The ring size of monocyclic ET-1 controls selectivity and signaling efficiency at both endothelin receptor subtypes

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Cardiovascular diseases (CVDs) like hypertension are a major cause for death worldwide. In the cardiovascular tissue, the endothelin system—consisting of the receptor subtypes A (ETAR) and B (ETBR) and the mixed agonist endothelin 1 (ET-1)—is a major key player in the regulation of vascular tone and blood pressure. Tight control of this system is required to maintain homeostasis; otherwise, the endothelin system can cause severe CVDs like pulmonary artery hypertension. The high sequence homology between both receptor subtypes limits the development of novel and selective ligands. Identification of small differences in receptor–ligand interactions and determination of selectivity constraints are crucial to fine-tune ligand properties and subsequent signaling events. Here, we report on novel ET-1 analogs and their detailed pharmacological characterization. We generated simplified ET-1-derived monocyclic peptides to provide an accessible synthesis route. By detailed in vitro characterization, we demonstrated that both G protein signaling and the subsequent arrestin recruitment of activated ETBR remain intact, whereas activation of the ETAR depends on the intramolecular ring size. Increasing of the intramolecular ring structure reduces activity at the ETAR and shifts the peptide toward ETBR selectivity. All ET-1 analogs displayed efficient ETBR-mediated signaling by G protein activation and arrestin 3 recruitment. Our study provides in-depth characterization of the ET-1/ETAR and ET-1/ETBR interactions, which has the potential for future development of endothelin-based drugs for CVD treatment. By identification of Lys9 for selective labeling, novel analogs for peptide-mediated shuttling by ET-1 are proposed.
1 | INTRODUCTION

Cardiovascular diseases (CVDs) comprise a huge variety of defects and dysregulations of the cardiovascular system like congenital heart defects, coronary heart disease, hypertension, and peripheral artery diseases and remain the number one cause of death worldwide, accounting for ~18 million annual deaths.\(^1\) Drugs to combat CVDs rely on target selectivity. The endothelin system is a multi-ligand/multi-receptor system. It consists of two G protein-coupled receptor (GPCR) subtypes—the endothelin receptor A (ET\(_{\text{AR}}\)) and B (ET\(_{\text{BR}}\))—and three endogenously expressed peptide agonists: endothelin 1, 2, and 3 (ET-1/2/3).\(^2\),\(^3\) There is high sequence homology between both GPCRs and also between the three ligands. Characteristic for all three 21 amino acid ligands is their C-terminal carboxyl function, necessary for receptor activation, and two intramolecular disulfide bridges (Cys\(^1\)–Cys\(^{15}\) and Cys\(^2\)–Cys\(^{14}\)), which stabilize an α-helical region, leading to challenges in production of high amount of ligand.\(^4\)–\(^6\) Agonist binding induces intracellular G\(_Q\) protein activation, followed by phosphorylation-dependent desensitization.\(^7\),\(^8\) Tight control of receptor activation, intracellular signaling, and signal termination is required for balancing the physiological effect of both receptor species. In the blood system, the interplay between ET\(_{\text{AR}}\), ET\(_{\text{BR}}\), and ET-1 is a crucial regulator of cardiovascular homeostasis. ET-1-mediated receptor activation leads to long-lasting vasoconstriction (ET\(_{\text{AR}}\) activation) or vasodilatation (ET\(_{\text{BR}}\) activation).\(^9\),\(^10\) Dysregulation of the endothelin signaling axis can cause severe CVDs like congenital heart defects, pulmonary fibrosis, and pulmonary artery hypertension.\(^11\)–\(^13\) Thus, the endothelin system is an interesting target for the development of novel treatment options due to its limited distribution in the human body. Because the endothelin receptor subtypes share high sequence homology but convert vastly different physiological effects, the development of selective ligands is of great pharmacological interest. Precise pinpointing of the structural requirements for both ET\(_{\text{AR}}\) and ET\(_{\text{BR}}\) activation can reveal novel differences between these closely related GPCRs. This is vital to the development of novel and potentially selective agonists for each receptor. Previous structure–activity relationship (SAR) studies were able to reveal the importance of different ET-1 elements like the compact secondary structure due to intramolecular disulfide bonds.\(^14\)

Here, we report on the synthesis of different monocyclic ET-1 derivatives and ET\(_{\text{BR}}\)-selective peptide agonists. By substitution of amino acid residues, one intramolecular disulfide bridge was selectively removed and the ring size of the remaining disulfide bridge was stepwise adjusted, keeping the characteristic structure of the wild-type (wt) endothelin peptides. The pharmacology of both ET\(_{\text{AR}}\) and ET\(_{\text{BR}}\) was investigated in response to the ET-1 derivatives. We applied detailed analysis of GPCR signaling by studying the G protein activation profiles and the arrestin recruitment and internalization of activated receptors. We demonstrate that the size of the Cys\(^1\)–Cys\(^{15}\) intramolecular disulfide acts as crucial selectivity determinant between the endothelin receptor subtypes. Increasing the size of this bond reduced ET\(_{\text{AR}}\) activity, pushing the peptide selectivity toward the ET\(_{\text{BR}}\). Furthermore, by introducing a fluorescent label, the receptor-mediated internalization of the peptides into ET\(_{\text{AR}}\)- and ET\(_{\text{BR}}\)-expressing cells was monitored, identifying Lys\(^5\) as potential attachment site for different cargos.

2 | MATERIALS AND METHODS

2.1 | Peptide synthesis

2.1.1 | Materials

All amino acids, 1-hydroxybenzotriazole (HOBt), ethyl 2-cyano-2-(hydroxyimino)acetate (Oxyma), and N,N’-disopropylcarbodiimide (DIC) were purchased from Iris Biotech (Marktredwitz, Germany), NovaSyn® W-TGA resin and O-(7-azabenzotriazolyl)-tetramethyluronium hexafluorophosphate (HATU) were supplied from Novabiochem (Darmstadt, Germany). 6-carboxytetramethylrhodamine (TAMRA) was purchased from ChemPep, Inc. (Wellington, Florida), and acetanitride (ACN) was obtained from VWR (Darmstadt, Germany). Dimethylformamide (DMF) and dichloromethane (DCM) were obtained from Biosolve ( Valkenswaard, The Netherlands). N,N-diisopropylethylamine (DIPEA), 1,2-ethanedithiol (EDT), 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES), hydrazine monohydrate, piperidine, tetrahydrofuran (THF), thioanisole (TA), trifluoroacetic acid (TFA), and trisopropylsilane (TIS) were purchased from Sigma-Aldrich ( Taufkirchen, Germany). Diethyl ether and diiodomethane were from Merck (Darmstadt, Germany). Potassium carbonate and triethylamine were purchased from Thermo Fischer Scientific ( Waltham, Massachusetts) and tris(2-carboxyethyl)phosphine (TCEP) was obtained from Carl Roth GmbH & Co. KG ( Karlsruhe, Germany).

2.1.2 | Solid phase peptide synthesis

Peptides were synthesized by solid-phase peptide synthesis (SPPS), using the 9-fluorenylmethyloxycarbonyl (Fmoc)/tert-butyl (tBu)
strategy. For automated SPPS, performed on a SYRO I peptide synthesizer, pre-loaded NovaSyn W-TGA resins (15-μmol scale), using an eightfold molar excess of N-Fmoc-protected amino acids (and N'-Boc-protected Boc-Cys(Trt)-OH and Boc-Ala-OH at Position 1), Oxyma, and DIC, dissolved in DMF. All coupling steps were carried out twice with a reaction time set to 40 min. For Fmoc cleavage, a solution of 40% (v/v) piperidine in DMF was applied for 3 min and 20% (v/v) piperidine in DMF for 10 min. Fluorescent labeling of the peptides was performed after complete synthesis of the peptide chain. Side-chain modification of Fmoc-Lys (Dde)-OH for 4,4-dimethyl-2,6-dioxocyclohex-1-ylidenethyl (Dde) cleavage, or Mmt group, the resin was treated with ultraviolet (UV) spectroscopy. For cleavage of the Dde in DMF for 10 min. For full cleavage of the peptides and simultaneous deprotection of all acid-labile protection groups, the resin was incubated with TFA/EDT/TA (90:3:7, v/v/v) for 3 h followed by precipitation from ice-cold diethyl ether. To induce the formation of disulfide bonds, crude peptides were dissolved in 10 mM HEPES, adjusted to pH 7.8, at a concentration of 0.5 mg/ml. Disulfide formation was allowed for 24 h under constant shaking. Crude peptides were purified by preparative reverse-phase high-performance liquid chromatography (RP-HPLC) on a Shimadzu system equipped with a Phenomenex Kinetex C18 100-Å column by applying a linear binary gradient system of eluent A and B (0.1% TFA in water, v/v, and 0.08% TFA in ACN, v/v). For peptides 2–5, displaying low solubility under acidic conditions, a binary gradient of 10 mM (NH4)HCO3 (aqueous solution), pH 8.5, and 10 mM (NH4)HCO3 in 80% ACN was applied. Purification was monitored by UV absorption (λ = 220 and 280 nm). The purity of the synthesized peptides was confirmed by two different analytical RP-HPLC systems. For peptide detection, the UV absorption was measured at λ = 220 and 280 nm, and for TAMRA-labeled peptides, fluorophore emission was monitored (excitation: 525 nm, emission: 572 nm). Peptide identity was verified by matrix-assisted laser desorption/ionization time of flight (MALDI-ToF) mass spectrometry (UltraflexIII, Bruker Daltonics) and ESI-ion trap mass spectrometry (HCT, Bruker). Pure peptides were lyophilized and salt adducts (depending on the purification system) were deducted from the amino acid sequence prior to experimental application.

2.1.3 Methylene thioacetal formation

The formation of methylene thioacetals is based on previously published protocols.15 Purified [Ala3,11, Nle7, (Cys7-Cys15)]-ET-1 (peptide 2) or [Ala3,11, Nle7, Lys5(TAMRA), (Cys7-Cys15)]-ET-1 (peptide 7) were dissolved in 3 M K2CO3 aqueous solution, containing 1.5 equiv TCEP. The peptide/TCEP solution was incubated for 2 h at RT under constant shaking. Subsequently, a solution of 12 equiv triethylamine and 8 equiv diiodomethane in THF was added dropwise, and the reaction mix was incubated for 12 h at RT under constant shaking. The peptide was purified as described in 2.1.2 SPPS.

2.1.4 Circular dichroism spectroscopy

Circular dichroism (CD) measurements were recorded on a JASCO J-715 spectropolarimeter (JASCO GmbH, Pfinztstadt, Germany). Peptides were dissolved in 10 mM sodium phosphate buffer (pH 7.4) and analyzed at a final concentration of 20 μM at 20°C. Sodium dodecylsulfate (SDS) was added to a final concentration of 20 mM, and samples were incubated at RT for 5 min prior to measurement. Three consecutive CD scans (resolution = 0.5 nm) were averaged and background-corrected with phosphate buffer.

2.2 Molecular biological methods

2.2.1 Materials

Lysogeny broth (LB) was sourced from Sigma-Aldrich (Taufkirchen, Germany). Ampicillin, Phusion polymerase, the enzymes AsISI, HindIII, and T4 ligase were purchased from Thermo Fisher Scientific (Waltham, Massachusetts). Oligonucleotides were purchased from biomers.net GmbH (Ulm, Germany). The pNL1.3-secLuc plasmid, containing the nanoluciferase (Nluc) coding sequence, was kindly provided by A. Kaiser (Leipzig University). The plasmids pCMV3-ETAR-GFPspark and pCMV3-ETBR-GFPspark were purchased from Sino Biological Inc. (Beijing, China).

2.2.2 Generation of the nanoluciferase-arrestin 3 construct

Overlap extension (OE) polymerase chain reaction (PCR) was used to fuse the Nluc to the N-terminus of arr3. The arr3 cDNA was amplified and modified with from the pcDNA-Rluc3-Arr3 plasmid.16 The Nluc vector and OE PCR product were digested using AsiSI and HindIII restriction site. The generated PCR products were fused by OE PCR. Vector and OE PCR product were digested using AsISI and HindIII prior to ligation with T4 ligase and transformation into Escherichia coli DH5α. Single clone selection was carried out on LB agar plates by
ampicillin resistance. Positive clones were verified by Sanger sequencing using an in-house facility.

2.3 Biological methods

2.3.1 Materials

Dulbecco’s modified Eagle’s medium (DMEM), Dulbecco’s phosphate-buffered saline (DPBS), Ham’s F12, Hank’s balanced salt solution (HBSS), and trypsin/ethylenediaminetetraacetate were purchased from Lonza (Basel, Switzerland). Fetal calf serum (FCS) was obtained from Biochrom (Berlin, Germany). Poly-D-lysine was obtained from Merck (Darmstadt, Germany). OptiMEM was purchased from Life Technologies (Carlsbad, California). Hoechst 33342 was supplied by Sigma-Aldrich (Taufkirchen, Germany). Fura-2 AM was sourced from Sigma-Aldrich (Madison, Wisconsin). Endothelin 1 (ET-1) was obtained from Bachem Holding (Bubendorf, Switzerland). 8-Well ibiTreat μ-slides were obtained from IBIDI (Martinsried, Germany). Lipofectamine® 2000 was supplied by Invitrogen (Carlsbad, California) and Metafectene® Pro was obtained from Biontex Laboratories GmbH (Munich, Germany). COS-7 and HEK293 cells were purchased from DSMZ (Deutsche Sammlung von Mikroorganismen und Zellkulturen, Braunschweig, Germany) and cultivated in cell culture flasks from TPP (Trasadingen, Switzerland). CELLSTAR 96-well plates, black and white μ Germany) and cultivated in cell culture flasks from TPP (Trasadingen, Switzerland). CELLSTAR 6-well plates, black and white μ Germany) and cultivated in cell culture flasks from TPP (Trasadingen, Switzerland). CELLSTAR 6-well plates, black and white μ Germany) and cultivated in cell culture flasks from TPP (Trasadingen, Switzerland). CELLSTAR 6-well plates, black and white μ Germany) and cultivated in cell culture flasks from TPP (Trasadingen, Switzerland). CELLSTAR 6-well plates, black and white μ Germany) and cultivated in cell culture flasks from TPP (Trasadingen, Switzerland). CELLSTAR 6-well plates, black and white μ Germany) and cultivated in cell culture flasks from TPP (Trasadingen, Switzerland). CELLSTAR 6-well plates, black and white μ Germany) and cultivated in cell culture flasks from TPP (Trasadingen, Switzerland). CELLSTAR 6-well plates, black and white μ Germany) and cultivated in cell culture flasks from TPP (Trasadingen, Switzerland). CELLSTAR 6-well plates, black and white μ Germany) and cultivated in cell culture flasks from TPP (Trasadingen, Switzerland). CELLSTAR 6-well plates, black and white μ Germany) and cultivated in cell culture flasks from TPP (Trasadingen, Switzerland). CELLSTAR 6-well plates, black and white μ Germany) and cultivated in cell culture flasks from TPP (Trasadingen, Switzerland). CELLSTAR 6-well plates, black and white μ Germany) and cultivated in cell culture flasks from TPP (Trasadingen, Switzerland). CELLSTAR 6-well plates, black and white μ Germany) and cultivated in cell culture flasks from TPP (Trasadingen, Switzerland).

2.3.2 Cell culture

All cell lines were cultured at 37°C, 95% humidity, and 5% CO2 (standard conditions). COS-7 cells were grown in DMEM containing 10% FCS (v/v). HEK293 cells were grown in DMEM/Ham’s F12 (1:1, v/v) containing 15% FCS (v/v). After cells reached full confluency, they were split into new cell culture flasks for further cultivation, transient transfection, or seeding into cell culture vessels for assays.

2.3.3 Inositol phosphate accumulation assay

COS-7 were grown in 25 cm² culture flasks until 70% confluency was reached. Transient transfection was performed using Metafectene® Pro transfection reagent (according to the manufacturer’s protocol) with either 4000 ng/25 cm² flask pCMV3-ET₄R-GFPspark or pCMV3-ET₃R-GFPspark. One day post transfection, cells were seeded into 384-well plates (15,000 cells/well) and cultured at standard conditions overnight. To detect receptor activation, cells were stimulated with different peptide concentrations in a range of 10⁻⁶ to 10⁻¹² M in assay buffer (HBSS containing 20 mM LiCl) for 60 min at standard conditions. Afterward, cell lysis and inositol phosphate detection was performed as described by the manufacturer using a Tecan Spark plate reader (Tecan Group, Männedorf, Switzerland). Assays were performed in triplicates, which were averaged and normalized to ET-1 (bottom value = 0%; top value = 100%). Concentration–response curves represent the mean ± standard error of the mean (SEM), calculated as average of the assays repetitions (n ≥ 3). Determination of EC₅₀/pEC₅₀ and E₅₀ values was performed with the software GraphPad PRISM 5.0 (San Diego, USA).

2.3.4 Arrestin 3 recruitment assay

HEK293 cells were transiently transfected using Metafectene® Pro transfection reagent (according to the manufacturer’s protocol) after reaching 70% confluency in either six-well plates (kinetic experiments) or 75 cm² cell culture flasks (concentration–response curves). Co-transfection of ET₄R-GFPspark or ET₃R-GFPspark and Nluc-ar3 was performed at a ratio of 20/1 with 4000 ng per well (kinetic experiments) or 12,000 ng/75 cm² flask (concentration–response curves) total plasmid DNA. One day post transfection, cells were seeded into poly-D-lysine-coated μClear 96-well plates (100,000 cells per well) and cultured at standard conditions. The medium was replaced with bioluminescence resonance energy transfer (BRET) buffer (25 mM HEPES in HBSS, pH 7.3), and experiments were performed at 37°C.

For BRET experiments, the luciferase substrate furimazine was added to a final concentration of 2.1 μM. For kinetic BRET studies, the baseline was measured for 5 min before 100 nM peptide was added. The BRET signal was measured for 30 min using a Tecan Spark plate reader (Tecan Group, Männedorf, Switzerland) (luminescence 400–440 nm; fluorescence: 505–590 nm). For concentration–response curves, peptides were added in a concentration range of 10⁻⁶ to 10⁻¹² M, and the BRET signal was measured after 7.5 min stimulation. The BRET ratio was calculated as a quotient of fluorescence to luminescence values, and the netBRET was determined by subtraction of BRET signals from unstimulated cells. Assays were performed in triplicates (concentration–response curves), which were averaged and normalized to ET-1 (bottom value = 0%; top value = 100%) or quadruplicates (recruitment kinetics). Concentration–response curves represent the mean ± SEM, calculated as average of the assays repetitions (n ≥ 3). Determination of EC₅₀/pEC₅₀ and E₅₀ values was performed with the software GraphPad PRISM 5.0 (San Diego, USA).
OptiMEM and incubated at standard conditions for 20 min. To induce receptor internalization, the starvation medium was aspirated and 200 μl OptiMEM, containing 100 nM peptide, was added. Stimulation was performed for 1 h at standard conditions. Cell nuclei were stained with 1 μl of Hoechst 33342 (0.5 mg/ml) 30 min prior to image acquisition. After stimulation, cells were washed twice with 20 mM HEPES/ HBSS (pH 8.5) to remove excessive peptide and maintained in OptiMEM. For microscopy analysis a Zeiss Axio Observer.Z1 microscope including an ApoTome.2 Imaging System, AxioCamMRRm camera, incubation chamber, and C-Apochromat 63x/1.20 W objective, a Zeiss Aplanotm.2 Imaging System, AxioCamMRm camera, incubation chamber, and C-Apochromat 63x/1.20 W objective, was performed with Zeiss ZEN 2 software.

3 | RESULTS

3.1 | Synthesis of monocyclic ET-1 derivatives

Endothelin 1 (ET-1) is a bicyclic peptide, which acts at both endothelin receptor subtypes with low nanomolar potency. By removal of one disulfide bridge and increase of the ring size, we aimed to develop novel monocyclic ET-1 analogs, which are easier to synthesize while maintaining the ET-1-like fold and retaining downstream signaling properties at the respective endothelin receptor subtype. ET-1 analogs (Table 1) were synthesized by automated and manual SPPS.

First, the four cysteines were substituted by alanine, removing the bicyclic structure of the peptide. To further simplify the synthesis and increase the stability toward oxidation of peptide 1, we replaced Met7 with isosteric norleucine. Next, we included one disulfide bridge (Cys1–Cys15; peptide 2). For derivative 3, we increased the size of the intramolecular bridge from Cys1–Cys15 by thioacetal formation (Figure 1). We incorporated an additional methylene moiety between the sulfur atoms, forming the disulfide bond. To test whether the distance of the sulfur atoms is important for peptide–receptor interaction, we replaced Cys3 with hCys (peptide 4), as well as Cys5 and Cys15 (peptide 5) to further increase the size of the disulfide bridge. To visualize receptor-mediated peptide uptake into cells by fluorescence microscopy, peptides 1–5 were additionally labeled with 6-carboxytetramethylrhodamine (TAMRA) moieties (peptides 1F–5F) at Lys9 to identify a potential modification site for cargo loading. The homogeneity of all synthesized peptides was assessed by RP-HPLC, and the molecular identity was verified by MALDI-ToF- and ESI-MS (Table S1 and Figures S1–S10). All peptides were biologically characterized and compared to wt ET-1 and the linearized ET-1 derivative (peptide 1).

3.2 | Identical folding properties of ET-1 derivatives in comparison to the wt peptide

Because all members of the endothelin peptides share high sequence identity, we used CD spectroscopy to estimate the structural similarities of the synthesized ET-1 analogs in comparison to ET-1 (Figure 2). For ET-1, characteristics of a partial α-helical fold was detected in aqueous solution, indicated by an ellipticity maximum at ~190 nm and a minimum at 205 nm (Figure 2A). No minimum at 220 nm was detected. Contrary, a random coil was determined for the linear

| Table 1 | Sequence and structural information of the synthesized peptides |
|---------|-----------------------------|
| **No.** | **Sequence** | **Cyclization** | **Sizea** |
| ET-1 (wt) | C1SC5SSLML0DK6EC11VYFCH15LDH1W | S−S | 4 |
| 1 | A1SA3SSLX7DKEA15VYFCH15LDH1W | / | / |
| 2 | CSA3SSLX7DKEA15VYFCH15LDH1W | S−S | 4 |
| 3 | CSA3SSLX7DKEA15VYFCH15LDH1W | S−CH2−S | 5 |
| 4 | hC1SA3SSLX7DKEA15VYFCH15LDH1W | S−S | 5 |
| 5 | hC1SA3SSLX7DKEA15VYFCH15LDH1W | S−S | 6 |
| 1F | A1SA3SSLX7DKEA15VYFCH15LDH1W | / | / |
| 2F | CSA3SSLX7DKEA15VYFCH15LDH1W | S−S | 4 |
| 3F | CSA3SSLX7DKEA15VYFCH15LDH1W | S−CH2−S | 5 |
| 4F | hC1SA3SSLX7DKEA15VYFCH15LDH1W | S−S | 5 |
| 5F | hC1SA3SSLX7DKEA15VYFCH15LDH1W | S−S | 6 |

Note: Modified residues are highlighted in superscript by their respective position in the sequence. Bold, substitution of cysteine by alanine; X, substitution of methionine; red, TAMRA; blue, substitution of cysteine by homocysteine. hC/hCys, L-homocysteine; X, L-norleucine; TAMRA, 6-carboxytetramethylrhodamine. “F” depicts fluorescently labeled peptides. Abbreviations: TAMRA, 6-carboxytetramethylrhodamine; wt, wild-type.

*aIntramolecular ring size depicts the number of atoms between the peptide backbones.*
peptide 1 by a dominant minimum at 195–200 nm. Surprisingly, for peptides 2, 4, and 5, no wt-like fold was detectable under aqueous conditions. These peptides resembled peptide 1. However, by adding 20 mM SDS, a membrane mimetic, a change in secondary structure was induced in all synthesized peptides (Figure 2B). The characteristic random coil minimum was replaced by an intensive ellipticity maximum at ~190 nm and minimum at ~206 nm, similar to ET-1. The most extensive change was observed for the linear peptide 1,
indicating the high flexibility of the peptide chain. For peptide 3, containing the methylene thioacetal instead of a regular disulfide bridge, the presence of the membrane mimetic induced an α-helical secondary structure (Figure 2C) highlighted by the intense maxima around 190 nm. This indicates that the global structure of the ET-1 derivatives does not differ from the wt peptide under membrane mimicking conditions.

### 3.3 Monocyclization of ET-1 analogs increases selectivity toward ET<sub>B</sub>R

We investigated the capability of the synthesized ET-1 analogs to induce different signaling pathways of the endothelin receptor subtypes. Signal transduction of membrane-embedded GPCRs can be conveyed through two distinct downstream effectors: G proteins and arrestins. The G<sub>q</sub> signaling was studied by inositol phosphate accumulation assays, and the subsequent recruitment of arrestin 3 (arr3) was analyzed through kinetic and concentration-dependent analyses. Receptor-arrestin interactions were investigated by bioluminescence resonance energy transfer (BRET) studies using nanoluciferase (Nluc)-equipped arr3 at both ET<sub>A</sub>R and ET<sub>B</sub>R, to gain more insight into receptor selectivity (Figure 3).

ET-1 was used as positive control and linear peptide 1 was used as selectivity control in both signaling assays. For ET-1, low nanomolar EC<sub>50</sub> values for G protein activation were determined for both ET<sub>A</sub>R (0.7 nM) and ET<sub>B</sub>R (2.6 nM) (Figure 3A and Table 2). ET-1 analog 1 displayed ET<sub>B</sub>R selectivity with a wt-like EC<sub>50</sub> at the ET<sub>B</sub>R, whereas no EC<sub>50</sub> value could be determined at the ET<sub>A</sub>R (ligands tested up to 1 μM). Monocyclization and Met-substitution of ET-1 (derivative 2) led to a receptor-unselective and ET-1-like agonist with comparable EC<sub>50</sub> values (ET<sub>A</sub>R: 0.2 nM; ET<sub>B</sub>R: 1.1 nM). Increasing the size of the disulfide bridge by one CH<sub>2</sub> unit between the sulfur atoms of the disulfide bridge (peptide 3) and elongation of the Cys<sup>1</sup> side chain (peptide 4) or both—Cys<sup>1</sup> and Cys<sup>15</sup>—side chains (peptide 5) did not decrease G protein activity at the ET<sub>B</sub>R. In contrast, all three peptides containing extended disulfide bridges lost activity at the ET<sub>A</sub>R. All three concentration–response curves show a right-shift compared to ET-1. Because no signal saturation was detected (at a maximum applied concentration of 1 μM) for peptides 3, 4, and 5, no EC<sub>50</sub> value could be determined for these analogs. Monocyclization of ET-1 induces an intrinsic 100-fold selectivity of ET<sub>B</sub>R-mediated G protein activation over ET<sub>A</sub>R-mediated signaling.

Because GPCR activation does lead not only to the activation of G protein signaling but also the recruitment of other downstream effectors like arrestins, we investigated the effects of the peptide modifications on arr3 as subsequent effector of the endothelin receptors. The interaction of the GPCRs and arr3 was investigated by BRET assay, for which a C-terminally GFP-tagged GPCR (BRET acceptor) was co-transfected with an N-terminal Nluc-tagged arr3 (BRET donor). First, we investigated the time dependency of the arr3 translocation from the cytoplasm to the membrane (Figure 3B). ET-1 and peptide 2 showed the same behavior for the recruitment of arr3 to ET<sub>A</sub>R. Peptides 1, 3, 4, and 5 did not induce arr3-association with ET<sub>A</sub>R, indicated by netBRET signals similar to the buffer condition (negative control). For the arr3 recruitment to activated ET<sub>B</sub>R, all tested ligands showed quick and ET-1 comparable recruitment kinetics of arr3 to the GPCR. However, in comparison to ET-1, only the monocyclic analog 2 reached a netBRET maximum similar to ET-1-induced netBRET values. To determine whether the reduced netBRET values were due to decreased ligand potency or induced signaling bias, we performed concentration–response analyses of the arr3-receptor interaction (Figure 3C). Validating the G protein data, only ET-1 and its derivative 2 showed a concentration-dependent

![Figure 3](image_url)
increase in BRET signal after ligand addition to the ET₄R. ET-1 exhibited a nanomolar EC₅₀ of 7.8 nM, whereas peptide 2 had a threefold decreased potency (19.2 nM) and reduced maximum arr3 recruitment (84% of ET-1), highlighting the importance of the bicyclic nature of ET-1 for efficient ET₄R-signaling by arr3. This indicates that monocyclic peptide 2 displays a minor favoring of G protein signaling over arr3 recruitment compared to ET-1 at this receptor. The remaining peptides (1, 3, 4, and 5) did not induce recruitment of arr3 to ET₄R until a concentration of at least 1 μM. For ET₅R, ET-1 exhibits an EC₅₀ of 19.1 nM, which was an approximately threefold shift compared to ET₄R (7.8 nM). The shifted peptide potency of arr3 recruitment was similar to the decreased potency of ET-1-induced G protein signaling at both receptors (ET₄R protein: 0.7 nM, ET₅R protein: 2.6 nM). Peptide 2, the monocyclic peptide with the unmodified disulfide bridge from Cys₁ to Cys₁₅, displayed ET-1-like potency and efficacy concerning arr3 recruitment to activated ET₄R. For the ET₅R-selective peptide 1 a similar ligand potency was determined for arr3, but a reduction of maximum BRET signal to 57% was detected, resulting in a small G protein signaling bias. This bias was also detected for the monocyclic peptides 3, 4, and 5, containing elongated disulfide bonds. These derivatives showed ET-1-like potencies; however, the efficacy was reduced to 70% for peptide 3, 64% for peptide 4 and 61% for peptide 5. Overall, our ET-1 analogs, containing only the cyclization Cys₁₋ Cys₁₅ with an increased length of one or two methylene units (derivatives 3, 4, and 5), exhibited a 100-fold signaling selectivity toward ET₄R over ET₅R, indicating their potential application.

Next, we investigated receptor internalization into endosomes. In case of GFP-tagged ET₄R/ET₅R, this led to a decrease of membrane fluorescence and an increase of intracellular fluorescence. We used transiently transfected HEK293 cells expressing either ET₄R-GFPspark or ET₅R-GFPspark to investigate the translocation of the receptor from the membrane in cytoplasmic vesicles depending on the applied ligand (Figure 4). For unstimulated ET₄R-GFPspark, a distinct membrane localization was detectable with only minor intracellularly retained vesicular fluorescence. When stimulated with 100 nM ET-1 (Figure 4A, wt) for 60 min, the intracellular fluorescence in vesicles was increased and the membrane fluorescence was reduced. This indicated the ET-1-dependent internalization of the ET₄R. For peptides 1, 3, 4, and 5, which displayed no recruitment of arr3 to ET₄R (see Figure 4C), no ET₄R internalization was detectable using fluorescence microscopy. Contrary, peptide 2 induced internalization of the ET₅R. Because the ET-1 derivative 2 showed G protein activation and retained arr3 recruitment similar to the wt ligand (Figure 3), the internalization of the ET₅R induced by peptide 2 was distinct from the remaining ET-1 analogs but displayed receptor internalization to a lower extent than the wt ligand.

In addition to the membrane fluorescence, a high amount of intracellular vesicles was already displayed by unstimulated cells that transiently express the ET₅R. When ET-1 was administered, the receptor was completely internalized within 60 min, indicated by the complete absence of membrane fluorescence. Application of the linear peptide 1 and the monocyclic peptides 2–5 also leads to an abolished membrane fluorescence after the stimulation period. This effect was similar to the wt agonist, but the effect was seemingly less pronounced for the peptide analogs. Interestingly, peptide ligands 1, 3, 4, and 5 led to complete receptor internalization despite the reduced efficacy determined in the arr3 recruitment assays.

### 3.4 | Lys⁹—A promising modification site for cargo attachment

To investigate the trafficking of the ET-1-derived peptides, fluorescently labeled analogs were used. Lys⁹, which is present in wt ET-1, was chosen for side-chain modification. We attached TAMRA at the Lys⁹ N⁺ by SPPS proceedings, generating peptides 1F–5F. To validate the compatibility of this modification with receptor–ligand interaction, we determined the G protein activation mediated by the TAMRA-labeled analogs in inositol phosphate accumulation assays (Figure 5). Similar to the G protein activation of the nonfluorescent ET-1-analogs, no nanomolar ET₄R activation was detectable for linear analogs.
peptide 1F, the thioacetal bridged peptide 3F, and the hCys-containing peptides 4F and 5F. Derivative 2F exhibited an EC50 of 1.7 nM, which was 2.5-fold reduced compared to the endogenous ligand ET-1 (0.7 nM; see Table 3) and approximately eightfold reduced compared to the nonfluorescent peptide 2 (0.2 nM) but fully activated the receptor. All TAMRA analogs activated the ETAR at low nanomolar concentrations. Surprisingly, the fluorescently labeled peptides were less potent to induce intracellular G protein signaling for both receptor species compared to the unlabeled peptides.

Next, we studied the cellular uptake of the TAMRA-labeled analogs 1F–5F into transiently transfected HEK293 cells (Figure 6). Because ET-1 was not available with a fluorescent label as external control peptide, we used peptide 1F as control for the internalization studies. In agreement with the G protein activation studies, this peptide did not induce internalization of the ETAR (as shown in Figure 6A), because of the low receptor activation at the applied concentration of 100 nM. Neither intracellular receptor subpopulations nor peptide accumulation was determinable. The same was found for peptides 4F and 5F. TAMRA-peptide 2F showed distinct intracellular colocalization of green fluorescence (ETaR-GFPspark) and red (TAMRA) fluorescence (peptide 2F) after stimulation for 60 min (indicated by yellow vesicles due to overlapping of GFPspark fluorescence with TAMRA fluorescence), indicating the receptor-mediated cellular uptake of this compound. Analog 3F showed activation of the ETaR in the G protein signal transduction assay (Figure 5) but did not reach maximum signal intensity even at 1 μM ligand concentration, intracellularly colocalized with the receptor only to little extent. The observed intracellular TAMRA fluorescence in vesicular structures was less compared to peptide 2F, which displayed a better receptor activation potency in contrast to peptide 3F at the ETaR (EC50(peptide 2F): 1.7 nM; EC50(peptide 3F): no signal saturation at 1 μM peptide).

For ETBR-mediated peptide uptake, all TAMRA-peptides showed intracellular accumulation after 60 min stimulation. No membrane fluorescence was detectable, displaying the complete translocation of ETBr species from the membrane into endosomes. The fluorescent analogs showed exclusively colocalization with internalized ETBr (depicted in yellow). TAMRA fluorescence was localized to the green GFP fluorescence from the GPCR.

**DISCUSSION**

Selective activation of the endothelin receptors is of pharmacological interest due to their opposing effects in vivo. High homology between both receptor subtypes and their ligands limits, however, the generation of selective compounds. Although one ETaR-selective agonist has been developed—\([-\text{Lys}^9]\text{cyclo}^{11-15}\text{ET-1(9-21)}\)—this compound displays only weak, micromolar activity at the receptor (EC50: 23 μM). Contrary to the ETaR, several ETBr-selective agonists have been described in literature, for example, the linear
[4Ala1,3,11,15]-ET-1, sarafotoxin 6c, IRL1620, and BQ3020. By removal of one disulfide bridge and modification of the ring size of the remaining disulfide bond, we aimed to investigate receptor selectivity and the effects on the downstream signaling profiles at both endothelin receptor subtypes. To simplify the synthesis of ET-1 analogs, we substituted the endogenous Met residue, which is sensitive to oxidation, by the isosteric norleucine. Peptide 1, which was based on the ETBR-selective [4Ala1,3,11,15]-ET-1, displayed no activity at the ETAR and showed ET-1-like activation of the endogenous signaling pathways of the ETBR in both G protein signaling and arr3 recruitment assays. This indicates the compatibility of the replacement with the receptor–ligand interaction. Thus, Met residues were exchanged to norleucine in all our peptide analogs. Next, we investigated the structural effect of monocyclization on ET-1-derived peptides. Because all members of the endothelin peptides and the related sarafotoxins share a common secondary structure motif, CD spectroscopy was applied to elucidate the structural integrity of the synthesized analogs. The α-helical character of wt ET-1, constrained by two disulfide-bridges, was detectable in buffered aqueous solution. The helix of ET-1 is located from Lys9 to Cys15 in aqueous solution (30% of all ET-1 residues) but can extent to the C-terminal Ile20 (as shown for the crystal structure of ET-1), whereas the remaining peptide backbone (Cys1 to Asp8) does not adopt this secondary structure. Due to structural flexibility of the C-terminus and the N-terminal peptide sequence not being folded in a specific manner (approximately random coil), ET-1 does not display the characteristics of a pure α-helix and rather is a mixture of secondary structure elements (random coil and α-helix).

The helical character was further enhanced by addition of SDS as membrane mimetic. This is in agreement with previous results on ET-1 folding, which report on the induction of secondary structures by cell membranes. Similar effects were demonstrated for other peptides hormones like the neuropeptide Y, which displays different folding patterns depending on the absence or presence of hydrophobic environments. Contrary to the wt peptide, the linearized ET-1 (peptide 1) showed only random coil structures in aqueous solution, although addition of the membrane mimetic induced an ET-1-like helical fold. We removed the disulfide bridge (Cys3–Cys11) to investigate whether one disulfide bridge (peptide 2) is sufficient for ET-1-like folding in the absence of a membrane mimic. Surprisingly, the constriction of the backbone flexibility of the peptide chain by one disulfide did not suffice to induce wt-like folding in aqueous

**FIGURE 5** Characterization of ET<sub>A</sub>R- and ET<sub>B</sub>R-mediated G protein activation induced by 6-carboxytetramethylrhodamine (TAMRA)-labeled ET-1 analogs. G protein activation was investigated for ET<sub>A</sub>R (A) or ET<sub>B</sub>R (B) in inositol phosphate accumulation assays (concentration range: 10<sup>-6</sup> to 10<sup>-12</sup> M; n ≥ 2). Data were normalized to ET-1. Concentration–response curves represent mean ± standard error of the mean (SEM).

**TABLE 3** In vitro characterization of ET<sub>A</sub>R- and ET<sub>B</sub>R-mediated G protein activation induced by TAMRA-labeled ET-1 analogs by inositol phosphate accumulation (n ≥ 3)

|     | G protein activation |     | G protein activation |
|-----|----------------------|-----|----------------------|
|     | ET<sub>A</sub>R      |     | ET<sub>B</sub>R      |
|     | EC<sub>50</sub> (nM) (pEC<sub>50</sub> ± SEM) | E<sub>max</sub> (%) (±SEM) | EC<sub>50</sub> (nM) (pEC<sub>50</sub> ± SEM) | E<sub>max</sub> (%) (±SEM) |
| wt  | 0.7 (9.17 ± 0.03)    | 100 | 2.6 (8.58 ± 0.04)    | 100 |
| 1F  | n.d.                 | n.d.| 10.6 (7.97 ± 0.12)   | 90 ± 7 |
| 2F  | 1.7 (8.77 ± 0.11)    | 100 ± 6 | 6.6 (8.18 ± 0.11)    | 87 ± 6 |
| 3F  | n.d.                 | n.d.| 5.8 (8.24 ± 0.13)    | 97 ± 7 |
| 4F  | n.d.                 | n.d.| 11.5 (7.94 ± 0.12)   | 96 ± 7 |
| 5F  | n.d.                 | n.d.| 8.6 (8.07 ± 0.18)    | 91 ± 9 |

Note: Data were normalized to ET-1 (wt) and are represented as mean value over all assay replicates (performed in triplicates) ± standard error of the mean (SEM). n.d., not detected up to an applied ligand concentration of 10<sup>-6</sup> M. Abbreviations: SEM, standard error of the mean; TAMRA, 6-carboxytetramethylrhodamine.
solution. Adding SDS, however, induced the typical ET-1 fold with a partial α-helical character in peptide 2. Increasing the flexibility of the peptide by increasing the length of the disulfide bridge by either one methylene unit (peptides 3 and 4) or two methylene units (peptide 5) did not allow for specific secondary structures under aqueous conditions. All three peptides were random coil and similar to the linear peptide 1, but adopted a wt-like structure after SDS addition, but did not exhibit the typical double minima of pure α-helices similar to ET-1. However, the compact secondary structure of ET-1, which is a requirement for ET₄R and ET₅R activation, was preserved in all generated derivatives, independent of the modification size of the intramolecular cyclization.

The receptor activation potentials of the ET-1 analogs were determined to characterize their ability to stimulate both G protein activation and arrestin recruitment of ET₄R and ET₅R. The bicyclic ET-1 and the monocyclic peptide 2-induced Gₛ signaling of both endothelin receptor subtypes. However, increasing the disulfide bridge by incorporation of one homocysteine (peptide 4), two homocysteines (peptide 5), or an additional methylene functionality in the disulfide (peptide 3) induced a 100-fold ET₅R selectivity compared to ET-1. The ET₄R- and ET₅R-mediated G protein activation profiles align well with previous SAR studies of ET-1 carried out on tissue preparations, demonstrating that the disulfide bridge formed by Cys¹ and Cys¹⁵ is sufficient to activate ET₄R.¹⁴ Contrary, linear peptide

| (A) | unstim | 1F | 2F | 3F | 4F | 5F |
|-----|--------|----|----|----|----|----|
| merge | ![Image](image1.png) | ![Image](image2.png) | ![Image](image3.png) | ![Image](image4.png) | ![Image](image5.png) | ![Image](image6.png) |
| ET₄R-GFPspark | ![Image](image7.png) | ![Image](image8.png) | ![Image](image9.png) | ![Image](image10.png) | ![Image](image11.png) | ![Image](image12.png) |
| 520-550 nm | ![Image](image13.png) | ![Image](image14.png) | ![Image](image15.png) | ![Image](image16.png) | ![Image](image17.png) | ![Image](image18.png) |
| ET₅R-GFPspark | ![Image](image19.png) | ![Image](image20.png) | ![Image](image21.png) | ![Image](image22.png) | ![Image](image23.png) | ![Image](image24.png) |
| 590-650 nm | ![Image](image25.png) | ![Image](image26.png) | ![Image](image27.png) | ![Image](image28.png) | ![Image](image29.png) | ![Image](image30.png) |

FIGURE 6  6-carboxytetramethylrhodamine (TAMRA)-peptide internalization studies of ET-1 analogs in transiently transfected cells, expressing either ET₄R-GFPspark (A) or ET₅R-GFPspark (B) (respective receptor shown in green). Cells were stimulated with 100 nM TAMRA-peptide (red) in OptiMEM for 60 min at 37°C. Cell nuclei were stained with Hoechst34442 (blue). GFP detection: excitation 488–512 nm; emission 520–550 nm; TAMRA detection: excitation 550–580 nm; emission 590–650 nm. Scale bar: 20 μm
1 activates only the ET\(_{B}\)R with nanomolar potency. Complete removal of the intramolecular disulfides by quadruple alanine substitution abolishes vasoconstriction by the ET\(_{A}\)R, but keeps the ET-1-like affinity at the ET\(_{B}\)R.\(^{19}\) The presence of both disulfide bridges (Cys\(^1\)-Cys\(^{15}\) and Cys\(^3\)-Cys\(^{11}\)) is required for the extensive vasoconstriction induced by ET-1 and its homologous peptide family of sarafotoxins.\(^{27}\) Removal of the disulfide from Cys\(^1\) to Cys\(^{15}\) abolishes vasoconstriction in rat artery ring preparations, whereas removal of the disulfide between Cys\(^3\) and Cys\(^{11}\) still leads to vasoactive effects.\(^{30–32}\) However, previous studies on the SARs of the endothelin peptides were acquired in biological assays in rodent-derived tissue. Ligand-induced effects were measured as a system response (e.g., the constriction of prepared vessel rings), which integrates multiple, intracellular signaling cascades. Thus, we tested the compounds in well-defined transiently transfected COS-7 cells, expressing either ET\(_{A}\)R or ET\(_{B}\)R.

In addition to G protein-mediated signals, the recruitment of arr3 to both endothelin receptor subtypes was investigated. Arrestins recognize GPCRs, which were phosphorylated by GPCR kinases after activation by their respective ligand. The recruitment of arrestins to these receptors induces the internalization of the GPCR from the membrane into intracellular vesicles and, thus, terminates G protein signaling of the receptor (receptor desensitization).\(^{33,34}\) For peptide 1, linearization abolished G protein-mediated responses and subsequent arr3 recruitment at the ET\(_{A}\)R, but retained ET-1-like signaling at the ET\(_{B}\)R. However, compared to ET-1, peptide 1 displayed a reduced maximum recruitment of arr3, indicating a minor signaling bias for this agonist in comparison to the wt ligand, which we report for the first time. Biased agonism has been discovered for other peptide-binding GPCRs like the angiotensin II receptor type 1 and the members of the neuropeptide Y receptor family.\(^{35–37}\) Monocyclic peptide 2 exhibited ET-1-like G protein signaling, arr3 recruitment and cellular internalization of both ET\(_{A}\)R and ET\(_{B}\)R. Peptides with increased ring sizes (peptides 3–5) behaved similar to the ET\(_{B}\)R-selective peptide 1 concerning the fast recruitment kinetics of arr3 to activated ET\(_{B}\)R and recruitment potency and efficacy. ET\(_{A}\)R internalization was observed for all peptides. Yet, it was most pronounced for the wt ligand ET-1, which correlates to the decreased arr3 recruitment efficacies of the ET-1 analogs. Nevertheless, receptor activation led to complete depletion of the cellular membrane from ET\(_{A}\)R populations and intracellular accumulation of GFP-positive vesicles.

The ET\(_{A}\)R and ET\(_{B}\)R can be distinguished by their kinetics of ligand binding and internalization behavior. Binding of ET-1 to the ET\(_{B}\)R induces quasi-irreversible trapping of the peptide in the transmembrane bundle of the GPCR, forming a stable complex.\(^{38}\) Depending on the endothelin receptor subtype, the protein complex is subsequently targeted toward different trafficking routes. Whereas ET\(_{A}\)R subpopulations internalize in an agonist-induced manner and recycle back to the cell membrane, internalized ET\(_{B}\)R do not recycle back to the cell membrane and are directed toward lysosomal degradation.\(^{39,40}\) In the absence of agonist, membrane-embedded ET\(_{A}\)R subpopulations remain at the cell surface, whereas ET\(_{B}\)R subpopulations undergo constitutive internalization.\(^{41,42}\) To discriminate between ligand-independent and ligand-dependent ET\(_{B}\)R internalization and to verify cellular uptake of the ET-1 analogs, the TAMRA fluorophore was introduced into the peptides as orthogonal fluorescent reporter. The organic dye was linked to the side chain of the endogenous Lys\(^9\) of ET-1 (peptides 1F–5F), which circumvents the need for additional amino acid substitution. The fluorescent ET-1 analogs 1F–5F displayed a minor loss of ET\(_{A}\)R and ET\(_{B}\)R activation potency compared to the respective nonfluorescent peptides (~5- to ~12-fold decrease concerning ET\(_{B}\)R activation), but EC\(_{50}\) values for these peptides still remained in the low nanomolar range. The loss of potency was likely caused by the attachment of the fluorophore as it removed the positive charge at Lys\(^9\) and added the bulky structure of the reporter. Lys\(^9\) is localized in the loop region of ET-1 at the beginning of the α-helix.\(^{4,25,26}\) In the ET-1/ET\(_{B}\)R complex, this residue interacts with the extracellular loop 2, due to its upward position in the transmembrane bundle.\(^{5,43–45}\) Alanine substitution of this residue does not alter both ET\(_{A}\)R- and ET\(_{B}\)R-induced effects in tissue preparations, indicating only a minor role in ligand recognition by the receptor.\(^{46}\) Only peptide 2F was able to activate the ET\(_{B}\)R in an ET-1-like manner, whereas all of the fluorescently labeled peptides induced ET\(_{B}\)R-selective signaling. Ligand efficacy at the ET\(_{B}\)R was not affected by the dye for all TAMRA-ET-1 analogs. We used the TAMRA fluorophore for tracking the selective cellular uptake of peptides 1F–5F in transiently transfected HEK293 cells to study receptor-mediated internalization of the receptor-ligand complex.

The efficient internalization machinery of GPCRs can be used for cell-specific drug delivery by peptide-drug conjugates, which can be easily and site-specifically equipped with different linker structures for intracellular cargo release.\(^{47–50}\) Taking advantage of ligand-induced internalization of GPCRs for intracellular cargo delivery has been demonstrated for targeting cancer-related receptors like the gastrin-releasing peptide receptor and the neuropeptide Y receptor 1 (Y\(_1\)R).\(^{51–53}\) Recently, the successful delivery of organic drugs into adipose tissue was demonstrated in a rodent model by targeting the Y\(_1\)R.\(^{54}\) For cardiovascular application, different drug delivery systems have been reported to increase the effectiveness and decrease off-target effects of drugs like aliskiren and cerivastatin.\(^{55–57}\) Due to its limited distribution in the periphery, the endothelin system provides an interesting target option for the development of a peptide shuttle to treat CVD.\(^{58–63}\) ET\(_{B}\)R-targeting by [4Ala\(^1,3,11,15\)]-ET-1 to treat CVDs is limited due to linear peptides being prone to enzymatic degradation in the blood stream.\(^{64–66}\) Metabolic stabilization can be achieved by incorporation of unnatural amino acids and artificial intramolecular bridges, resistant toward degradation.\(^{67}\) We propose that monocyclic peptides, displaying ET\(_{B}\)R-selectivity, are promising vectors to combat CVDs due to the favorable physiological responses of the ET\(_{B}\)R (e.g., ligand clearing). Furthermore, Lys\(^9\) is a suitable position for drug attachment as demonstrated by fluorescence labeling.

### 5 | CONCLUSION

Here, we report on the development of new ET\(_{B}\)R-selective peptides with minimal modifications compared to the wt agonist ET-1 and their
detailed characterization with regard to endothelin receptor signaling. Monocyclization of ET-1 by removal of the Cys3–Cys11 disulfide bridge and increase of the size of the monocyte from Cys3–Cys15 to hCys1–hCys15 led to a loss of potency at the ETAR but kept activity at the ETBR. We established a methylene thioacetal formation of monocyclic ET-1 analogs to probe the acceptance of artificial bonds. Additionally, by introduction of a fluorescent label, we identified Lys9 as a potential modification site for the attachment of fluorophores and other cargos in the future. Receptor-mediated peptide internalization in ETAR- and ETBR-expressing cells was studied by TAMRA-labeled compounds.

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SUPPORTING INFORMATION

Additional supporting information may be found online in the Supporting Information section at the end of this article.