk in TBS containing 0.05% Tween-20 for 60 min, followed by incubation with primary antibodies at 4 °C overnight (phospho-PDGFR α [EP2478]:(Y720) and (Y742) were from Abcam Inc. (Waltham, Massachusetts); [23B2]:(Y754) and phospho-PDGFR β [88H8]:(Y751) from Cell Signaling Technology (Danvers, Massachusetts)). Protein bands were detected by incubation with horseradish peroxidase-conjugated antibodies (Pierce Biotechnology, Rockford, Illinois) and visualized with the SuperSignal West Pico chemiluminescence substrate (Thermo Scientific, Rockford, Illinois). To assess cell migration after treatment with cyclic peptides, 8 μm polycarbonate membranes were incubated in fibronectin (10 $\mu\text{g}/\text{mL}$ in PBS, at 4 °C, overnight). Serum-starved cells were seeded at a density of 5×10^4 cells/well after 20 min of cyclic peptide exposure (60–0.1 μM). Migration toward PDGF-AA (100 nM) as a

chemoattractant was measured after 4 h by crystal blue staining of the membrane.

Radiolabeling of PDGFRA-Targeting Peptides: ^{68}Ga -Labeling of NOTA-CP18.5 ($n = 32$). Radionuclide ^{68}Ga was eluted with ~ 5 mL of 0.6 N HCl (trace-metal grade) from the $^{68}\text{Ge}/^{68}\text{Ga}$ generator via an automated GRP module (Scintomics GmbH, Fuerstenfeldbruck, Germany). The high activity fraction of the ^{68}Ga -eluate (1.5 mL) was collected in the plastic reaction vessel, while the rest of the radioactivity was dispensed in the 20 mL syringe of the GRP module dispenser unit. 50 μg of lyophilized NOTA-CP18.5 was reacted with 1000 μL of ^{68}Ga -GaCl₃ and 500 μL of 2 M NaOAc buffer (pH 8.0) at 60 °C (21 min, pH 4.5) in a LoBind Eppendorf tube using a Thermoshaker. ^{68}Ga -incorporation was monitored by Radio-TLC (silica TLC plates, mobile phase: 0.1 M citric acid, R_f (free ^{68}Ga) = 0.9–1.0, R_f (^{68}Ga -NOTA-CP18.5) = 0.0–0.1). The reaction mixture was diluted with 9 mL of DI H₂O and subjected to solid-phase extraction using a Sep-Pak C18 Plus cartridge (preconditioned with 5 mL of MeCN and 10 mL of DI H₂O). ^{68}Ga -NOTA-CP18.5 was eluted with EtOH in fractions of 2 drops. Typically, ^{68}Ga -NOTA-CP18.5 was recovered in fractions 4–6. For subsequent *in vitro* and *in vivo* evaluation, 50 μL of the ^{68}Ga -NOTA-CP18.5 fraction was formulated with 200 μL of saline. Quality control was performed using analytical Radio-HPLC at a constant flow rate of 1 mL/min and the following gradient with water/0.2% TFA as solvent A and acetonitrile as solvent B: 0–3 min 10% B, 10 min 30% B, 17 min 50% B, 23 min 70% B, 27–30 min 90% B (t_R (^{68}Ga -NOTA-CP18.5) = 16.0 min, radiochemical purity: 95–98%). Total synthesis time was 30–40 min. radiochemical yield: 16–20% (dc) for one injectable ^{68}Ga -NOTA-CP18.5 product fraction.

The following ^{68}Ga -labeled PDGFRA-targeting peptides have been synthesized according to the protocol mentioned above for ^{68}Ga -NOTA-CP18.5.

^{68}Ga -NOTA-CP18 ($n = 5$). Total synthesis time: 30–49 min. Radiochemical yield: 11–31% (dc) for one injectable ^{68}Ga -NOTA-CP18 product fraction. Radiochemical purity: >95%, t_R (HPLC) = 16.2 min.

^{68}Ga -NOTA-CP16 ($n = 3$). Total synthesis time: 30–34 min. Radiochemical yield: 11–16% (dc) for one injectable ^{68}Ga -NOTA-CP16 product fraction. Radiochemical purity: >95%, t_R (HPLC) = 18.6 min.

^{68}Ga -NOTA-CP16.2 ($n = 1$). Total synthesis time: 34 min. Radiochemical yield: 22% (dc) for one injectable ^{68}Ga -NOTA-CP16.2 product fraction. Radiochemical purity >95%, t_R (HPLC) = 19.1 min.

Determination of Lipophilicity of ^{68}Ga -NOTA-CP18.5. Lipophilicity was determined according to the shake-flask method by determining the partition coefficient of ^{68}Ga -NOTA-CP18.5 in *n*-octanol and PBS buffer (pH 7.4) as the aqueous phase. The organic and the aqueous phases were presaturated 24 h before the actual start of the experiment. Five hundred microliters of each layer was added to 0.9–2 MBq of ^{68}Ga -NOTA-CP18.5 in a LoBind Eppendorf tube, and the mixture was mixed vigorously for 3 min. The layers were allowed to separate by centrifugation at 2000 rpm for 5 min. Aliquots of 100 μL were removed from each phase and measured in a Wizard γ counter (Wallac 1480 Wizard-3, PerkinElmer, Woodbridge, Ontario, Canada). Calculated $\log D_{7.4}$ values are expressed as mean $\pm$ SD from 3 experiments, each performed in triplicate.

In Vitro Cell Uptake Studies. BCPAP(+), BCPAP(−), and 8305C cells were grown in 12-well plates (100 000 cells/well). BCPAP(+) and BCPAP(−) cells were treated with doxycycline (2 $\mu\text{g}/\text{mL}$) for at least 48 h before the *in vitro* experiment. The medium was removed 1 h prior to the experiment, and cells were washed 2 times with PBS. Next, 500 μL of Krebs-Ringer solution with 0.1–0.5 MBq ^{68}Ga -NOTA-CP18.5 (^{68}Ga -NOTA-CP18 or ^{68}Ga -NOTA-CP16) was added to each well. Plates were incubated at 37 $^{\circ}\text{C}$ for specific time points (5, 15, 30, and 60 min). Radiotracer uptake was stopped with 1 mL of ice-cold PBS, and cells were washed 2 times with PBS and lysed in 0.4 mL of lysis buffer (50 mM Tris, 150 mM NaCl, 0.1% SDS, 0.5% sodium deoxycholate, 0.5% Triton X). Radioactivity in cell lysates was measured using Hidex Automatic γ Counter (Turku, Finland via GTL, Mississauga, Ontario, Canada). Total protein concentration in the samples was determined using a Pierce Bicinchoninic Acid—based Protein Assay (Thermo Fisher Scientific). Data were calculated as the percentage of total added radioactivity per milligram of protein.

Dynamic PET Imaging Studies. PET imaging of PDGFRA-targeting ^{68}Ga -NOTA-peptides was performed on an INVEON PET/CT scanner (Siemens Preclinical Solutions, Knoxville, Tennessee). Prior to radiotracer injection, mice were anesthetized through inhalation of isoflurane in 40% oxygen/60% nitrogen (gas flow 1 L/min), and body temperature was kept constant at 37 $^{\circ}\text{C}$. Mice were placed in a prone position in the center of the field of view. A transmission scan for attenuation correction was not acquired. Mice were injected with 4–8 MBq of ^{68}Ga -NOTA-CP18.5 (^{68}Ga -NOTA-CP18 or ^{68}Ga -NOTA-CP16 or ^{68}Ga -NOTA-CP16.2) in 150 μL of 20% EtOH/saline solution through a tail vein catheter. For blocking studies with peptide, ^{68}Ga -NOTA-CP18.5 BCPAP(+)-tumor-bearing NSG mice were predosed i.v. with 300 μg of cyclic CP18.5 (15 min prior to radiotracer injection) in 50 μL of saline. Data acquisition was performed over 60 min in 3D list mode. The dynamic list mode data were sorted into sinograms with 54-time frames (10 s $\times$ 2 s, 8 s $\times$ 5 s, 6 s $\times$ 10 s, 6 s $\times$ 20 s, 8 s $\times$ 60 s, 10 s $\times$ 120 s, 6 s $\times$ 300 s). The frames were reconstructed using maximum a posteriori (MAP) as reconstruction mode. No correction for partial volume effects was applied. The image files were processed using ROVER v2.0.51 software (ABX GmbH, Radeberg, Germany). Masks defining 3D regions of interest (ROI) were set, and the ROIs were defined by thresholding. ROIs covered all visible tumor mass of the subcutaneous tumors, and the thresholds were defined by 50% of the maximum radioactivity uptake level. Mean standardized uptake values [$\text{SUV}_{\text{mean}} = (\text{activity}/\text{mL tissue})/(\text{injected activity}/\text{body weight}), \text{mL}/\text{g}$] were calculated for each ROI, and time–activity curves (TAC) were generated. All semiquantified PET data are presented as means $\pm$ SEM. Statistical differences were tested by Student's *t*-test and were considered significant for $p < 0.05$.

Metabolic Stability In Vivo. For metabolic stability studies *in vivo*, control BALB/c mice were anesthetized through inhalation of isoflurane in 40% oxygen/60% nitrogen (gas flow 1 L/min) prior to i.v. radiotracer injection via the tail vein. Mice were injected with 25–30 MBq of ^{68}Ga -NOTA-CP18.5 or ^{68}Ga -NOTA-CP18 in 150 μL of 20% EtOH/saline. Venous blood samples were collected at 5, 15, 30, and 60 min post-injection via the mouse tail vein and further processed. Blood cells were separated by centrifugation (13 000 rpm $\times$ 5

min). The supernatant was removed, and the contained proteins were precipitated by the addition of 2 volume parts of methanol (2 vol of MeOH/1 vol of sample). Another centrifugation step (13 000 rpm $\times$ 5 min) was performed to obtain plasma. The clear plasma supernatant was injected into a Shimadzu HPLC system. The samples were analyzed using a Phenomenex Luna 10u C18(2) 100A, 250 mm $\times$ 4.6 mm column at a constant flow rate of 1 mL/min, and the following gradient with water/0.2% TFA as solvent A and acetonitrile as solvent B: 0–3 min 10% B, 10 min 30% B, 17 min 50% B, 23 min 70% B, 27–30 min 90% B.

RESULTS AND DISCUSSION

Biopanning and Peptide Selection. The general biopanning strategy is outlined in Figure 1. In short, phage-

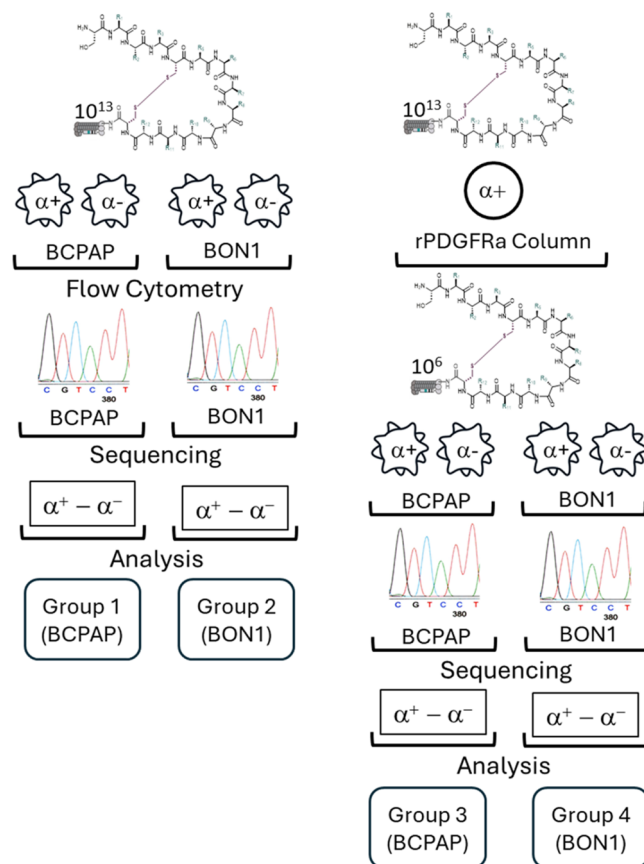


Figure 1. Scheme for the biopanning strategy of phage library MBX-SXC6C against BCPAP and BON1 cells grouped according to cell line and panning method.

display library Cy5-MBX-SXC6C, expressing 10¹³ different cyclic peptide sequences, were panned for PDGFRA-specific binding against two pairs of cell lines (BCPAP and BON1) genetically modified to express the receptor or empty vector. Each group of cell lines underwent three rounds of panning, and after deep sequence (Illumina platform and MatLab software analysis),²⁹ sequences specific for PDGFRA binding for each group were selected. Sequences were grouped according to the cell line they were selected from. “Group 1” corresponded to BCPAP hits, and “Group 2” corresponded to sequences from panning BON1 cells. The phage library MBX-SXC6C was panned for binding to recombinant PDGFRA-His protein, and the bound phage was eluted and amplified to

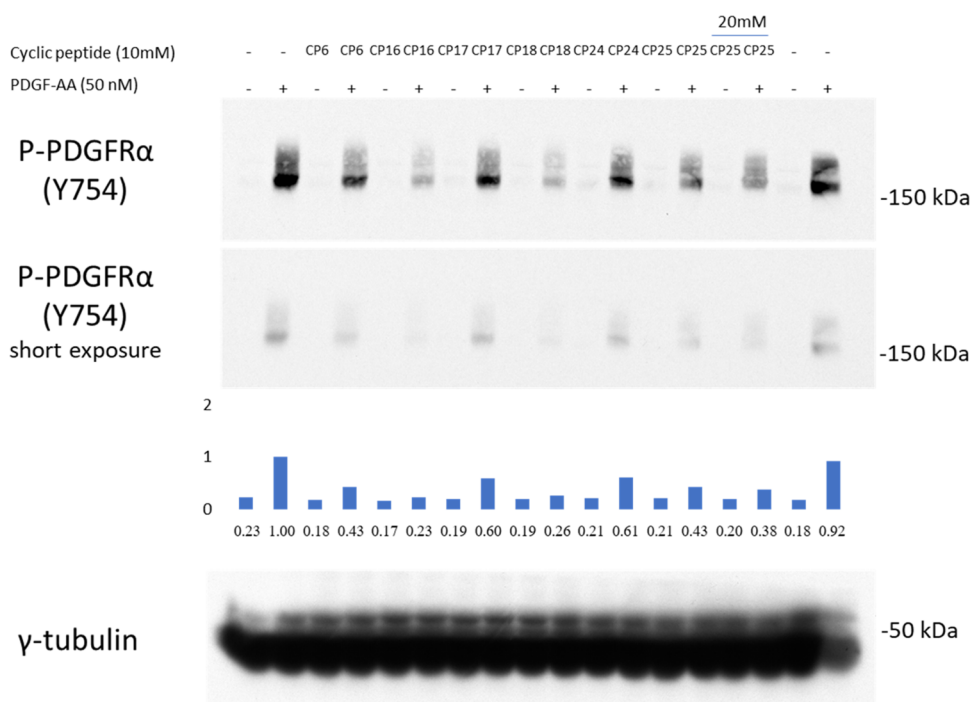


Figure 2. Screening of selected peptide sequences (CP6, CP16, CP17, CP18, CP24, CP25) in the absence and presence of 50 nM PDGF-AA from the biopanning experiment for effects on the phosphorylation of PDGFRA. Data are shown from protein analysis from Western blot gels at longer (top) and shorter (bottom) exposure times. Quantified data from a single gel for the expression of phosphorylated PDGFRA normalized to γ -tubulin is shown between the gel blots.

create a subset library (10^6 clones). This subset-phage-display library was used for a second set of panning with BCPAP and BON1 sets of cell lines. After three rounds of panning with the subset library, two new groups of sequences were generated, ‘Group 3’ for BCPAP and ‘Group 4’ for BON1 hits. The phage bound to PDGFR α beads vs control beads was also sequenced to generate ‘Group 5’ of sequences.

Supporting Figure S1 shows the strategy for sequence selection. If sequences were common between Groups 1–4 and Group 5 and those common sequences were also top 26 in relevance within their groups, then peptides were synthesized, cyclized, and tested. The complex strategy, using multiple cell lines and cross-referenced with recombinant protein, was designed to provide multiple and overlapping epitopes for presentation.^{30,31} Given the differences in surface binding and cell-based binding that can influence binding, we chose multiple formats with the goal of providing a wide selection of potential peptides to target the α subunit of PDGFR.

Phenotypic Screening. The 26 peptide sequences were selected (Supporting Figure S3), and their effect on PDGFR α phosphorylation was investigated as outlined for a selection in Figure 2. The greatest effects were from CP16 (77% reduction) and CP18 (74% reduction). Cell migration was also assessed to reveal that changes in phosphorylation were also reflected in altered migration for CP16 and CP18 (Supporting Figure S2).

Both peptides exhibited the –PQXW– sequence motif. A new subset of peptide sequences was derived by way of single amino substitutions (alanine) within this motif that were tested for changes in PDGFR α phosphorylation and cell migration (Figure 3a).

From these screening experiments, 4 peptide sequences were identified as the strongest candidates to continue studying *in vitro* and *in vivo* experiments (cyclic CP18, cyclic CP18.5,

cyclic CP16, cyclic CP16.2); these peptides were also found to be antagonists of PDGF-AA-induced phosphorylation (Figure 3a), subsequent migration (Figure 3b), and growth in Matrigel (Figure 4). The phenotypic approach to screening allowed us to identify the first peptides to target PDGFR α and downstream signaling. By use of a phenotypic approach to screening, we helped identify physiologically relevant peptides. They are useful in targeting and providing an opportunity to modulate PDGFR α function.

Peptide Synthesis. PDGFR α -targeting peptides were synthesized in multiple steps from linear peptide substrates. An automated peptide synthesizer was utilized to generate the linear versions of peptides CP18.5, CP18, CP16, and CP16.2 with the Fmoc/^tBu solid-phase strategy. After cleavage from the solid support, HPLC purification, and lyophilization, 19–23.8 mg of the linear peptides were obtained in good yields of 37–50%. A small linchpin in the form of α,α' -dibromo-*m*-xylene was inserted to access the cyclized peptides according to Timmerman et al.²⁶ in a fast and clean reaction. Here, the free thiol groups of the Cys residues in the peptide were reacted with the bromo residues of the linchpin with conversion yields of 95–97% for the cyclized CP18.5, CP16, and CP16.2 and 84% for cyclized CP18 after 15–20 min at r.t. that correlate with reported yields.²⁶ Isolated yields were in the range of 60–81%. Cyclized PDGFR α -targeting peptides were further modified with bifunctional macrocyclic chelator NOTA-Bn-NCS to obtain precursor peptides with a thiourea linkage between the peptide and chelator for radiolabeling with ⁶⁸Ga. In the case of CP18.5, a fluorescent-labeled cyclic CP18.5 (FITC–CP18.5) was synthesized for *in vitro* flow cytometry studies. While NOTA-modified PDGFR α -targeting peptides were obtained in moderate yields of 47–55%, FITC–CP18.5 yielded 70%. Table 1 gives an overview of all synthesized peptides, including names, sequences, mass, and isolated yields.

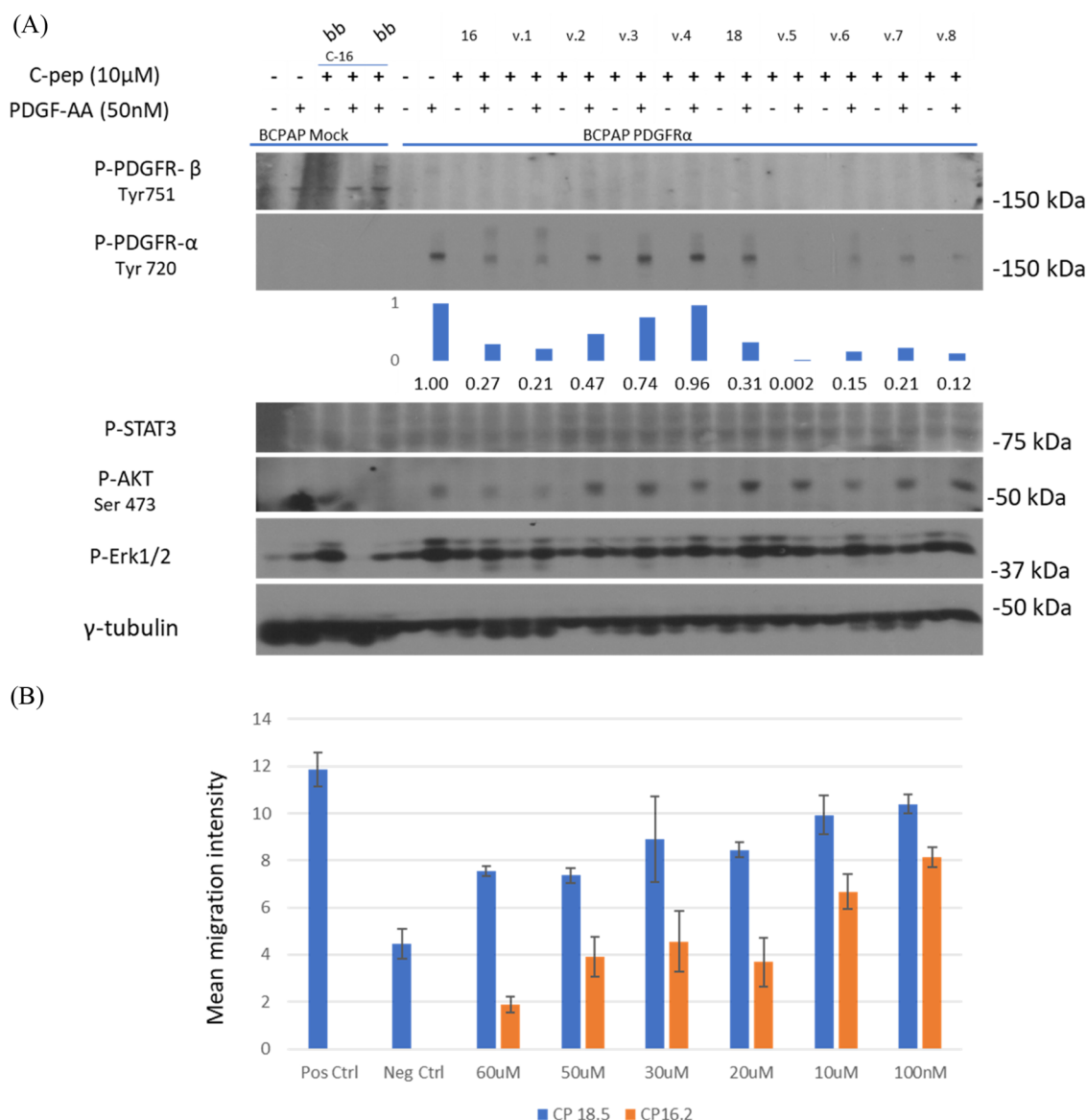


Figure 3. (A) Protein analysis of the effect of single amino acid substitutions on the phosphorylation of PDGFR α in comparison to effects on the phosphorylation of PDGFR β as well as STAT-3, AKT, and Erk1/2. Quantified data from a single gel for the expression of phosphorylated PDGFR α normalized to γ -tubulin is shown between the gel blots. (B) Concentration-dependent effects of increasing amounts of cyclic CP18.5 and cyclic CP16.2 peptides on BCPAP(+) cell migration. Data are shown as mean $\pm$ SEM from $n = 3$ experiments.

Microscale Thermophoresis. The PDGFR α -peptide library, with and without NOTA functional groups and as linear and cyclic peptides (Table 1), was assessed first for direct binding using microscale thermophoresis. This immobilization-free technique follows the directional movement of biomolecules along a temperature gradient established by an IR laser.³² Figure 5 displays the dose–response curves, where the thermophoresis signal is plotted against ligand concentration. Binding affinity was deduced from the dose–response curves and expressed as the dissociation constant (K_D values). The PDGF-AA protein was used as positive control and revealed high binding affinity with a K_D of 9 nM. Peptide Ava-BBN2 was selected as a negative control as a stable bombesin peptide analogue that binds to G-protein coupled receptor gastrin-releasing peptide receptor (GRPR) with high affinity but not to tyrosine-kinase receptor proteins. Peptides of the cyclic CP18 series exhibited a higher binding affinity than peptides of the cyclic CP16 series. The NOTA-modified cyclic

peptides, which we generated for radiolabeling, demonstrated a different trend following the order $\text{NOTA-CP18} > \text{NOTA-CP16} > \text{NOTA-CP18.5} \approx \text{NOTA-CP16.2}$.

We classified **NOTA-CP18.5** as the lead peptide with a K_D of 18 μ M showing high binding affinity to PDGFR α . Alternate peptides reported in the literature target PDGFR β . Comparably, the IC_{50} value for PDGFR β -targeting ^{125}I -PDGFR-P1 was also in the μ M range based on a different assay (radiometric competitive binding assay) in human pancreatic carcinoma BxPC3.²² Alongside the PDGFR α -targeting peptide library, we also investigated PDGFR β -targeting nonradioactive peptide **PDGFR-P1**. The peptide showed very low binding to PDGFR α with a K_d of 552 μ M, potentially attributable primarily to nonspecific binding.

Visual Interference Color Assay. We used a visual interference color assay (VICA)²⁸ to assess the ability of the core and alanine-scan variant peptide sequences (cyclic CP16, CP16.2, CP18.0, and CP18.5) to bind soluble, recombinant

Matrigel 3D growth BCPAP-Ala scan in C-peptides 16&18: 6 days at 1μM

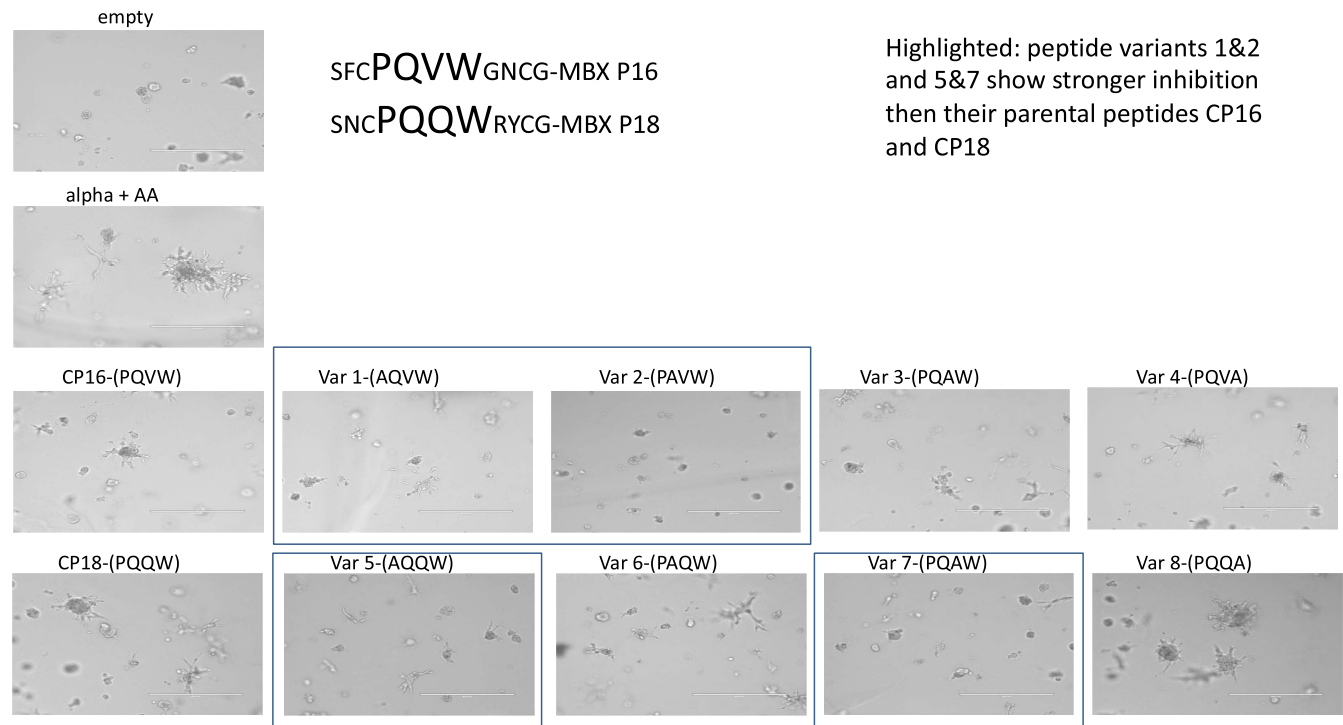


Figure 4. Original Matrigel 3D growth BCPAP-Alanine scans in CP16 and CP18, including different variants from alanine substitution (CP16.1, CP16.2, CP16.3, CP16.4 and CP18.5, CP18.6, CP18.7, CP18.8) in comparison to PDGF-AA peptide and empty controls.

Table 1. Name, Sequence, Molecular Weight (MW), Yielded Mass, and Isolated Yield of PDGFRA-Targeting Peptides

Name	Sequence	MW	Mass	Yield (isolated)
Linear CP18.5	SNCAQQWRYCG-NH ₂	1314.46 g/mol	23.8 mg	47%
Linear CP18	SNCPQQWRYCG-NH ₂	1340.50 g/mol	19.0 mg	37%
Linear CP16	SFCPQVWGNCG-NH ₂	1196.37 g/mol	21.6 mg	48%
Linear CP16.2	SFCPAVWGNCG-NH ₂	1139.32 g/mol	22.0 mg	50%
Cyclic CP18.5	SNCAQQWRYCG-NH ₂	1416.60 g/mol	6.1 mg	81%
Cyclic CP18	SNCPQQWRYCG-NH ₂	1442.64 g/mol	4.3 mg	60%
Cyclic CP16	SFCPQVWGNCG-NH ₂	1298.50 g/mol	4.6 mg	61%
Cyclic CP16.2	SFCPAVWGNCG-NH ₂	1241.45 g/mol	4.6 mg	60%
NOTA-CP18.5	NOTA-SNCAQQWRYCG-NH ₂	1867.11 g/mol	1.3 mg	47%
NOTA-CP18	NOTA-SNCPQQWRYCG-NH ₂	1893.15 g/mol	1.9 mg	49%
NOTA-CP16	NOTA-SFCPQVWGNCG-NH ₂	1749.01 g/mol	2.2 mg	55%
NOTA-CP16.2	NOTA-SFCPAVWGNCG-NH ₂	1691.96 g/mol	2.2 mg	54%
Ga-NOTA-CP18.5	Ga-NOTA-SNCAQQWRYCG-NH ₂	1933.81 g/mol	0.5 mg	42%
FITC-CP18.5	FITC-SNCAQQWRYCG-NH ₂	1805.98 g/mol	2.5 mg	70%
PDGFR-P1 (PDGFRβ peptide)	IPLPPSRPFFKY-NH ₂	1557.88 g/mol	21.7 mg	36%

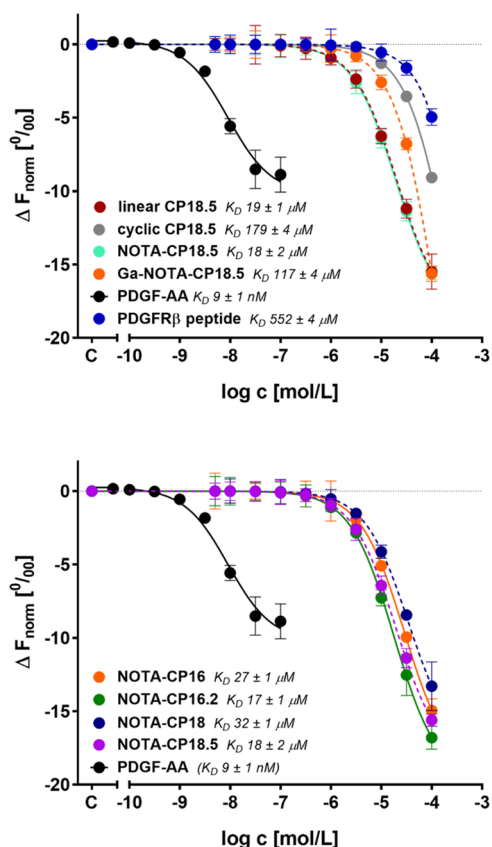


Figure 5. Microscale thermophoresis (MST). Titration concentration–response curves of increasing concentrations of tested target peptides against the His-tag-PDGFR α protein in comparison to PDGF-AA and PDGFR β peptides. The x - and y -axes of each graph show the change (Δ) $F_{\text{norm}} (I_{00})$ vs increasing ligand concentrations. Determined K_D values are given in the graphs. Data are shown as mean $\pm$ SEM from $n = 3$ experiments.

PDGFR α . Briefly, macromolecular complexes of varying sizes will shift the observed colors as a function of the affinity of binding for the different compounds.²⁸ Shown in Figure 6 are the resulting interference colors after binding of PDGFR α to different peptides on the surface in a qualitatively and quantitatively similar sequence to that seen by the microscale thermophoresis.

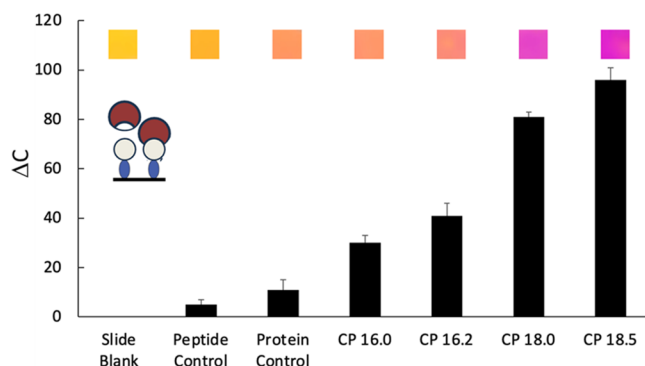


Figure 6. Visual interference color assay for the analysis of binding of cyclic CP16, CP16.2, CP18, and CP18.5 to PDGFR α via color distance (ΔC). Controls were protein PDGFR α only and peptide only. Data are shown as mean $\pm$ SEM from $n = 3$ experiments.

We demonstrated that the relative binding of matched levels of cyclic peptides to PDGFR α is at a maximum with the cyclic CP18.5 peptide variant. Nonsense peptides added to PDGFR α (data not shown), or PDGFR α alone, induced only small changes in the interference color pattern compared to the presence of the cyclic peptides immobilized on the aluminum oxide surface. Overall, this data facilitated the process of identification of PDGFR α -binding peptides, and the results were qualitatively consistent among the phenotypic and molecular studies. The ability to use this approach for comparative studies allowed us to demonstrate the important changes in the behavior of the peptides with respect to protein binding as a consequence of changes in charge and size.

Flow Cytometry. Flow cytometry was performed to elucidate the binding of lead peptide CP18.5 in its fluorescent-labeled version to PDGFR α receptors in BCPAP(+) vs BCPAP(−) cells, outlined in Figure 7. FITC-labeled CP18.5 produced a significant shift in BCPAP(+) cells compared to the negative control (PBS) and in BCPAP(−) cells, which was indicative of increased binding of FITC-CP18.5 in the PDGFR α expressing BCPAP(+) cell line. Pretreatment with a pharmacological concentration of 2 mM cyclic CP18.5 led to the same shift in both cell lines BCPAP(+) (15.0 ± 3.3 ; $n = 3$) and BCPAP(−) (15.1 ± 4.0 ; $n = 3$) compared with the negative control. In contrast, when treating BCPAP(−) and BCPAP(+) cells with 100 μg of PDGFR α -binding monoclonal antibody Lartruvo (Eli Lilly) prior to the addition of FITC-CP18.5, significant blocking was observed especially in BCPAP(+) cells. This could be an indicator that cyclic CP18.5 peptide indeed binds to the binding site of the PDGFR α antibody on the receptor site. Lastly, pretreatment with PDGFR β binding peptide PDGFR-P1 also resulted in blocking in BCPAP(+) cells, which may also contribute to a possible heterodimeric PDGFR α /B mediated binding of CP18.5 peptide in BCPAP(+) cells. Overall, results from the flow cytometry experiments were indicative of some binding of cyclic FITC-CP18.5 toward PDGFR α with potential heterodimeric binding, with significantly less avidity for the β subunit of PDGFR.

Radiolabeling of Cyclic Peptides. Radiolabeling with positron emitter Ga-68 ($t_{1/2} = 68$ min) was achieved by reacting the high activity fraction of $^{68}\text{Ga-Cl}_3$ from a 50 mCi $^{68}\text{Ge}/^{68}\text{Ga}$ generator with 50 μg of each of the NOTA-functionalized cyclic peptides (NOTA-CP18.5, NOTA-CP18, NOTA-CP16, and NOTA-CP16.2) in 2 M sodium acetate buffer to give a pH of 4.5. ^{68}Ga -labeled peptides were obtained after 21 min at 60 $^\circ\text{C}$ monitored by Radio-TLC. Purification with solid-phase extraction (SPE) and reformulation in 20% EtOH/saline delivered ^{68}Ga -NOTA-peptides in high radiochemical purity >95% determined by Radio-HPLC for subsequent *in vitro* and *in vivo* testing. Total synthesis time was 30–40 min for each of the 4 ^{68}Ga -peptides. Lipophilicity of ^{68}Ga -NOTA-CP18.5 was determined as the partition coefficient in *n*-octanol and PBS (pH 7.4) with $\log D_{7.4} = -2.77 \pm 0.16$ ($n = 9/3$). Supporting Figure S4 exemplifies the ^{68}Ga -labeling reaction of NOTA-CP18.5.

In Vitro Cell Uptake. Radiolabeled ^{68}Ga -NOTA-CP18.5, ^{68}Ga -NOTA-CP18, and ^{68}Ga -NOTA-CP16 were tested for their *in vitro* cell uptake properties in native PDGFR α cell line 8305C vs nonexpressing BCPAP(−) cells. Figure 8 summarizes the cell uptake results for those peptides. ^{68}Ga -NOTA-CP18.5 revealed highest uptake in both 8305C cells at both 15

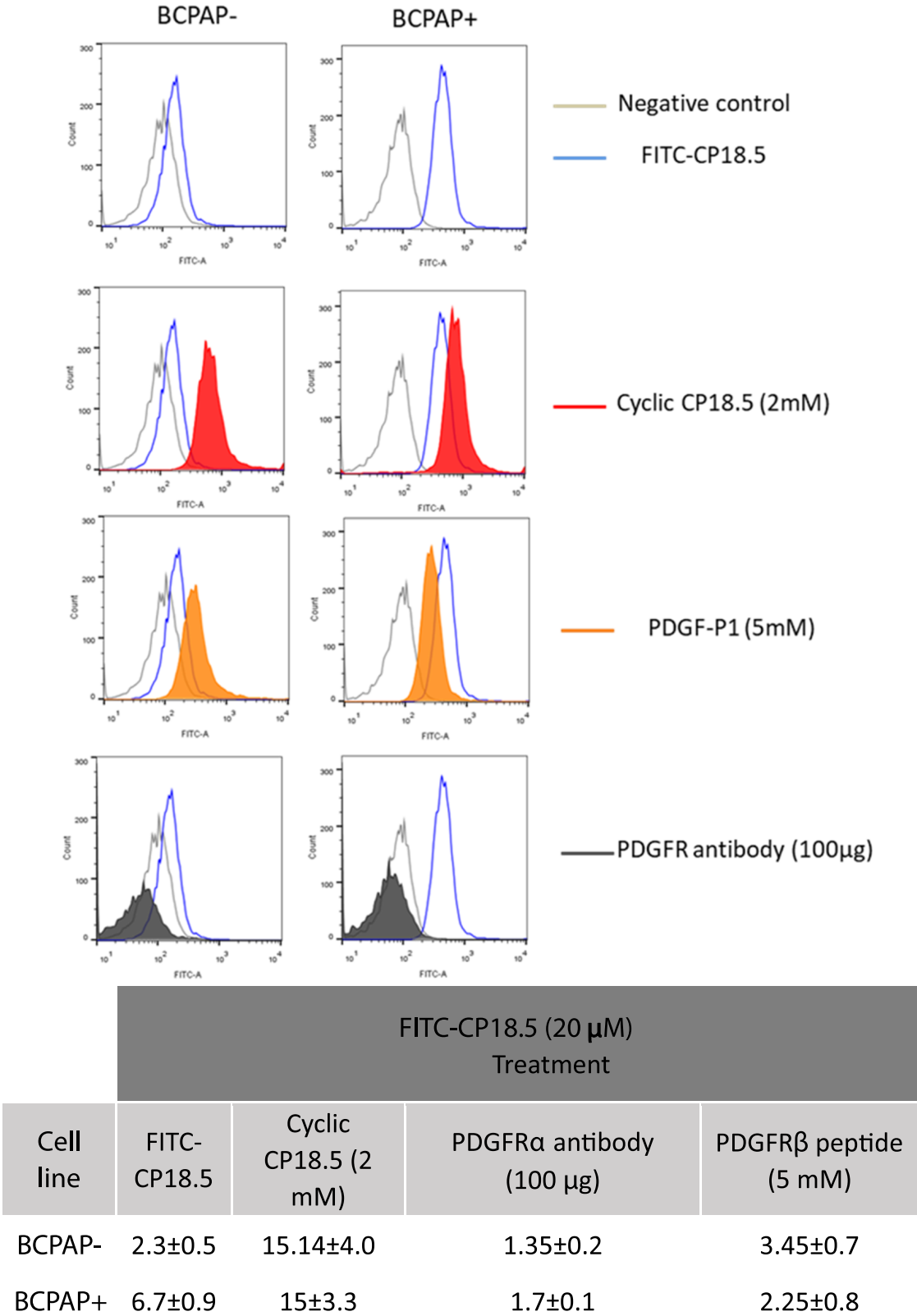


Figure 7. Flow cytometry of FITC–CP18.5 in BCPAP(–) (left) and BCPAP(+) cells (right). The top line shows the initial signal for FITC–CP18.5 in both cell lines. The next three lines show the effect of pretreatment with 2 mM cyclic CP18.5, 5 mM PDGFR-P1 peptide, or 100 μg of PDGFRα monoclonal antibody on the FITC–CP18.5 signal. The x- and y-axes of each graph show the fluorescence intensity and the number of cells, respectively. The table below presents the ratios of the basis FITC–CP18.5 signal vs pretreated cells calculated based on the mean fluorescence intensity. Data are shown as mean ± SEM from *n* = 3 experiments.

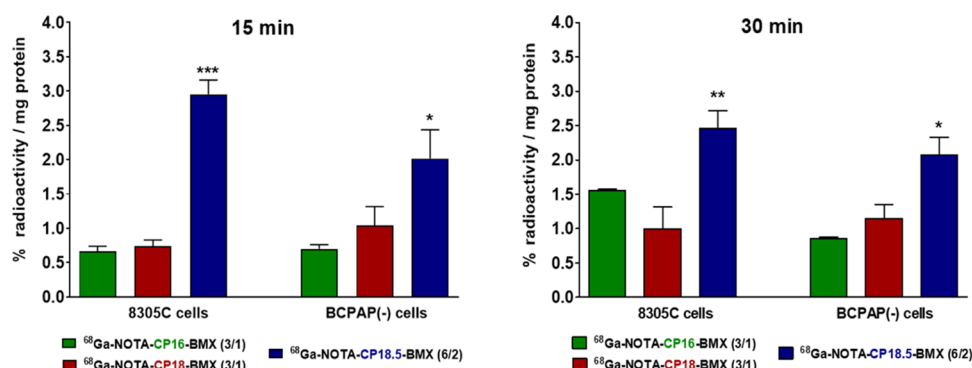


Figure 8. *In vitro* cell uptake of ^{68}Ga -labeled PDGFRA-targeting peptides CP16, CP18, and CP18.5 into 8305C and BCPAP(−) thyroid cancer cell lines after 15 min (left) and 30 min (right) incubation time. Data are presented as % radioactivity uptake per milligram of protein and as mean $\pm$ SEM from 3 to 6 data points from $n = 12$ experiments.

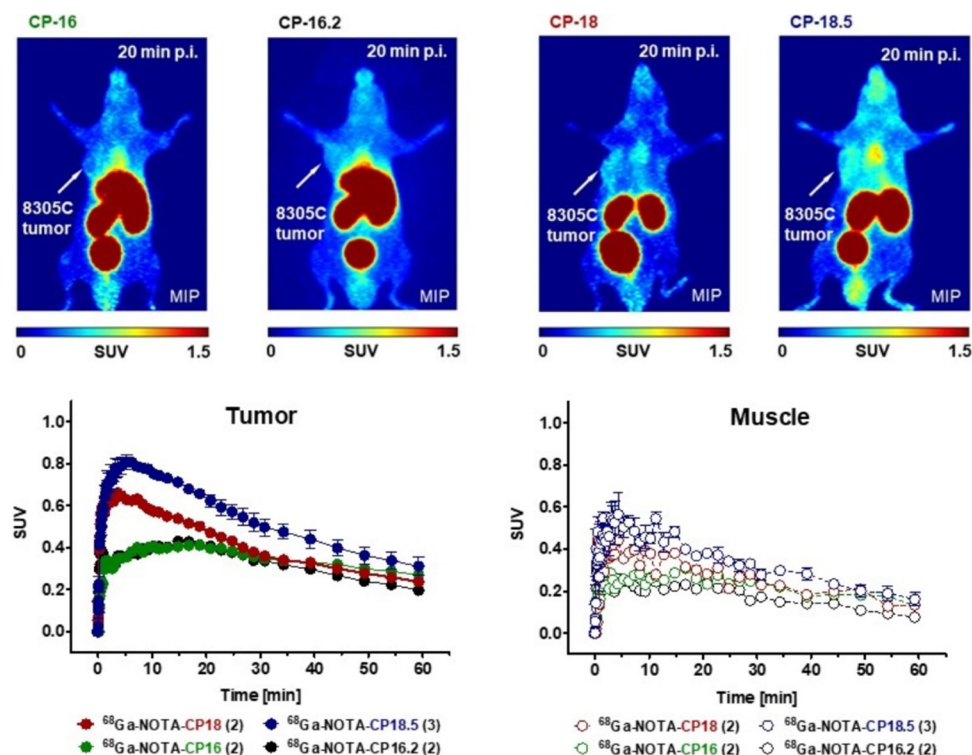


Figure 9. *In vivo* PET images from dynamic experiments after injection of ^{68}Ga -labeled PDGFRA-peptides CP16, CP16.2, CP18, and CP18.5 into 8305C thyroid tumor-bearing NSG mice. Depicted images are shown as maximum intensity projections (MIP) at 20 min post injection time. Below are the corresponding time-activity curves (TACs) for radioactivity uptake levels into the 8305C tumor tissue (left) and the contralateral nontargeting muscle tissue (right). Data are shown as standardized uptake values (SUV) over the time course of 60 min as mean $\pm$ SEM values from $n = 2$ –3 mice.

and 30 min vs ^{68}Ga -NOTA-CP18 and ^{68}Ga -NOTA-CP16 (both $<1.6\%$ ID/mg), respectively. These novel cyclic peptides show higher uptake in a PDGFRA(+) cell line compared to a PDGFR(−) cell line, similar to that shown for PDGFRA-targeting antibody ^{64}Cu -NOTA-D13C6 in a previously published study.¹³ The uptake values for our cyclic peptides are in the same range as ^{67}Ga -labeled PDGFRB-targeting PDGFR-P1 peptide analogues (1.29–3.09% ID/mg protein) in BxPC3-luc cells.²³ The difference between PDGFRA expressing vs nonexpressing cells amounted to $\sim 30\%$. As ^{68}Ga -NOTA-CP18.5 uptake in 8305C cells was also slightly lower ($\Delta 17\%$) at 30 min compared to 15 min, this was indicative of some levels of a reversible process in binding to the target proteins. Overall, ^{68}Ga -NOTA-CP18.5 did perform

better *in vitro* than the other two peptides, but some interactions were observed with ^{68}Ga -NOTA-CP18.5 to PDGFRB. We note that different cell lines will present unique cell line turnover and cellular internalization of PDGFRA with resulting variations in receptor binding kinetics and peptide retention.²⁷

PET Imaging Experiments. ^{68}Ga -labeled PDGFRA-targeting peptides were also evaluated *in vivo*. For comparison, all four peptides were investigated for their *in vivo* pharmacokinetics and tumor uptake in the 8305C thyroid cancer xenograft model with native PDGFRA expression by using small animal PET. Figure 9 displays representative PET images of all four ^{68}Ga -labeled PDFGRA peptides at 20 min p.i. with the corresponding time-activity curves (TACs) in the

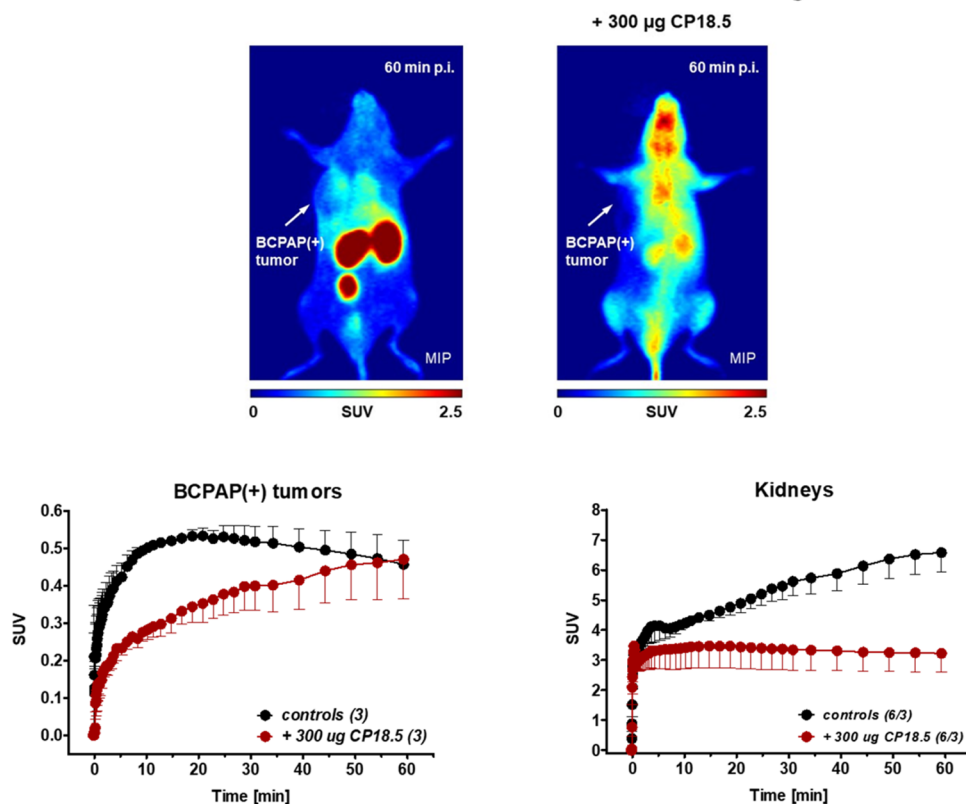
^{68}Ga -NOTA-CP18.5 – self blocking

Figure 10. *In vivo* PET image data from dynamic experiments after injection of ^{68}Ga -labeled PDGFRA-peptide CP18.5 into BCPAP(+) thyroid tumor-bearing NSG mice. Depicted images are shown as maximum intensity projections (MIP), and at 60 min post injection time from a control mouse (left) and mouse co-injected with 300 μg cyclic CP18.5 (right). Below are the corresponding time-activity curves (TACs) for radioactivity uptake levels into the BCPAP(+) tumor tissue (left) and the clearance through the kidneys (right). Data are shown as standardized uptake values (SUV) over the time course of 60 min as mean $\pm$ SEM values from $n = 3$ mice.

8305C tumors and muscle tissues below. ^{68}Ga -NOTA-CP18.5 and ^{68}Ga -NOTA-CP18 show clear tumor delineation in the left upper flanks of the mice, with ^{68}Ga -NOTA-CP18.5 being superior to ^{68}Ga -NOTA-CP18. Lower tumor uptake is visible with the ^{68}Ga -NOTA-CP16 and ^{68}Ga -NOTA-CP16.2 peptides. While tumor uptake into a PDGFRA(+)-mouse model has been demonstrated with the PDGFRA-targeting antibody ^{64}Cu -NOTA-D13C6 before,¹³ the novel cyclic peptides represent a valid alternative for PDGFRA-targeting. Tumor uptake over time demonstrated rapid radioactivity uptake during the initial phase of 5 min after injection, which would correspond to the distribution phase with ^{68}Ga -NOTA-CP18.5 > ^{68}Ga -NOTA-CP18 > ^{68}Ga -NOTA-CP16 = ^{68}Ga -NOTA-CP16.2 followed by a subsequent washout.

This profile is typical for radiolabeled peptides, including a fast but also somewhat reversible receptor interaction.^{33,34} At 60 min p.i. considering the equilibration phase, ^{68}Ga -NOTA-CP18.5 uptake was still 0.31 ± 0.05 , while ^{68}Ga -NOTA-CP16.2 revealed only the lowest uptake of 0.20 ± 0.02 (60 min p.i.). Muscle uptake and clearance followed a similar profile, albeit at an overall lower radioactivity level. Tumor-to-muscle ratios for ^{68}Ga -labeled peptides in the 8305C model were calculated at 10 min p.i. CP16 1.54, CP16.2 1.58, CP18 1.50, and CP18.5 1.67 and at 60 min p.i. CP16 2.07, CP16.2 2.5, CP18 1.85, and CP18.5 1.94. For a more detailed analysis of CP18.5 in BCPAP(+) tumors, we determined a tumor-to-muscle ratio of 1.18 ± 0.07 after 10 min p.i. and 2.15 ± 0.29

(both $n = 3$) after 60 min p.i. Overall, tumor accumulation significantly exceeds healthy tissue, as seen in PDGFRB-targeting peptides.²² Heart uptake due to perfusion was also detected for all 4 ^{68}Ga -PDGFRA-targeting peptides, which is typical for ^{68}Ga -labeled peptides.³⁴ For the CP16 series, both hepatobiliary and renal clearance were noted; however, the CP18 series demonstrated predominant renal clearance. As demonstrated with ^{67}Ga -labeled PDGFR-P1 peptide analogues,²³ our PDGFRA-targeting peptides also revealed fast clearance after 60 min in nontargeted tissues but the kidneys.

While data obtained from PET studies *in vivo* do not always correlate with 2D *in vitro* cell uptake studies, the overall biochemical parameters favoring ^{68}Ga -NOTA-CP18.5 uptake are consistent with the *in vivo* uptake profile. We further examined the target specificity for ^{68}Ga -NOTA-CP18.5 through blocking experiments in the BCPAP(+) xenograft model (Figure 10). For self-blocking, pharmacological doses of 300 μg (10 mg/kg) of the cyclic CP18.5 peptide were used. During the early receptor interaction and binding phase, a blocking effect of approximately 40% was detected strongly, in support of a direct receptor binding interaction of ^{68}Ga -NOTA-CP18.5. However, at this point we cannot distinguish between binding on the dimers and potential PDGFRA/PDGFRB heterodimers. Interestingly, the self-blocking resulted in a strong blocking effect of 50% in the kidneys at 60 min p.i. ($\text{SUV}_{60\text{min}}$ from 6.58 ± 0.63 (control) to 3.22 ± 0.62 (blocking), both $n = 6/3$; Figure 10). Interestingly, the observed renal clearance of CP18.5 in the kidneys is consistent

with the known expression of PDGFRA in the renal tubular system, one of the few places where the α subunit is found in normal tissue.³⁵ Also, radioactivity muscle uptake and retention were strongly elevated in the presence of the pharmacological dose of cyclic CP18.5, as well as blood clearance. This effect is in line with the observation of Effendi et al. and their PDGFRB-targeting peptide ⁶⁷Ga-PDGFR-P1 analogue.²³

Metabolic Stability *In Vivo*. Lastly, the ⁶⁸Ga-labeled peptides of the CP18 series (⁶⁸Ga-NOTA-CP18.5 and ⁶⁸Ga-NOTA-CP18) were examined for metabolic stability *in vivo* (Figure 11). Over the time course of 60 min, ⁶⁸Ga-NOTA-

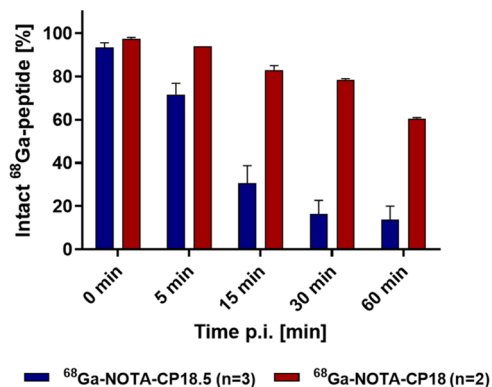


Figure 11. *In vivo* metabolic stability of ⁶⁸Ga-labeled CP18 and CP18.5 over 60 min post injection in control BALB/c mice. Data was collected from 2 to 3 mice, and it is displayed as a percentage of intact ⁶⁸Ga-labeled peptide and as mean $\pm$ SEM for every time point.

CP18 integrity was consistently higher for each time point compared to ⁶⁸Ga-NOTA-CP18.5 with 60.5% ⁶⁸Ga-NOTA-CP18 vs 14% ⁶⁸Ga-NOTA-CP18.5 at 60 min p.i. This was indicative of the much lower metabolic stability of ⁶⁸Ga-NOTA-CP18.5 and accounts, in part, for the observed washout profile from the tumor uptake curves. So far, only *in vitro* metabolic stability has been described for PDGFR (PDGFRB)-targeting peptides, with ¹²⁵I-PDGFR-P1 exhibiting a low serum half-life of 4 min,²² and ⁶⁷Ga-labeled PDGFR-P1 analogues with 75–80% intact after 1 h in mouse plasma.²³

Overall, while ⁶⁸Ga-NOTA-CP18.5 reveals a lower metabolic stability *in vivo* than its ⁶⁸Ga-NOTA-CP18 counterpart, tumor uptake remained higher for ⁶⁸Ga-NOTA-CP18.5 in comparison to ⁶⁸Ga-NOTA-CP18 indicative of a higher affinity for the α subunit of PDGFRA. The two peptides differ in only one amino acid at position 4 (Ala for ⁶⁸Ga-NOTA-CP18.5 and Pro for ⁶⁸Ga-NOTA-CP18). Radiometabolites were not identified, but the main radiometabolite in ⁶⁸Ga-NOTA-CP18.5 is slightly more hydrophilic than the parent compound, and the difference of one amino acid in CP18.5 vs CP18 indicates that the metabolite may be a truncated peptide of ⁶⁸Ga-NOTA-CP18.5 cleaved at the Ala position. Similar results were observed with minigastrin peptides by von Guggenberg et al.³⁶ Proline-substituted minigastrin analogues exhibited a higher metabolic stability *in vivo* than minigastrin analogues with alanine with no influence on their tumor uptake.^{36,37} Future studies will address the metabolic instability of ⁶⁸Ga-NOTA-CP18.5 to improve its metabolic profile. Beyond the cyclization and the amidated N-terminus of the radiopeptide, the Ala residue could be *N*-methylated or exchanged for a D-amino acid, or dimerization/multimeriza-

tion^{38,39} could complement targeting the dimeric PDGFRA receptor through multivalent interaction and improve binding affinity.

CONCLUSIONS

This study describes radiolabeled cyclic peptides targeting PDGFRA, a driver of metastatic thyroid cancer and other tumors. *In vitro* and *in vivo* evaluation of a series of peptides derived from phage-display libraries used both phenotypic screening and direct binding methods to identify a lead candidate, ⁶⁸Ga-NOTA-CP18.5, with favorable tumor targeting and pharmacokinetic profile despite suboptimal metabolic stability *in vivo*. As a first-generation PDGFRA-targeting peptide, CP18.5 was able to significantly influence the behavior of immortal cell lines expressing PDGFRA while also demonstrating direct interactions to PDGFRA heterodimers and homodimers. The complexity of PDGFRA signaling and other potential heterodimers with PDGFRB and EGFR requires additional research as we continue to improve target specificity as well as metabolic stability.

ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge at <https://pubs.acs.org/doi/10.1021/acs.molpharmaceut.4c00549>.

Sequence selection strategy; effects on PDGF-AA-induced migration; PDGFRA-binding antibody (Lartruvo) specificity in various thyroid cancer cell lines; ⁶⁸Ga-labeling of cyclic PDGFRA-targeting peptides; and HPLC QC chromatograms of final peptides and ⁶⁸Ga-labeled peptides (PDF)

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Author Contributions

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Notes

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