Supplementary Information

Second-Generation of Prokineticin PKR1 Receptor Agonists: Advancing Cardioprotection Against Chemotherapy-Induced Toxicity

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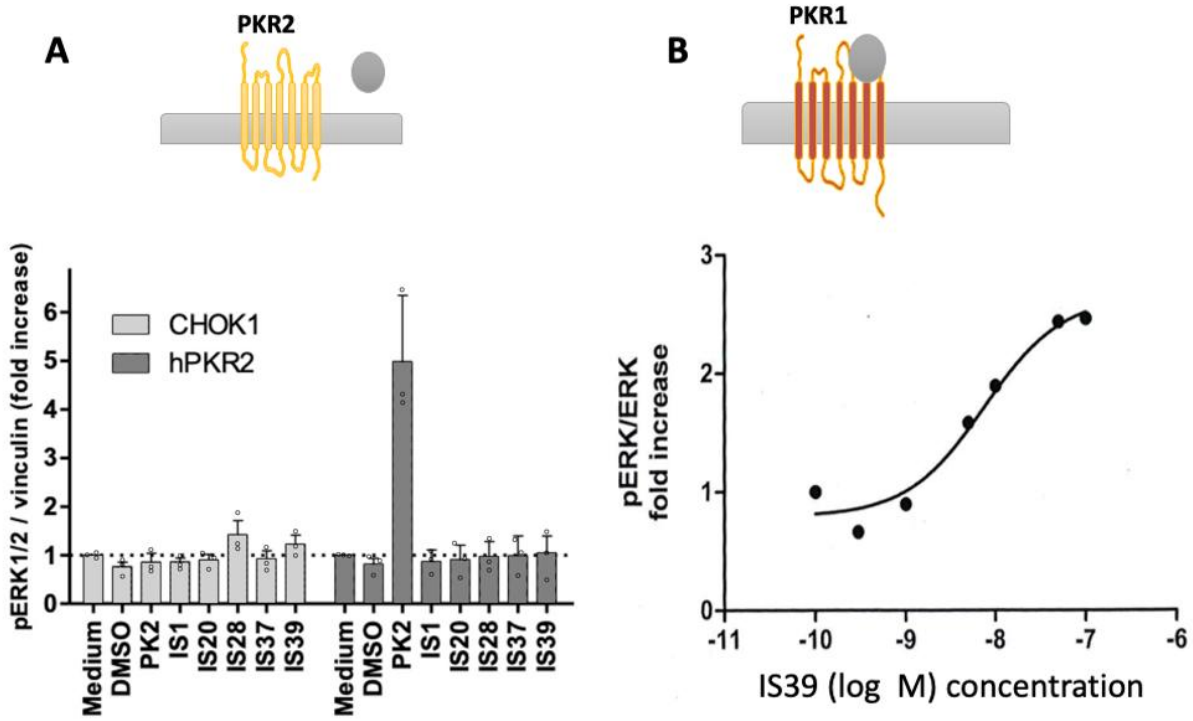
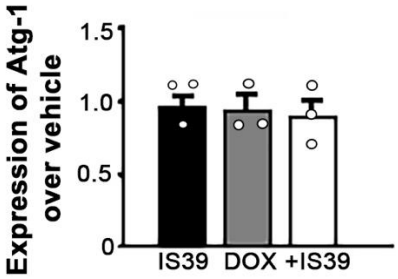
Content



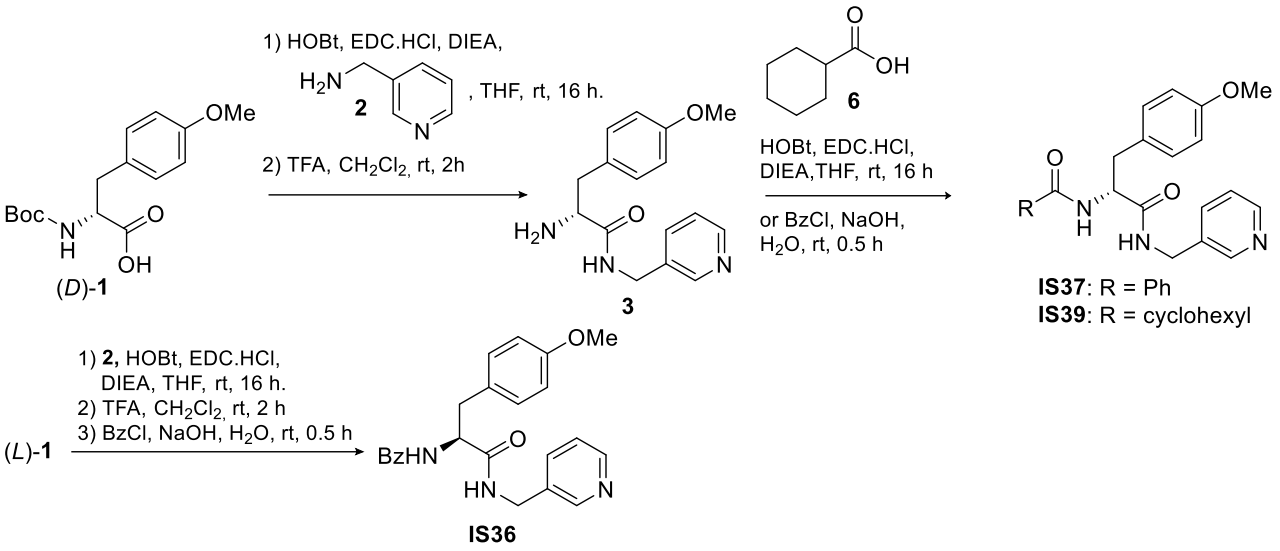
Supplementary Figure S1. Specificity and selectivity of IS39: The CHO cells were transfected with plasmid carrying PKR1 or PKR2 as previously described (Gasser, *et al*., 2015). **(A)** The effect of IS37 and IS39 on ERK activity in the CHOK cells without both PKR1 and PKR2 (left) showing that endogenous ligand prokineticin-2 (PK2) , IS37 and IS39 have no interaction of the endogenously expressing other GPCRs (at least <70 GPCRs express in CHO cells). In the CHO cells expressing PKR2 (CHOK-PKR2 right) in S1A, right, only PK2 but not IS37 and IS39 have promote ERK activity. . Data were represented as fold increase vs control (DMSO), mean ± SEM, n = 3 per group, each triplicated). **(B)** IS39 and IS37 were able to increase ERK activity in the CHO-PKR1 cells (S1B), indicating that they are PKR1 selective ligands. In CHO-PKR1 cells IS39 increased ERK activity in concentration-dependent manner. Data were represented as fold increase vs control (DMSO), mean ± SEM, n = 3 per group, each triplicated).



Supplementary Figure S2. DOX did not alter cardiac atrophy marker Atrogen-1 in the chronic mice models of cardiotoxicity. RT–PCR analysis of cardiac atrophy marker, Atg-1 gene in mouse hearts. mRNA levels were quantified by the $2^{-}\Delta\Delta Ct$ method after normalization to GAPDH and β-actin

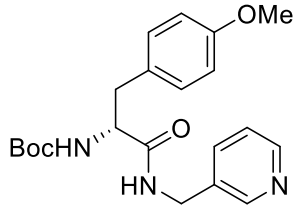
and are expressed as fold change relative to the vehicle group (mean ± SEM, n = 3 hearts per group, each measured in technical duplicate). Dots shows individual data.

Chemical synthesis



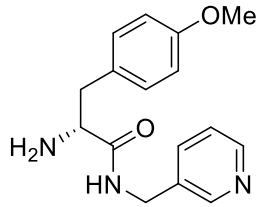
Scheme 1 : Synthesis of *O*-methyl-tyrosine derivatives IS36, IS37 and IS39.

***Tert*-butyl (*R*)-(3-(4-methoxyphenyl)-1-oxo-1-((pyridin-3-ylmethyl)amino)propan-2-yl)carbamate**



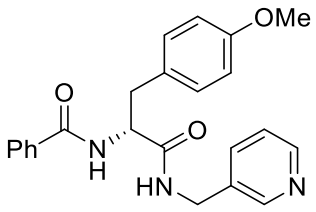
3-(Methylamino)pyridine **2** (40 µL, 0.41 mmol), triethylamine (50 µL, 0.34 mmol) and HOBt (55.4 mg, 0.41 mmol) in THF (5.1 mL) were added to a solution of Boc-O-Me-*D*-Tyr (*D*)-**1** (100 mg, 0.34 mmol), at 0°C, EDC (130.4 mg, 0.68 mmol). After stirring at room temperature overnight the solution was purified by flash chromatography eluting with DCM/ *i*-PrOH (95:5 to 90:10) to afford the title compound (110 mg, 84%) as a white solid. **^{1}H-NMR** (400 MHz, $CDCl_3$) : δ 1.36 (s, 9H), 2.91-3.09 (m, 2H), 3.74 (s, 3H), 4.24-4.29 (m, 1H), 4.33 (t, J= 5.9 Hz, 2H), 6.73-6.77 (m, 2H), 7.03 (d, J= 8.7 Hz, 2H), 7.17 (dd, J= 7.7-4.9 Hz, 1H), 7.36-7.47 (m, 1H), 8.37 (s, 1H), 8.45 (d, J= 4.6 Hz, 1H). **^{13}C-NMR** (100 MHz, $CDCl_3$): δ 25.18, 28.12, 37.31, 40.72, 45.67, 55.21, 114.1, 123.40, 128.46, 130.23, 133.53, 135.53, 148.6, 148.95, 158.61, 171.45. **HRMS** calcd for $C_{21}H_{28}N_3O_4^+$ $[M + H]^+$ 386.2075; found 386.2072.

(*R*)-2-Amino-3-(4-methoxyphenyl)-*N*-(pyridin-3-ylmethyl)propenamide (3)



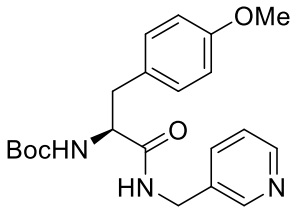
A solution of the previous adduct (110 mg; 0.34 mmol) was stirred at room temperature for 3 h in TFA (2.2 mL). The solution was concentrated in vacuo to quantitatively afford the title as a yellow oil that was used in the following step without further purification. **^{1}H-NMR** (400 MHz, $CDCl_3$) : δ 2.71 (dd, J= 14.1-8.7 Hz, 1H), 3.16 (dd, J= 14.1-4.2 Hz, 1H), 3.61 (dd, J= 8.9-4.2 Hz, 1H), 3.77 (s, 3H), 4.42 (t, J= 7.2 Hz, 2H), 6.80 (dt, J= 9.5-2.4 Hz, 2H), 7.07-7.11 (m, 2H), 7.54 (d, J= 8.0 Hz, 1H), 7.70 (s, 1H), 8.48 (d, J = 2.6 Hz, 2H). **^{13}C-NMR** (100 MHz, $CDCl_3$): δ 39.91, 40.50, 55.31, 56.29, 114.02, 123.49, 129.32, 130.40, 134.05, 135.63, 148.95, 149.25, 158.62, 174.41. **HRMS** calcd for $C_{16}H_{20}N_3O_2^+$ $[M + H]^+$ 286.1551; found 286.1543.

(*R*)-*N*-(3-(4-Methoxyphenyl)-1-oxo-1-((pyridin-3-ylmethyl)amino)propan-2-yl)benzamide (IS37)



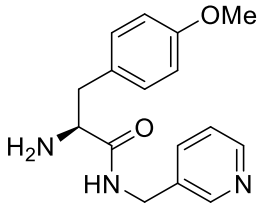
Benzoyl chloride (0.11 mL; 0.96 mmol) was added to a solution of the previous compound (229 mg; 0.8 mmol) in NaOH (5%, 4.6 ml). The mixture war vigorously stirred at room temperature during 30min. After stirring vigorously at room temperature for 30 min, the solution was diluted in DCM, washed with water, dried ($MgSO_4$), concentrated *in vacuo* and crystallized from *i*-PrOH to afford the adduct **IS37** (196 mg, 63% yield) as a white solid. **^{1}H-NMR** (400 MHz, $CDCl_3$) : δ 3.06 (dd, J= 13.7-8.3 Hz, 1H), 3.18 (dd, J= 13.6-5.6 Hz, 1H), 3.74 (s, 3H), 4.25 (dd, J= 14.6-5.4 Hz, 1H), 4.40 (dd, J= 14.9-5.9 Hz, 1H), 4.86 (q, J= 6.9 Hz, 1H), 6.75 (d, J= 8.0 Hz, 2H), 7.10 (d, J= 8.8 Hz, 2H), 7.15 (t, J= 6.9 Hz, 1H), 7.37 (t, J= 7.5 Hz, 3H), 7.48 (t, J= 7.4 Hz, 1H), 7.66 (d, J= 7.5 Hz, 2H), 8.38 (s, 1H), 8.45 (d, J= 4.5 Hz, 1H). **^{13}C-NMR** (100 MHz, $CDCl_3$): δ 29.92, 37.96, 41.14, 55.44, 114.35, 123.72, 127.24, 128.53, 128.85, 130.57, 132.16, 133.73, 135.81, 148.84, 149.21, 158.89, 167.55, 171.38. **HRMS** calcd for $C_{23}H_{24}N_3O_2^+$ $[M + H]^+$ 390.1813; found 390.1814.

Tert-butyl (_S_)-(3-(4-methoxyphenyl)-1-oxo-1-((pyridin-3-ylmethyl)amino)propan-2-yl)carbamate



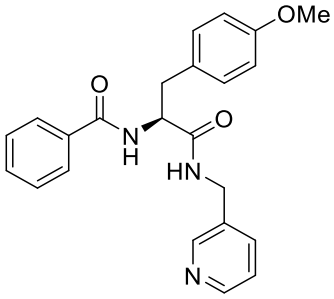
To a solution of Boc-O-Me-*L*-Tyr (100 mg, 0.34 mmol), 3-(methylamino)pyridine **2** (40 µL, 0.41 mmol), triethylamine (50 µL, 0.34 mmol) and HOBt (55.4 mg, 0.41 mmol) in THF (5.1 mL) was added, at 0°C, EDC (130.4 mg, 0.68 mmol). After stirring at room temperature overnight the solution was purified by Flash chromatography eluting with 5:95/10:90 iso-propanol: DCM affording the title compound (130 mg, 99% yield) as a white solid. **^{1}H-NMR** (400 MHz, $CDCl_3$) : δ 1.32 (s, 9H), 2.92 (d, J= 6.5 Hz, 2H), 3.71 (s, 3H), 4.22-4.35 (m, 3H), 5.19 (s_l, 1H), 6.71 (dd, J= 8.5-2.5 Hz, 2H), 6.99 (dd, J= 9.2-2.3 Hz, 2H), 7.10-7.16 (m, 1H), 7.32-7.39 (m, 1H), 8.29 (s, 1H), 8.40 (s, 1H). **^{13}C-NMR** (100 MHz, $CDCl_3$): δ 25.18 (CH_3 Boc), 28.12, 37.31, 40.72, 45.67, 55.21, 114.1, 123.40, 128.46, 130.23, 133.53, 135.53, 148.6, 148.95, 158.61, 171.45. **HRMS** calcd for $C_{21}H_{28}N_3O_4^+$ $[M + H]^+$ 386.2074; found 386.2076.

(_S_)-2-Amino-3-(4-methoxyphenyl)-_N_-(pyridin-3-ylmethyl)propenamide



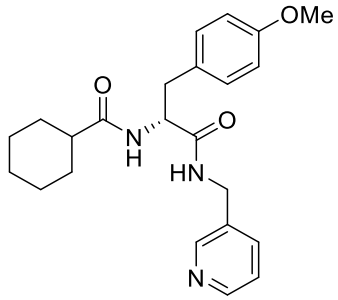
A solution of the previous adduct (110 mg; 0.34 mmol) was stirred at room temperature for 6 h in TFA (2.6 mL). The solution was concentrated in vacuo to afford quantitatively the deprotected amine as a yellow oil that was used in the following step without further purification. **^{1}H-NMR** (400 MHz, $CDCl_3$): δ 2.71 (dd, J= 14.1-8.7 Hz, 1H), 3.16 (dd, J= 14.1-4.2 Hz, 1H), 3.61 (dd, J= 8.9-4.2 Hz, 1H), 3.77 (s, 3H), 4.42 (t, J= 7.2 Hz, 2H), 6.80 (dt, J= 9.5-2.4 Hz, 2H), 7.07-7.11 (m, 2H), 7.54 (d, J= 8.0 Hz, 1H), 7.70 (s, 1H), 8.48 (d, J= 2.6 Hz, 2H). **^{13}C-NMR** (100 MHz, $CDCl_3$): δ 39.91, 40.50, 55.31, 56.29, 114.02, 123.49, 129.32, 130.40, 134.05, 135.63, 148.95, 149.25, 158.62, 174.41. **HRMS** calcd for $C_{16}H_{20}N_3O_2^+$ $[M + H]^+$ 286.1551; found 286.1550.

(_S_)-_N_-(3-(4-methoxyphenyl)-1-oxo-1-((pyridin-3-ylmethyl)amino)propan-2-yl)benzamide (IS36)



Benzoyl chloride (0.11 mL; 0.96 mmol) was added to a solution of the previous amine (229 mg; 0.8 mmol) in NaOH (5%, 4.6 ml). The mixture war vigorously stirred at room temperature during 30min. After stirring vigorously at room temperature for 30 min, the solution was diluted in DCM, washed with water, dried ($MgSO_4$), concentrated *in vacuo* and crystallized from *i*-PrOH to afford the adduct **IS36** (190 mg, 61 % yield) as a white solid. **^{1}H-NMR** (400 MHz, $CDCl_3$) : δ 3.06 (dd, J= 13.9-8.0 Hz, 1H), 3.18 (dd, J=, 13.3-6.4 Hz, 1H), 3.74 (s, 3H), 4.26 (dd, J= 14.4-4.8 Hz, 1H), 4.41 (dd, J= 14.4-4.3 Hz, 1H), 4.86 (q, J= 6.9 Hz, 1H), 6.75 (d, J= 8.3 Hz, 2H), 6.95 (d, J= 8.4 Hz, 2H), 7.37 (t, J= 7.0 Hz, 3H), 7.48 (t, J= 7.4 Hz, 1H), 7.66 (d, J= 7.5 Hz, 2H), 8.39 (s_l, 1H, NH), 8.46 (s_l, 1H, NH). **^{13}C-NMR** (100 MHz, $CDCl_3$): δ 29.92, 37.96, 41.14, 55.44, 114.35, 123.72, 127.24, 128.53, 128.85, 130.57, 132.16, 133.73, 135.81, 148.84, 149.21, 158.89, 167.55, 171.38. **HRMS** calcd for $C_{23}H_{24}N_3O_2^+$ $[M + H]^+$ 390.1813; found 390.1810.

(*R*)-*N*-(3-(4-methoxyphenyl)-1-oxo-1-((pyridin-3-ylmethyl)amino)propan-2yl) cyclohexanecarboxamide (IS39).



The amine **3** (142 mg, 0.5 mmol) was added to a solution of the carboxylic acid cyclohexanoic acid (64 mg, 0.5 mmol) and HOBt (155 mg, 1.0 mmol) in THF (7.5 mL) under argon and cooled down to 0 ˚C. Then, EDC.HCl (115 mg, 0.6 mmol, 1.2 equiv.) and *N*,*N*-diisopropylethylamine (220 µL, 161 mg, 1.25 mmol, 2.5 equiv.) were added and the medium was stirred at room temperature for 16 h. The reaction was quenched with water (10 mL), concentrated in vacuo to remove THF and extracted with dichloromethane (3 × 25 mL). The combined organic phases were washed with 25 mL of saturated aqueous $NaHCO_3$, 25 mL of brine and dried over Na_2SO_4, filtered, concentrated by rotary evaporation in vacuo. Purification by flash column chromatography on silica gel with AcOEt: MeOH (100:0 and 80: 20) provided **IS39** (160 mg, 81%) as a white solid. **^{1}H-NMR** (400 MHz, $CDCl_3$) : δ 8.46 (dd, J= 4.9-1.6 Hz, 1H), 8.37 (d, J= 2.0 Hz, 1H), 7.17 (dd, J= 7.9-4.9 Hz, 1H), 7.03 (d, J= 8.5 Hz, 2H), 6.74 (d, J= 8.7 Hz, 2H), 6.74 (t, J= 5.5 Hz, 1H), 6.09 (d, J= 7.7 Hz, 1H), 4.59 (q, J= 8.0 Hz, 1H), 4.24-4.38 (m, 2H), 3.74 (s, 3H), 2.90-3.02 (m, 2H), 1.99-2.06 (m, 1H), 1.70-1.73 (m, 4H), 1.59-1.63 (m, 1H), 1.11-1.34 (m, 6H). **^{13}C-NMR** (100 MHz, $CDCl_3$): δ 25.60, 29.32, 29.45, 37.27, 40.76, 45.22, 54.60, 55.32, 114.19, 123.69, 128.39, 130.20, 135.49, 148.73, 149.21, 176.41, 176.53. **HRMS** calcd for $C_{23}H_{30}N_3O_3^+$ $[M + H]^+$ 396.2282; found 396.2288.

Annex

Original Western Blot corresponding to Figure 2C and D

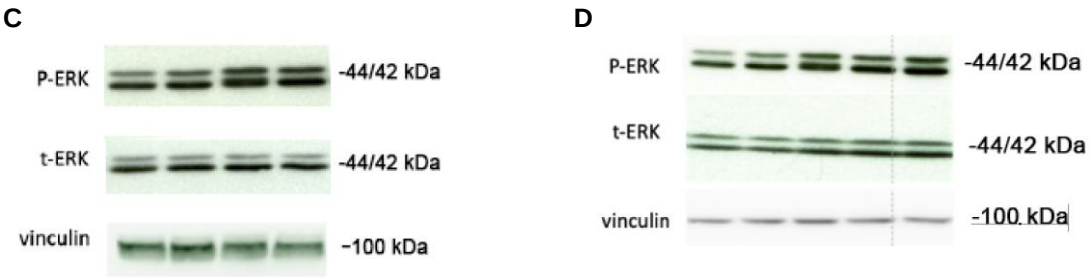


Figure 2 : ERK activity assay by phosphorylation

Activation of ERK signaling pathways was assessed by measuring their phosphorylation status. Cells were lysed under denaturing conditions, and equal amounts of total protein were subjected to SDS-PAGE followed by immunoblotting. Membranes were probed with phospho-specific antibodies recognizing ERK1/2 phosphorylated at Thr202/Tyr204 (42/44kDa). Antibodies against total ERK and vinculin were used as internal loading controls were used as loading and normalization controls. Signal detection was performed using chemiluminescence, and band intensities were quantified by densitometry using ImageJ software. Phosphorylation levels were expressed as the ratio of phosphorylated protein to total protein and normalized to control conditions.

Original Western Blot corresponding to figure 4D

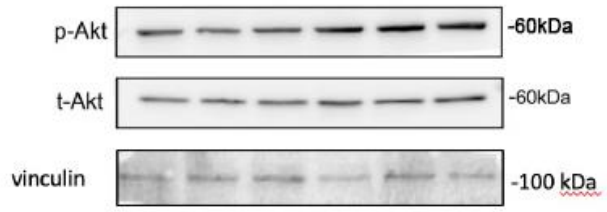


Figure 4D: AKT activation assay by phosphorylation.

Activation of AKT pathways was assessed by measuring their phosphorylation status. Cells were lysed under denaturing conditions, and equal amounts of total protein were subjected to SDS-PAGE followed by immunoblotting. Membranes were probed with phospho-specific antibodies recognizing AKT phosphorylated at Ser473. Antibodies against total AKT, total ERK, and vinculin were used as internal loading controls. Signals were detected by chemiluminescence, band intensities were quantified by densitometry using ImageJ software. Phosphorylation levels were expressed as the ratio of phosphorylated protein to total protein and normalized to vinculin and control conditions.

FIG 5A and 5B: Ponceau stained Western Blots used to Fig. 5 for the detection of γ-H2AX and GAPDH before cropping.