

Article

Small Peptides Able to Suppress Prostaglandin E₂ Generation in Renal Mesangial Cells

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Abstract: Peptide drug discovery may play a key role in the identification of novel medicinal agents. Here, we present the development of novel small peptides able to suppress the production of PGE₂ in mesangial cells. The new compounds were generated by structural alterations applied on GK115, a novel inhibitor of secreted phospholipase A₂, which has been previously shown to reduce PGE₂ synthesis in rat renal mesangial cells. Among the synthesized compounds, the tripeptide derivative **11** exhibited a nice dose-dependent suppression of PGE₂ production, similar to that observed for GK115.

Keywords: inhibitors; mesangial cells; peptides; prostaglandin E₂; secreted phospholipase A₂

1. Introduction

Prostaglandin E₂ (PGE₂) is one of the most important lipid mediators [1] playing a pivotal role in inflammatory diseases [2] including glomerulonephritis. Glomerulonephritis is initiated by the invasion of activated immune cells into the renal glomerulus and the release of pro-inflammatory cytokines such as TNF α and interleukin (IL) 1 β to activate the local resident mesangial cells [3,4]. This activation includes three main responses, i.e., increased proliferation, increased matrix production, and increased inflammatory mediator production, which are all hallmarks of glomerulonephritis, finally resulting in glomerulosclerosis and renal failure [4]. Due to this central involvement of mesangial cells in the pathogenesis of chronic inflammatory kidney diseases, they serve as a useful cellular model system to test for new therapeutic compounds to treat such diseases.

The biosynthesis of PGE₂ is initiated by the action of phospholipases A₂ (PLA₂), which cleave the *sn*-2 ester bond of membrane glycerophospholipids to release arachidonic acid [5,6]. Then, arachidonic acid is converted to PGH₂ and the terminal step of PGE₂ generation is catalyzed by microsomal prostaglandin synthase-1 [7]. Four types of PLA₂ [6] are expressed at mesangial cells: cytosolic (cPLA₂) GIVA, calcium-independent (iPLA₂) GVIA and secreted (sPLA₂) GIIA and GV [8–10]. It has been demonstrated that the progression of glomerular inflammatory processes is a result of a network of interactions between different PLA₂s [9] and a cross-talk between cPLA₂ and sPLA₂ has been reported in hydrogen peroxide-induced arachidonic acid release in murine mesangial cells [11]. In addition, sPLA₂ GIIA seems to have multiple roles in mesangial cells inducing the production of pro-inflammatory mediators [12].

We have recently demonstrated that inhibitors of sPLA₂ suppress the release of PGE₂ formation in renal mesangial cells [13]. Four such inhibitors presented an interesting effect on the suppression of PGE₂ release and their structures are shown in Figure 1. Inhibitors **1** (GK115) [14] and **2** [15] are amides based on the non-natural amino acids γ -norleucine and γ -tyrosine, respectively, while inhibitors **3**

(GK126) [16] and 4 (GK241) [17] are 2-oxoamides based on the natural amino acids leucine and valine, respectively. As depicted in Figure 1, all of them are characterized by high lipophilicity (ClogP value higher than 5), an unfavorable property according to Lipinski's rule of 5 [18]. The ClogP values of the inhibitors were calculated using ChemOffice Ultra 11.00.

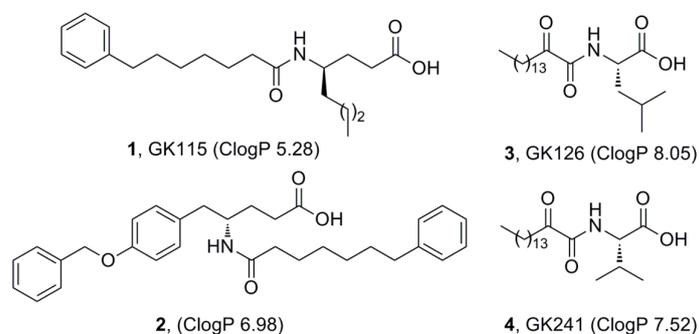


Figure 1. Inhibitors of secreted PLA₂ able to suppress the formation of PGE₂ in mesangial cells.

Over the last years, peptide drug discovery has experienced a revival of interest, as the pharmaceutical industry has come to appreciate the role that peptides may play in addressing unmet medical needs and how peptides can be an excellent complement to small molecule medicinal agents [19]. In an effort to identify novel compounds able to suppress the formation of PGE₂, in the current work we explored the possibility of small conjugated peptides to inhibit the release of PGE₂ at a cellular level. Herein, we report the structure-based design and synthesis of several small peptides derivatives (Figure 2), whose structures are inspired by the structures of sPLA₂ inhibitors described above, and the evaluation of their ability to suppress PGE₂ formation.

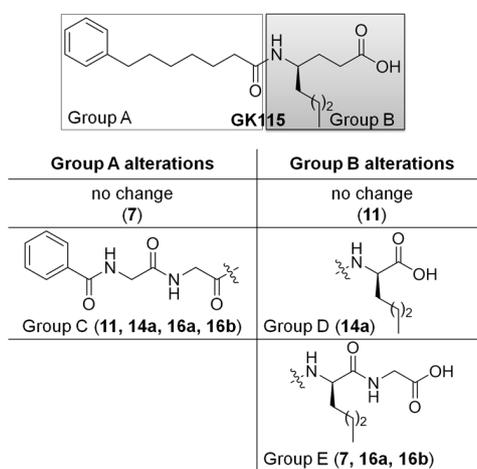


Figure 2. The design of new inhibitors was based on alterations of Groups A and B of GK115 structure. The table describes the alterations of Groups A and B and the corresponded generated compounds in brackets.

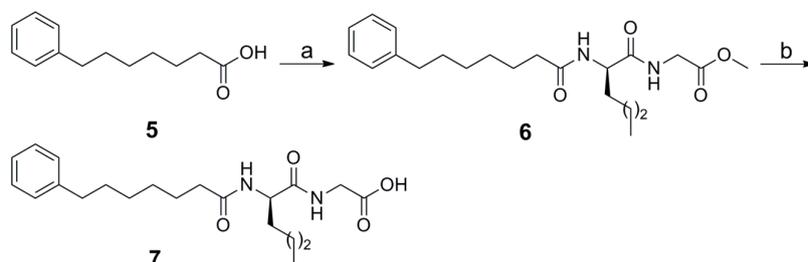
2. Results

2.1. Synthesis

Inspired by the structure of inhibitor GK115 and considering it as a lead compound, we designed derivatives replacing the γ-norleucine residue by α-norleucine or a norleucine based dipeptide. In addition, and in an effort to decrease the lipophilicity of the compounds, we replaced the acyl chain

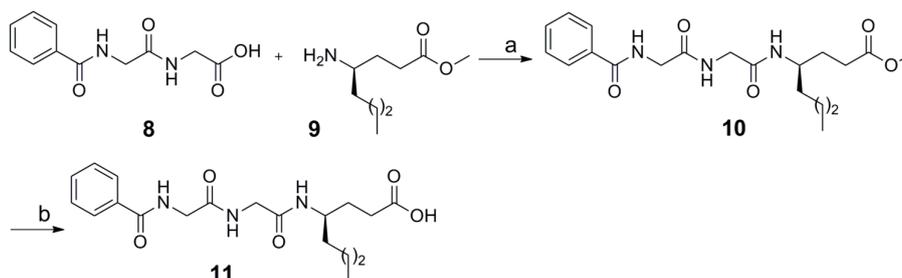
of GK115 (Group A, Figure 2) by a chain based on the benzoyl derivative of the peptide glycyl-glycine (Group C, Figure 2).

Compound 7 was synthesized by coupling of 7-phenyl-heptanoic acid 5 with the dipeptide norleucyl-glycine using 1-(3-dimethylaminopropyl)-3-ethyl carbodiimide (WSCl) as a condensing agent in the presence of 1-hydroxybenzotriazole (HOBt), followed by alkaline hydrolysis (Scheme 1).



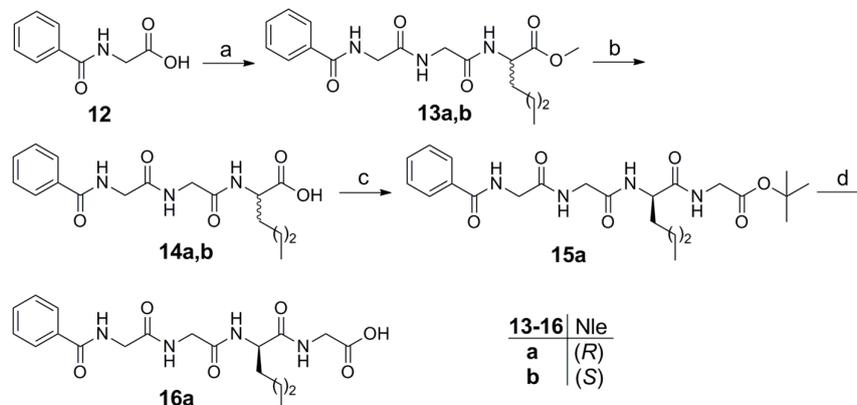
Scheme 1. Reagents and conditions: a. H-Nle-Gly-OMe, WSCI·HCl, HOBt, Et₃N, CH₂Cl₂; b. 1 N aq NaOH, MeOH.

Compound 11, where the acyl chain of GK115 was replaced by the benzoyl derivative of the dipeptide glycyl-glycine, was synthesized by coupling of benzoyl-glycyl-glycine 8 with methyl γ -norleucinate 9, as depicted in Scheme 2.



Scheme 2. Reagents and conditions: a. WSCI·HCl, HOBt, Et₃N, CH₂Cl₂; b. 1 N aq NaOH, MeOH.

To synthesize derivatives based on either α -norleucine or the dipeptide norleucyl-glycine, benzoyl-glycine 12 was coupled with methyl norleucyl-glycinate (Scheme 3). After alkaline hydrolysis, compounds 14a and 14b based on (R)- and (S)-norleucine, respectively, were obtained. Coupling of 14a with *tert*-butyl glycinate led to compound 16a after acidic hydrolysis.



Scheme 3. Reagents and conditions: a. H-Gly-Nle-OMe, WSCI·HCl, HOBt, Et₃N, CH₂Cl₂; b. 1 N aq NaOH, MeOH; c. H-Gly-*t*Bu, WSCI·HCl, HOBt, Et₃N, CH₂Cl₂; d. CF₃COOH, CH₂Cl₂.

2.2. Suppression of PGE₂ Release in Mesangial Cells

Cultures of rat renal mesangial cells were stimulated for 24 h with IL-1 β plus forskolin (Fk) to trigger a huge increase of PGE₂ synthesis, as previously described [20]. Cells were treated in the absence or presence of three different concentrations (1 μ M, 3 μ M and 10 μ M) of the various synthetic compounds. Supernatants were collected and subjected to PGE₂-ELISA to quantify PGE₂ released from the cells.

The results of the suppression of PGE₂ release in mesangial cells caused by the new peptides derivatives are shown in Figure 3. In the absence of IL-1 β plus Fk, none of the compounds, tested at the highest concentration of 10 μ M, changed the basal PGE₂ synthesis significantly (data not shown).

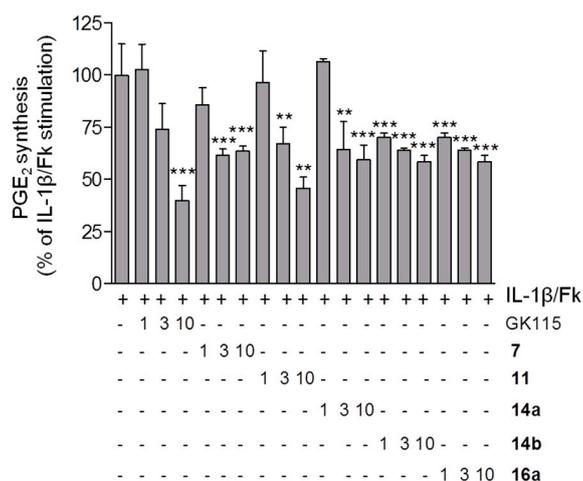


Figure 3. Effect of compounds **7**, **11**, **14a**, **14b** and **16a** on IL-1 β /Fk-stimulated PGE₂ formation in mesangial cells. Data are presented as % of maximal IL-1 β /Fk stimulation and are means \pm S.D. ($n = 3$). ** $p < 0.01$, *** $p < 0.001$ considered statistical significant when compared to the IL-1 β /Fk samples. Basal PGE₂ concentration was at 7.9 ± 5.3 pg/100 μ L supernatant; the maximal IL-1 β /Fk-stimulated PGE₂ concentration was at 685 ± 81 pg/100 μ L supernatant.

Most of the peptide derivatives tested were able to reduce the release of PGE₂. The replacement of γ -norleucine by the dipeptide Nle-Gly (compound **7**) did not cause any remarkable difference in activity. The tripeptide derivative **11** exhibited a nice dose-dependent curve similar to that observed for GK115. The replacement of the hydrocarbon acyl chain of GK115 by the dipeptide glycyl-glycine chain resulted in a considerable decrease of the lipophilicity, dropping the ClogP value from 5.28 to 1.73. It should be noticed that this derivative seems efficiently protected by the action of both aminopeptidases and carboxypeptidases. Peptides based on γ -amino acids are resistant to carboxypeptidases [21], while the benzoyl protecting group offers resistance to aminopeptidases. Tripeptides **14a** and **14b** and tetrapeptide **16a** derivatives exhibited similar ability to suppress PGE₂ formation irrespectively of either the stereochemistry of norleucine (*R* in **14a** and *S* in **14b**) or the size of the peptide (tripeptide versus tetrapeptide). Peptide incorporation of segments in **13a**, **14b** and **16a** resulted in reduced lipophilicities with ClogP values of 1.51, 1.51 and 0.94, respectively. These results confirm our hypothesis that we could improve compounds' lipophilicity, while preserve their potency to suppress PGE₂ release.

2.3. Docking Studies

Since we have shown that inhibition of sPLA₂ is a possible path via which our sPLA₂ inhibitors (GK115, GK126 and GK241) evoke the suppression of PGE₂ release [13], we performed docking calculations for the binding pose prediction of compounds GK115 and **11** in the active site of the enzyme.

Sybyl 8.0 [22] by TRIPOS was used for the design and the energy minimization of the structures. The carboxyl group was considered deprotonated and the Powell algorithm was used for the energy minimization. The crystal structure of sPLA₂ GIIA was retrieved from the Brookhaven Protein Databank (code: 1KQU) and used for the docking calculations. The protein's missing loops were added with the Prime module of Schrödinger Suite [23]. The molecules were placed in the active site of the enzyme manually in Chimera [24] and docked in AutoDock Vina [25].

By visual inspection of the docking results, docking poses which reproduced the known interactions between the co-crystallized ligands and the enzyme, were chosen and subjected to molecular dynamics (MD) simulations with AMBER14 [26], following a standard procedure [17]. MD simulations (100 ns) were run and the top cluster of each simulation is shown in Figures 4 and 5. For the clustering, the hieragglo algorithm was used and the *averagelinkage* option in *cptraj* [27] was defined using the Leu2, Phe5, His6, Ile9, Ala17-Gly32, Cys44, His47, Asp48, Lys62, Asp91 and Phe98 amino acids.

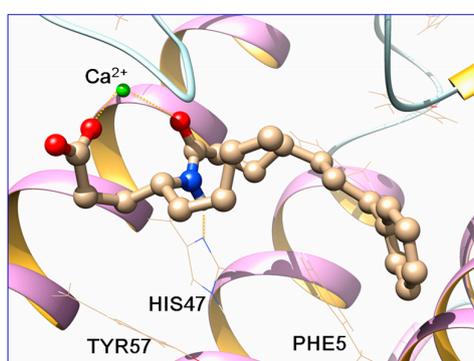


Figure 4. Binding pose prediction of GK115 in the active site of sPLA₂ GIIA (red: oxygen atoms, blue: nitrogen atoms, green: calcium atom).

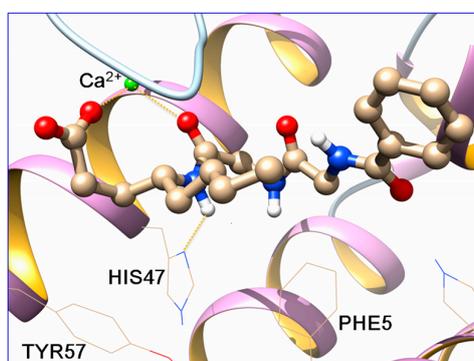


Figure 5. Binding pose prediction of **11** in the active site of sPLA₂ GIIA (red: oxygen atoms, blue: nitrogen atoms, green: calcium atom).

The carboxyl group of both molecules and the carbonyl group of the Nle-Gly peptide bond of **11** and the amide group of GK115 chelate the Ca²⁺ ion throughout the simulation time. Additionally, a hydrogen bond is formed between His47 and the -NH group of the peptide bond of **11** and the amide group of GK115, respectively. These interactions are present in the crystallized structures of sPLA₂ GIIA with the native ligands [15]. Both inhibitors adopt similar conformation in the active site and they preserve it, with small fluctuations, during the MD time (Figure 6).

This further contributes to the persistence of the interactions between the molecules and the residues of the active site. MM-PBSA [28] method was used for the estimation of the energetic terms of both systems. The ΔH value for inhibitor GK115 is -47.16 kcal/mol with a standard deviation error (SDE) 5.16 kcal/mol and for compound **11** is -38.22 kcal/mol with SDE of 6.26 kcal/mol. Since GK115

is a potent inhibitor of sPLA₂ GIIA, both the key interactions and the ΔH values calculated by the MD trajectories are a good indicator of a possible inhibition of sPLA₂ GIIA by **11** and biological assays will be performed in a future study to investigate this possibility.

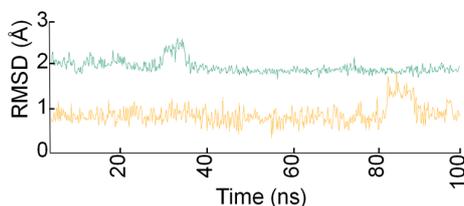


Figure 6. Root-mean-square deviation (RMSD) values of inhibitors **11** (green) and GK115 (orange) in the active site of sPLA₂ GIIA during the MD simulation time.

3. Discussion

Our studies led to the identification of a number of small peptides derivatives that present a significant suppression of PGE₂ release in renal mesangial cells. Among them, benzoyl-glycyl-glycyl- γ -(*R*)-norleucine (**11**) showed an interesting effect on the suppression of PGE₂ formation. This tripeptide derivative has a significant lower lipophilicity in comparison to the lead compound GK115 without loss of the ability to suppress PGE₂ release at a cellular level. Thus, new agents that can serve as leads to reduce pathological PGE₂ production, as seen in chronic inflammatory kidney diseases such as glomerulonephritis, have been identified.

4. Materials and Methods

4.1. Chemistry

Merck Silica Gel 60 (70–230 or 230–400 mesh) (Merck Co., Darmstadt, Germany) was used for the chromatographic purification of products and Silica Gel 60 F254 (Merck Co., Darmstadt, Germany) aluminum plates for the thin-layer chromatography (TLC). UV light and/or phosphomolybdic acid in EtOH was employed for visualizing shpots. A Büchi 530 apparatus (Büchi, Flawil, Switzerland) was used to estimate melting points and they were uncorrected. ¹H and ¹³C-NMR spectra were recorded on Varian Mercury (Varian Inc., Palo Alto, CA, USA) at 200 MHz and 50 MHz respectively. Samples were diluted in CDCl₃, CD₃OD, DMSO or D₂O. Chemical shifts are given in ppm, and coupling constants (*J*) in Hz. Peak multiplicities are typified as: s, singlet, d, doublet, t, triplet and m, multiplet. Electron spray ionization (ESI) mass spectra were recorded on a Finnigan, Surveyor MSQ Plus spectrometer (Thermo Finnigan, Ringoes, NJ, USA). Specific rotations of the compounds were measured at 25 °C on a Perkin-Elmer 343 polarimeter (PerkinElmer Inc., Shelton, CT, USA) using a 10 cm cell. Dichloromethane was dried by standard procedures and stored over molecular sieves. No further purification of other solvents and chemicals needed as they were reagent grade. HRMS spectra were recorded on a Bruker Maxis Impact QTOF Spectrometer (Bruker Daltonics, Billerica, MA, USA).

4.1.1. Coupling Method

To a stirred solution of hydrochloride amino component (1.0 mmol) in CH₂Cl₂ (10 mL), Et₃N (0.3 mL, 2.2 mmol) and subsequently WSCI·HCl (0.21 g, 1.1 mmol) and HOBt (0.14 g, 1.0 mmol) were added at 0 °C. The acid component (1.0 mmol) was added and the reaction mixture was stirred for 1 h at 0 °C and then overnight at room temperature. After the completion of the reaction, the solvent was evaporated under reduced pressure and EtOAc (20 mL) was added. The organic layer was washed consecutively with brine, 1 N aqueous HCl, brine, 5% NaHCO₃, and brine, dried over Na₂SO₄ and evaporated under reduced pressure. The residue was purified by column chromatography [EtOAc/petroleum ether (bp 40–60 °C), 2:8].

(*R*)-Methyl 2-(2-(7-phenylheptanamido)hexanamido)acetate (**6**). Yield 77%; Colorless oil; $[\alpha]_D^{20} + 33.0$ (c 1.00, CH₃OH); ¹H-NMR (CDCl₃, 200 MHz): δ 7.72 (t, *J* = 5.5 Hz, 1H, NH), 7.33–7.07 (m, 5H, arom), 6.91 (d, *J* = 8.3 Hz, 1H, NH), 4.63 (dt, *J* = 7.5 Hz, 1H, NHCH), 4.19–3.81 (m, 2H, NHCH₂), 3.68 (s, 3H, COOCH₃), 2.57 (t, *J* = 7.6 Hz, 2H, CH₂), 2.19 (t, *J* = 7.4 Hz, 2H, NHCOCH₂), 1.92–1.45 (m, 6H, CH₂), 1.44–1.16 (m, 8H, CH₂), 0.87 (t, *J* = 6.7 Hz, 3H, CH₃); ¹³C-NMR (CDCl₃, 50 MHz): δ 173.3, 172.8, 169.8, 142.4, 128.5, 128.0, 125.4, 52.6, 52.0, 40.9, 36.1, 35.7, 32.2, 31.6, 28.9, 28.8, 27.4, 25.4, 22.3, 13.8; MS (ESI) *m/z* (%): 391.14 (92) [M + H]⁺; Anal. Calcd for C₂₂H₃₄N₂O₄: C, 67.66; H, 8.78; N, 7.17. Found: C, 67.49; H, 8.97; N, 7.03.

(*R*)-Methyl 4-(2-(2-benzamidoacetamido)acetamido)octanoate (**10**). Yield 62%; Yellow oil; $[\alpha]_D^{20} + 1.5$ (c 1.00, CHCl₃); ¹H-NMR (CDCl₃, 200 MHz): δ 8.19–8.02 (m, 1H, NH), 7.87–7.67 (m, 3H, NH & arom), 7.51–7.24 (m, 3H, NH & arom), 7.03–6.86 (m, 1H, arom), 4.03 (d, *J* = 4.5 Hz, 2H, NHCH₂), 3.85 (d, *J* = 5.0 Hz, 2H, NHCH₂), 3.75–3.69 (m, 1H, NHCH), 3.53 (s, 3H, COOCH₃), 2.26 (t, *J* = 7.3 Hz, 2H, CH₂COO), 1.92–1.52 (m, 2H, NHCHCH₂), 1.48–1.03 (m, 6H, CH₂), 0.87–0.65 (m, 3H, CH₃); ¹³C-NMR (CDCl₃, 50 MHz): δ 174.1, 170.1, 168.8, 168.3, 132.9, 131.8, 128.4, 127.2, 51.5, 49.0, 43.9, 43.0, 34.5, 30.5, 29.7, 27.9, 22.4, 13.8; MS (ESI) *m/z* (%): 392.14 (90) [M + H]⁺; Anal. Calcd for C₂₀H₂₉N₃O₅: C, 61.36; H, 7.47; N, 10.73. Found: C, 61.15; H, 7.74; N, 10.54.

(*R*)-Methyl 2-(2-(2-benzamidoacetamido)acetamido)hexanoate (**13a**). Yield 53%; White solid; mp 140–142 °C; $[\alpha]_D^{20} + 18.6$ (c 1.02, CHCl₃); ¹H-NMR (CDCl₃, 200 MHz): δ 7.95–7.66 (m, 4H, NH & arom), 7.52–7.27 (m, 4H, arom), 4.45 (dt, *J* = 7.5 Hz, 1H, NHCH), 4.10 (d, *J* = 5.1 Hz, 2H, NHCH₂), 4.06–3.82 (m, 2H, NHCH₂), 3.63 (s, 3H, COOCH₃), 1.86–1.49 (m, 2H, NHCHCH₂), 1.34–1.09 (m, 4H, CH₂), 0.80 (t, *J* = 6.6 Hz, 3H, CH₃); ¹³C-NMR (CDCl₃, 50 MHz): δ 173.0, 170.2, 169.2, 168.1, 133.2, 131.7, 128.4, 127.2, 52.4, 52.2, 43.7, 42.8, 31.4, 27.4, 22.1, 13.7; MS (ESI) *m/z* (%): 364.09 (90) [M + H]⁺; Anal. Calcd for C₁₈H₂₅N₃O₅: C, 59.49; H, 6.93; N, 11.56. Found: C, 59.40; H, 7.03; N, 11.47.

(*S*)-Methyl 2-(2-(2-benzamidoacetamido)acetamido)hexanoate (**13b**). Yield 68%; White solid; mp 139–141 °C; $[\alpha]_D^{20} - 18.0$ (c 1.00, CHCl₃); ¹H-NMR (CDCl₃, 200 MHz): δ 8.04 (t, *J* = 4.9 Hz, 1H, NH), 7.88 (t, *J* = 5.1 Hz, 1H, NH), 7.83–7.69 (m, 2H, NH & arom), 7.62–7.46 (m, 1H, arom), 7.45–7.19 (m, 3H, arom), 4.40 (dt, *J* = 7.6 Hz, 1H, NHCH), 4.06 (d, *J* = 4.7 Hz, 2H, NHCH₂), 4.00–3.80 (m, 2H, NHCH₂), 3.59 (s, 3H, COOCH₃), 1.86–1.47 (m, 2H, NHCHCH₂), 1.32–1.05 (m, 4H, CH₂), 0.77 (t, *J* = 6.7 Hz, 3H, CH₃); ¹³C-NMR (CDCl₃, 50 MHz): δ 173.0, 170.2, 169.2, 168.1, 133.1, 131.6, 128.2, 127.2, 52.3, 52.0, 43.6, 42.7, 31.3, 27.4, 22.0, 13.6; MS (ESI) *m/z* (%): 364.04 (90) [M + H]⁺; Anal. Calcd for C₁₈H₂₅N₃O₅: C, 59.49; H, 6.93; N, 11.56. Found: C, 59.20; H, 7.15; N, 11.35.

(*R*)-tert-Butyl 9-butyl-1,4,7,10-tetraoxo-1-phenyl-2,5,8,11-tetraazatridecan-13-oate (**15a**). Yield 92%; White solid; mp 177–178 °C; $[\alpha]_D^{20} + 11.0$ (c 1.00, CH₃OH); ¹H-NMR (CDCl₃, 200 MHz): δ 8.25–8.07 (m, 1H, NH), 8.05–7.94 (m, 1H, NH), 7.93–7.76 (m, 3H, NH & arom), 7.75–7.58 (m, 1H, arom), 7.55–7.29 (m, 3H, arom), 4.96–4.74 (m, 1H, NHCH), 4.43–4.25 (m, 2H, NHCH₂), 4.22–4.03 (m, 2H, NHCH₂), 4.01–3.71 (m, 2H, NHCH₂), 1.94–1.49 (m, 2H, CH₂), 1.40 (s, 9H, CH₃), 1.33–1.10 (m, 4H, CH₂), 0.81 (t, *J* = 6.7 Hz, 3H, CH₃); ¹³C-NMR (CDCl₃, 50 MHz): δ 172.2, 169.6, 168.7, 168.5, 167.9, 133.5, 131.6, 128.3, 127.4, 81.9, 53.0, 43.5, 41.9, 33.1, 27.9, 27.5, 25.9, 22.4, 13.9; MS (ESI) *m/z* (%): 463.22 (90) [M + H]⁺; Anal. Calcd for C₂₃H₃₄N₄O₆: C, 59.72; H, 7.41; N, 12.11. Found: C, 59.44; H, 7.70; N, 11.93.

4.1.2. Saponification of Methyl Esters

To a stirred solution of a methyl ester (1.0 mmol) in water, 1 N aqueous NaOH (1 mL, 1.0 mmol) was added and the mixture was left under stirring overnight at room temperature. After the completion of the reaction, the mixture was washed with EtOAc (3 × 10 mL), acidified with 1 N aqueous HCl to pH 1 and extracted with EtOAc (3 × 10 mL). The combined organic layers were washed with brine, dried over Na₂SO₄ and evaporated under reduced pressure.

(*R*)-2-(2-(7-Phenylheptanamido)hexanamido)acetic acid (**7**). Yield 83%; White oil; $[\alpha]_D^{20} + 15.0$ (c 1.00, CH₃OH); ¹H-NMR (CDCl₃, 200 MHz): δ 9.67 (br s, 1H, COOH), 7.72–7.45 (m, 2H, NH), 7.36–6.99 (m, 5H, arom), 4.76–4.51 (m, 1H, NHCH), 4.13–3.86 (br s, 2H, NHCH₂), 2.69–2.39 (m, 2H, CH₂), 2.31–2.05 (m, 2H, NHCOCH₂), 1.92–1.45 (m, 6H, CH₂), 1.41–1.05 (m, 8H, CH₂), 0.96–0.69 (m, 3H, CH₃); ¹³C-NMR

(CDCl₃, 50 MHz): δ 174.3, 172.9, 172.0, 142.5, 128.2, 128.1, 125.5, 52.8, 41.2, 36.1, 35.7, 32.1, 31.2, 28.9, 28.9, 27.4, 25.5, 22.2, 13.8; HRMS (ESI) calcd for C₂₁H₃₃N₂O₄ [M + H]⁺: 377.2435. Found: 377.2438; Anal. Calcd for C₂₁H₃₂N₂O₄: C, 66.99; H, 8.57; N, 7.44. Found: C, 66.72; H, 8.86; N, 7.27.

(*R*)-4-(2-(2-Benzamidoacetamido)acetamido)octanoic acid (**11**). Yield 88%; White solid; mp 202–204 °C; [α]_D²⁰ + 4.0 (c 1.00, CH₃OH); ¹H-NMR (D₂O, 200 MHz): δ 7.89–7.70 (m, 2H, arom), 7.68–7.41 (m, 3H, arom), 4.07 (s, 2H, NHCH₂), 3.88 (s, 2H, NHCH₂), 3.82–3.64 (m, 1H, NHCH), 2.10 (t, *J* = 6.7 Hz, 2H, CH₂COOH), 1.93–1.06 (m, 8H, CH₂), 0.89–0.66 (m, 3H, CH₃); ¹³C-NMR (D₂O, 50 MHz): δ 182.9, 172.4, 171.4, 170.9, 132.7, 132.5, 128.9, 127.4, 50.0, 34.2, 33.6, 31.2, 27.5, 21.9, 13.4; HRMS (ESI) calcd for C₁₉H₂₈N₃O₅ [M + H]⁺: 378.2023. Found: 378.2025; Anal. Calcd for C₁₉H₂₇N₃O₅: C, 60.46; H, 7.21; N, 11.13. Found: C, 60.25; H, 7.42; N, 11.01.

(*R*)-2-(2-(2-Benzamidoacetamido)acetamido)hexanoic acid (**14a**). Yield 93%; White solid; mp 213–215 °C; [α]_D²⁰ + 8.6 (c 1.00, CH₃OH); ¹H-NMR (DMSO, 200 MHz): δ 8.84 (t, *J* = 5.6 Hz, 1H, NH), 8.19 (t, *J* = 5.5 Hz, 1H, NH), 8.05–7.70 (m, 3H, NH & arom), 7.58–7.24 (m, 3H, arom), 4.22–4.02 (m, 1H, NHCH), 3.85 (d, *J* = 5.6 Hz, 2H, NHCH₂), 3.72 (d, *J* = 5.6 Hz, 2H, NHCH₂), 1.76–1.43 (m, 2H, CH₂), 1.35–1.01 (m, 4H, CH₂), 0.79 (t, *J* = 6.5 Hz, 3H, CH₃); ¹³C-NMR (DMSO, 50 MHz): δ 173.6, 169.3, 168.8, 166.7, 133.9, 131.5, 128.3, 127.4, 51.8, 42.9, 41.8, 30.8, 27.5, 21.8, 13.8; HRMS (ESI) calcd for C₁₇H₂₄N₃O₅ [M + H]⁺: 350.1710. Found: 350.1711; Anal. Calcd for C₁₇H₂₃N₃O₅: C, 58.44; H, 6.64; N, 12.03. Found: C, 58.22; H, 6.86; N, 11.87.

(*S*)-2-(2-(2-Benzamidoacetamido)acetamido)hexanoic acid (**14b**). Yield 91%; White solid; mp 192–201 °C; [α]_D²⁰ – 8.1 (c 1.08, CH₃OH); ¹H-NMR (CDCl₃ and drops CD₃OD, 200 MHz): δ 7.82–7.58 (m, 1H, arom), 7.53–7.14 (m, 4H, arom), 4.38–4.21 (m, 1H, NHCH), 4.05 (s, 2H, NHCH₂), 3.96 (s, 2H, NHCH₂), 1.87–1.43 (m, 2H, CH₂), 1.34–1.09 (m, 4H, CH₂), 0.75 (t, *J* = 6.5 Hz, 3H, CH₃); ¹³C-NMR (DMSO, 50 MHz): δ 173.8, 168.9, 169.0, 166.5, 134.3, 131.8, 128.7, 127.0, 51.4, 43.2, 41.1, 31.2, 27.3, 21.2, 13.4; HRMS (ESI) calcd for C₁₇H₂₄N₃O₅ [M + H]⁺: 350.1710. Found: 350.1712; Anal. Calcd for C₁₇H₂₃N₃O₅: C, 58.44; H, 6.64; N, 12.03. Found: C, 58.24; H, 6.82; N, 11.90.

4.1.3. Cleavage of *tert*-Butyl Protecting Group

A solution of the *tert*-butyl ester derivative (1 mmol) in 50% TFA/CH₂Cl₂ (0.5 M) was stirred for 2 h at room temperature. After the completion of the reaction, the organic solvent was evaporated under reduced pressure and the residue was purified by recrystallization.

(*R*)-9-Butyl-1,4,7,10-tetraoxo-1-phenyl-2,5,8,11-tetraazatridecan-13-oic acid (**16a**). Yield 87%; White solid; mp 195–206 °C; [α]_D²⁰ + 20.0 (c 1.00, CH₃OH); ¹H-NMR (CD₃OD, 200 MHz): δ 7.91–7.77 (m, 2H, arom), 7.59–7.35 (m, 3H, arom), 4.45–4.32 (m, 1H, NHCH), 4.26 (s, 2H, NHCH₂), 4.05 (s, 2H, NHCH₂), 3.90 (s, 2H, NHCH₂), 1.97–1.57 (m, 2H, CH₂), 1.41–1.16 (m, 4H, CH₂), 0.86 (t, *J* = 6.6 Hz, 3H, CH₃); ¹³C-NMR (CD₃OD, 50 MHz): δ 173.2, 171.8, 171.2, 170.2, 169.2, 133.3, 132.2, 128.7, 127.5, 53.6, 43.7, 42.9, 31.6, 28.0, 22.4, 22.3, 13.9; HRMS (ESI) calcd for C₁₉H₂₇N₄O₆ [M + H]⁺: 407.1925. Found: 407.1927; Anal. Calcd for C₁₉H₂₆N₄O₆: C, 56.15; H, 6.45; N, 13.79. Found: C, 55.84; H, 6.62; N, 13.65.

4.2. Biology

4.2.1. Cell Culture

The methods used for the isolation, characterization and cultivation of rat renal mesangial cells have been previously described in details [29]. Confluent cells were incubated in 24-well-plates for 4 h in serum-free DMEM including 0.1 mg/mL of fatty acid-free bovine serum albumin. Subsequently, cells were preincubated for 20 min either with the vehicle or the indicated concentrations of the various inhibitors prior to stimulation with IL1 β (1 nM) plus Fk (5 μ M) in the presence of the inhibitors for 24 h. The total stimulation volume per well was 500 μ L. Cell supernatants were taken for PGE₂ quantification.

4.2.2. Quantification of Prostaglandin E₂

A PGE₂-specific enzyme-linked immunosorbent assay (ELISA) kit (from Enzo Life Science, Lörrach, Germany) was used for the quantification of PGE₂ in the cell culture supernatants. 100 µL of the supernatants were processed according to the manufacturer's instructions.

4.2.3. Statistical Analysis

Statistical analysis of data was performed using one-way ANOVA following Bonferroni post-hoc test for multiple comparisons.

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