A bifunctional biased mu opioid agonist - neuropeptide FF receptor antagonist as analgesic with improved acute and chronic side effects

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Supplemental Digital Content:

Materials and Methods Supplemental Digital Content 1, which describes peptide characterization and synthesis of the arginine mimetics and ornithine mimetics.

Table Supplemental Digital Content 2, which summarize affinity constant (K_i) values of KGFF compounds for MOPr, NPFF1R, and NPFF2R.

Table Supplemental Digital Content 3, which summarize agonist activity constants (Ec_{50} and E_{max}) values of KGFF compounds for MOPr, NPFF1R, and NPFF2R.

Figure Supplemental Digital Content 4, which shows in vitro characterization of KGFF03 and KGFF09 on NPFFRs.

Table Supplemental Digital Content 5, which shows the robustness of β-arrestin-2 recruitment to MOR induced by DAMGO.

Figure Supplemental Digital Content 6, which shows in vitro characterization of KGOP01, KGFF03 and KGFF09 on opioid receptors.

Table Supplemental Digital Content 7, which summarize affinity constant (K_i) values of KGFF compounds for GPR10, GPR54 and GPR103.

Figure Supplemental Digital Content 8, which shows effect of KGOP01, KGFF03 and KGFF09 on naltrexone-precipitated withdrawal signs after chronic exposure in mice.

Figure Supplemental Digital Content 9, which shows effect of KGOP01, KGFF03 and KGFF09 on gastro-intestinal motility and motor coordination.

Figure Supplemental Digital Content 10, which shows acute antinociceptive time-course of KGOP01, KGFF03 and KGFF09 in CFA-induced pain model.

SDC Material and Methods

Materials

Naltrexone hydrochloride, forskolin, 3-isobutyl-1-methylxanthine (IBMX), [D-Ala^2,Me-Phe^4,Gly-ol^5]enkephalin (DAMGO), probenecid and Complete Freund's Adjuvant (CFA) were purchased from Sigma-Aldrich (Saint Quentin Fallavier, France). Glass bead were purschased from SigmAldrich Chemicals (St; Louis, MO, USA). [D-Pen^2, D-Pen^5]enkephalin (DPDPE) and dynorphin were obtained from Abcam (Paris, France), nociception from Polypeptide (Strasbourg, France), morphine hydrochloride from Francopia (Paris, France) and the Fluo-4 acetoxymethyl ester from Molecular Probes (Invitrogen, Cergy Pontoise, France). Human RF-amide peptides were obtained from Genecust (Luxembourg; Kp-10, NPFF, QRFP26 or 26RFa, PrRP-20 and RFRP-3). [^{125}I]-1-DMe-NPFF (2200 Ci/mmol) and [^{3}H]-PrRP-20 (150 Ci/mmol) were obtained from Hartmann Analytic (Braunschweig, Germany). [^{35}S]Guanosine 5'-*O*-[γ-thio] triphosphate ([^{35}S]GTPγS; 1250 Ci/mmol), [^{3}H]-diprenorphine (42.3Ci/mmol), [^{3}H]-nociceptine (114.7Ci/mmol), [^{125}I]-Kp-10 (2200 Ci/mmol) and [^{125}I]-QRFP43 (2200 Ci/mmol) were purchased from Perkin Elmer Life and Analytical Sciences (Courtaboeuf, France) and Luciferin from Synchem UG & Co KG (Felsberg, Germany). All other chemicals were of analytical grade and obtained from standard commercial sources.

Synthesis and compound characterization

General

Thin-layer chromatography (TLC) was performed on glass plates precoated with silica gel 60F254 (Merck, Darmstadt, Germany) using the mentioned solvent systems. Mass Spectrometry (MS) was done on a Micromass Q-Tof Micro spectrometer with electrospray ionisation (ESI). Data collection and spectrum analysis was done with Masslynx software.

Analytical RP-HPLC was performed using a Waters 717plus Autosampler, a Waters 1525 Binary HPLC Pump and a Waters 2487 Dual Absorbance Wavelength Detector (Milford, MA), with a Grace (Deerfield, IL) Vydac RP C18 column (25 cm x 4.6 mm x 5 m) using UV detection at 215 nm. The mobile phase is a mixture of water and acetonitrile and contains 0.1% TFA. The used gradient runs from 3 to 100% acetonitrile in 20 minutes at a flow rate of 1 mL/min. Preparative RP-HPLC purification was done on a Gilson (Middleton, WI) HPLC system with Gilson 322 pumps, controlled by the software package Unipoint, and a reversed phase C18 column (Discovery®BIO SUPELCO Wide Pore C18 column, 25 cm x 2.21 cm, 5 mm) with a linear gradient of 1%/min increase of acetonitrile in water (both having 0.1% TFA). After purification, the purity of all compounds was evaluated as being more than 95% by analytical RP-HPLC. All fractions were lyophilized using a Flexy-Dry lyophilizer (FTS Systems, Warminster, PA). ^{1}H and ^{13}C NMR spectra were recorded at 500 and 125 MHz on a Bruker Avance II 500 or at 400 and 100.62 MHz on a Bruker Avance 400 (Bruker Corp, Billerica, MA). Tetramethylsilane (TMS) or residual solvent signals are used as internal standard. The solvent used is mentioned in all cases, and the abbreviations used are as follows: s (singlet), d (doublet), dd (double doublet), t (triplet), q (quadruplet) and m (multiplet).

Peptide characterization

H-Dmt-D-Arg-Aba-Gly-Arg-Phe-NH_2 (**KGFF01**). Preparative RP-HPLC yielded the desired compound (white powder, 15%). HPLC: t_R = 9.9 min. TLC Rf 0.69 (EBAW). HRMS (ESP^+) found m/z 884.4915 $[M+ H]^+$, $[C_{44}H_{61}N_{13}O_7 + H^+]$ required 884.4890.

H-Dmt-D-Arg-Aba-Gly-Arg-Phe-OH (**KGFF02**). Preparative RP-HPLC yielded the desired compound (white powder, 20%). HPLC: t_R = 10.4 min. TLC Rf 0.65 (EBAW). HRMS (ESP^+) found m/z 885.4764 $[M + H]^+$, $[C_{44} H_{60} N_{12}O_8 + H^+]$ required 885.4730.

H-Dmt-D-Arg-Aba-β-Ala-Arg-Phe-NH_2 (**KGFF03**). Preparative RP-HPLC yielded the desired compound (white powder, 18%). HPLC: t_R = 10.0 min. TLC Rf 0.67 (EBAW). HRMS (ESP^+) found m/z 898.5076 [M + H^+], [$C_{45}H_{63}N_{13}O_7$ + H^+] required 898.5046.

H-Dmt-D-Arg-Aba-Gly-Orn-Phe-NH_2 (**KGFF04**). Preparative RP-HPLC yielded the desired compound (white powder, 17%). HPLC: t_R = 9.9 min. TLC Rf 0.67(EBAW). HRMS(ESP^+) found m/z 842.4694 [M+ H]$^+$, [$C_{43}H_{59}N_{11}O_7$ + H^+] required 842.4672.

H-Dmt-D-Arg-Phe-Orn-Phe-NH_2 (**KGFF05**). Preparative RP-HPLC yielded the desired compound (white powder, 27%). HPLC: t_R = 10.1 min. TLC Rf 0.67 (EBAW). HRMS (ESP^+) found m/z 773.4477 [M + H]$^+$, [$C_{40}H_{56}N_{10}O_6$ + H^+] required 773.4457.

H-Dmt-Arg-Phe-NH_2 (**KGFF06**). Preparative RP-HPLC yielded the desired compound (white powder, 44%). HPLC: t_R = 10.1 min. TLC Rf 0.68 (EBAW). HRMS (ESP^+) found m/z 512.2994 [M + H^+], [$C_{26}H_{37}N_7O_4$ + H^+] required 512.2980.

H-Dmt-D-Arg-Phe-NH_2 (**KGFF07**). Preparative RP-HPLC yielded the desired compound (white powder, 63%). HPLC: t_R = 8.9 min. TLC Rf 0.71 (EBAW). HRMS (ESP^+) found m/z 512.2977 [M + H^+], [$C_{26}H_{37}N_7O_4$ + H^+] required 512.2980.

H-Dmt-D-Arg-Aba-β-Ala-Apa-Phe-NH_2 (**KGFF08**). Preparative RP-HPLC yielded the desired compound (white powder, 20%). HPLC: t_R = 10.2 min. TLC Rf 0.46 (EBAW). HRMS (ESP^+) found m/z 924.5476 [M + H^+], [$C_{49}H_{69}N_{11}O_7$ + H^+] required 924.5454.

H-Dmt-D-Arg-Aba-β-Ala-Bpa-Phe-NH_2 (**KGFF09**). Preparative RP-HPLC yielded the desired compound (white powder, 17%). HPLC: t_R = 12.0 min. TLC Rf 0.67 (EBAW). HRMS (ESP^+) found m/z 1014.5931 [M + H^+], [$C_{56}H_{75}N_{11}O_7$ + H^+] required 1014.5923.

H-Dmt-Apa-Phe-NH_2 (**KGFF10**). Preparative RP-HPLC yielded the desired powder, (25%). HPLC: t_R = 10.0 min. TLC Rf 0.43 (EBAW). HRMS (ESP^+) found m/z [$C_{30}H_{43}N_5O_4$ + H^+] required 538.3388.

H-Dmt-Bpa-Phe-NH_2 (**KGFF11**). Preparative RP-HPLC yielded the desired powder, (9%). HPLC: t_R = 12.0 min. TLC Rf 0.70 (EBAW). HRMS (ESP^+) found m/z [$C_{37}H_{49}N_5O_4$ + H^+] required 628.3857.

H-Dmt-D-Arg-Aba-NH (**KGFF12**). Preparative RP-HPLC yielded the desired powder, (15%). HPLC: t_R = 9.0 min. TLC Rf 0.66 (EBAW). HRMS (ESP^+) found m/z [$C_{27}H_{37}N_7O_4$ + H^+] required 524.2980.

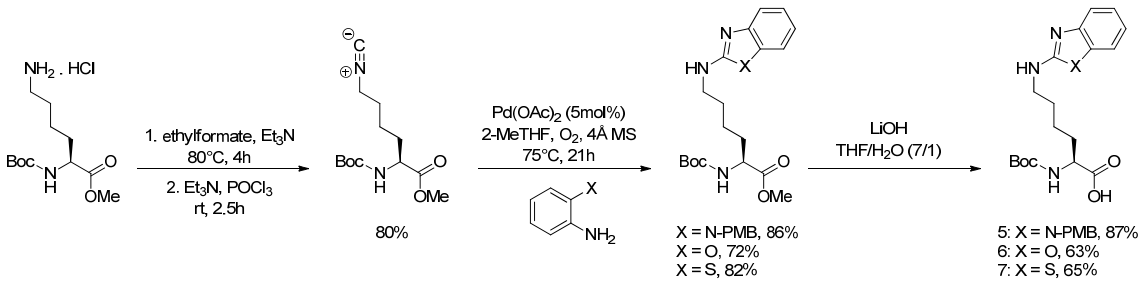
H-Dmt-D-Arg-Phe-NHMe (**KGFF13**). Preparative RP-HPLC yielded the desired compound (white powder, 8%). HPLC: t_R = 9.0 min. TLC Rf 0.66 (EBAW). HRMS (ESP^+) found m/z 526.3140 [M + H^+], [$C_{31}H_{39}F_6N_7O_6$ + H^+] required 526.3136.

H-Dmt-D-Arg-Aba-β-Ala-Lys(Bim)-Phe-NH_2 (**KGFF14**). Preparative RP-HPLC yielded the desired compound (white powder, 22%). HPLC: t_R = 11.2 min. TLC Rf 0.69 (EBAW). HRMS (ESP^+) found m/z 986.5317 [M + H^+], [$C_{52}H_{67}N_{13}O_7$ + H^+] required 986.5359.

H-Dmt-D-Arg-Aba-β-Ala-Lys(Box)-Phe-NH_2 (**KGFF15**). Preparative RP-HPLC yielded the desired compound (white powder, 43%). HPLC: t_R = 11.3 min. TLC Rf 0.74 (EBAW). HRMS (ESP^+) found m/z 987.5257 [M + H^+], [$C_{52}H_{66}N_{12}O_8$ + H^+] required 987.5200.

H-Dmt-D-Arg-Aba-β-Ala-Lys(Bth)-Phe-NH_2 (**KGFF16**). Preparative RP-HPLC yielded the desired compound (white powder, 39%). HPLC: t_R = 11.4 min. TLC Rf 0.70 (EBAW). HRMS (ESP^+) found m/z 1003.4985 [M + H^+], [$C_{52}H_{66}N_{12}O_7S$ + H^+] required 1003.4970.

Synthesis of the arginine mimetics



Synthesis of Boc-Lys(NC)-OMe

Boc-Lys-OMe hydrochloride (3.00 g, 10.1 mmol, 1.0 equiv.) was dissolved in ethylformate (28.8 mL, 35.5 mmol, 35 equiv.). To this solution, triethylamine (1.4 mL, 10.1 mmol, 1.0 equiv.) was added and stirred for 4 h at 80 °C. After cooling down to room temperature, the mixture was evaporated in vacuo. The crude formamide was obtained as a white solid and used without further purification. The formamide was dissolved in dry CH_2Cl_2 (20 mL) and triethylamine (7.0 mL, 50.5 mmol, 5.0 equiv.) was added to this solution. The solution was flushed with argon and cooled to 0 °C. Subsequently, phosphoryl chloride (1.4 mL, 15.2 mmol, 1.5 equiv.) was added dropwise while stirring. After the addition, the ice bath was removed and the mixture was stirred for an additional 2.5 h at room temperature. The mixture was poured in cold water (30 mL) and extracted with CH_2Cl_2 (3 x 30 mL). The combined organic layers were washed with water and brine (2 x 30 mL) and dried over $MgSO_4$. After concentration, the product was purified by flash chromatography using heptane/EtOAc (1/1) as eluent. (*S*)-methyl-2-((tert-butoxycarbonyl)amino)-6-isocyanohexanoate was obtained in 80 % yield. Yield: 80% (yellow oil, 3.64 g); Formula: $C_{13}H_{22}N_2O_4$; MW = 270.32 g/mol; TLC Rf: = 0.59 (EtOAc/heptane 1/1); 1H-NMR ($CDCl_3$, 400 MHz) in ppm δ: 1.44 (s, 9H), 1.48-1.55 (m, 2H), 1.63-1.72 (m, 3H), 1.82-1.89 (m, 1H), 3.39 (t, J =6.0 Hz, 2H), 3.75 (s, 3H), 4.31 (br s, 1H), 5.04 (br s, 1H); 13C-NMR ($CDCl_3$, 100 MHz) in ppm δ: 22.1, 28.3, 28.5, 32.0, 41.3 (t, J= 6.4 Hz), 52.4, 53.0, 80.1, 155.4, 156.4, 172.9.

Synthesis of Boc-Lys(Bim-PMB)-OMe

A 25 mL Kjeldahl flask was flame dried under vacuum and refilled with argon. Subsequently, the vial was charged with $Pd(OAc)_2$ (11 mg, 0.05 mmol, 0.05 equiv.), *N*-(p-methoxybenzyl)-o- phenylenediamine (228 mg, 1.00 mmol, 1.0 equiv.) and 4 Å molecular sieves (300 mg). The flask was equipped with a reflux condenser, evacuated and back filled with O_2 (three times). (*S*)-methyl-2-((tert-butoxycarbonyl)amino)-6-isocyanohexanoate (324 mg, 1.20 mmol,

1.2 equiv.) was dissolved in 2-MeTHF (1 mL) in a separate vial under argon. This mixture was added to the Kjeldahl flask followed by 2-MeTHF (1.5 mL). The reaction mixture was stirred at 75 °C under O_2 atmosphere for 21 h. After cooling down to room temperature, the solution was filtered through celite using EtOAc (50 mL) and concentrated in vacuo. The product was purified by automated flash chromatography applying a heptane/EtOAc gradient (from 100% heptane to 10% EtOAc, 25 mL/min). Methyl-2-((tert-butoxycarbonyl)amino)-6-((1-(4-methoxybenzyl)-1H-benzoimidazol-2-yl)amino)hexanoate was obtained in 86% (425 mg) yield. Yield: 86% (brown oil, 425 mg); Formula: $C_{27}H_{36}N_4O_5$; MW = 496.60 g/mol; TLC Rf = 0.61 (acetone/heptane 1/4); HPLC: t_R = 14.8 min; HRMS (ES^+): found 497.2744, calculated 497.2758 $[M+H]^+$; 1H-NMR ($CDCl_3$, 400MHz) in ppm δ: 1.23-1.40 (m,4H), 1.43(s,9H), 1.57-1.67 (m,4H), 1.75-1.81 (m, 2H), 3.47 (q, J =6.5 Hz, 2H), 3.70 (s, 3H), 3.78 (s, 3H), 4.01 (br s, 1H), 4.25 (br s, 1H), 5.02 (br s, 3H), 6.85-6.88 (m, 2H), 7.01-7.14 (m, 5H), 7.51 (d, J =7.8 Hz, 1H); 13C-NMR ($CDCl_3$, 100 MHz) in ppm δ: 22.5, 28.2, 29.1, 32.2, 42.9, 45.0, 52.0, 53.2, 55.1, 79.7, 107.1, 114.4, 116.2, 119.5, 121.1, 127.3, 127.7, 134.6, 142.1, 154.2, 155.3, 159.3, 173.1.

Synthesis of Boc-Lys(Box)-OMe

A 25 mL Kjeldahl flask was flame dried under vacuum and refilled with argon. Subsequently, the vial was charged with $Pd(OAc)_2$ (11 mg, 0.05 mmol, 0.05 equiv.), 2-aminophenol (109 mg, 1.0 mmol, 1.0 equiv.) and 4 Å molecular sieves (300 mg). The flask was equipped with a reflux condensor, evacuated and back filled with O_2 (three times). (S)-methyl-2-((tert-butoxycarbonyl)amino)-6- isocyanohexanoate (324 mg, 1.20 mmol, 1.2 equiv.) was dissolved in 2-MeTHF (1 mL) in a seperate vial under argon. This mixture was added to the Kjeldahl flask followed by 2-MeTHF (1.5 mL). The reaction mixture was stirred at 75 °C under O_2 atmosphere for 21 h. After cooling down to room temperature, the solution was filtered

through celite using EtOAc (50 mL) and concentrated in vacuo. The product was purified by an automated flash chromatography applying a heptane/EtOAc gradient (from 100% heptane to 10% EtOAc, 25 mL/min). Tert-butyl 6-(benzoxazol-2-ylamino)-2-((methoxycarbonyl)amino)hexanoate was obtained in 72% (270 mg) yield. Yield: 72% (brown solid, 270 mg); Formula: $C_{19}H_{27}N_3O_5$; MW = 377.43 g/mol; TLC Rf = 0.32 (EtOAc/heptane 1/1); HPLC t_R = 13.0 min; HRMS (ES^+): found 378.2000, calculated 378.2023 $[M+H]^+$; 1H-NMR(CDCl3,400MHz) in ppm δ =1.44-1.51(s,11H), 1.64-1.90(m,4H), 3.48(m,2H), 4.73(s,3H), 4.32 (br s, 1H), 5.08 (s, 2H), 7.02 (td, J =7.8, 1.0 Hz, 1H), 7.15 (td, J =7.7, 0.8 Hz, 1H), 7.22 (d, J =7.9 Hz, 1H), 7.36 (d, J =7.7 Hz, 1H); 13C-NMR (CDCl3, 100 MHz) in ppm δ: 22.6, 28.5, 29.2, 32.8, 43.0, 52.5, 53.2, 80.2, 108.8, 116.5, 121.0, 124.0, 143.2, 148.7, 155.7, 162.2, 173.3.

Synthesis of Boc-Lys(Bth)-OMe

A 25 mL Kjeldahl flask was flame dried under vacuum and refilled with argon. Subsequently, the vial was charged with $Pd(OAc)_2$ (11 mg, 0.05 mmol, 0.05 equiv.), 2-aminothiophenol (107µl, 1.00 mmol, 1.0 equiv.) and 4 Å molecular sieves (300 mg). The flask was equipped with a reflux condensor, evacuated and back filled with O_2 (three times). (S)-methyl-2-((tert-butoxycarbonyl)amino)-6-isocyanohexanoate (324 mg, 1.20 mmol, 1.2 equiv.) was dissolved in 2-MeTHF (1 mL) in a seperate vial under argon. This mixture was added to the Kjeldahl flask followed by 2-MeTHF (1.5 mL). The reaction mixture was stirred at 75 °C under O_2 atmosphere for 21 h. After cooling down to room temperature, the solution was filtered through celite using EtOAc (50 mL) and concentrated in vacuo. The product was purified by an automated flash chromatography applying a heptane / EtOAc gradient (from 100% heptane to 10% EtOAc, 25 mL/min). Tert-butyl 6-(benzothiazol-2-ylamino)-2-((methoxycarbonyl)amino)hexanoate was obtained in 80% yield. Yield: 80% (white solid,

316 mg); Formula: $C_{19}H_{27}N_3O_4S$; MW: 393.50 g/mol; TLC Rf: = 0.61 (EtOAc/heptane 1/2); HPLC: t_R = 13.1 min; HRMS (ES^+): found 394.1779, calculated 394.1795 $[M+H]^+$; 1H-NMR ($CDCl_3$, 400 MHz) in ppm δ: 1.44-1.48 (m, 10H), 1.50-1.67 (m, 3H), 1.84-1.89 (m, 1H), 3.44 (t, J =6.8 Hz, 2H), 3.73 (s, 3H), 4.32 (br s, 1H), 5.07 (br s, 1H), 5.41 (br s, 1H), 7.07 (td, J= 7.6, 0.9 Hz, 1H), 7.26-7.30 (m, 1H), 7.53 (d, J =7.8 Hz, 1H), 7.57 (d, J =7.8 Hz, 1H); 13C-NMR ($CDCl_3$, 100 MHz) in ppm δ: 22.8, 28.5, 29.1, 31.0, 32.8, 45.3, 52.5, 53.2, 80.2, 119.1, 120.9, 121.7, 126.1, 130.6, 152.7, 167.4, 173.3.

Boc-Lys(Bim-PMB)-OH

Boc-Lys(Bim,PMB)-OMe (250 mg, 0.50 mmol) was dissolved in a mixture of THF/H_2O (7:1, total 3.2 mL). Lithium hydroxide monohydrate (148 mg, 3.52 mmol, 7 equiv.) was added and continuously stirred for 16 h at room temperature. The reaction mixture was concentrated by evaporation and resuspended in H_2O (10 mL). The aqueous phase was washed with CH_2Cl_2 (2 x 5 mL) and carefully acidified to pH = 3, as indicated by pH paper, with 1N HCl. The aqueous layer was extracted with CH_2Cl_2 (4 x 10 mL). The combined organic layers were collected, washed with brine (1x 20 mL) and dried over $MgSO_4$, filtered and concentrated to obtain the corresponding carboxylic acid as a pink solid in 87% yield. The building block was used in Boc -based SPPS without further purification. Yield: 87% (211 mg); HPLC: t_R = 14.0 min; HRMS: (ES^+): found 483.2580, calculated 493.2602 $[M+H]^+$.

Boc-Lys(Box)-OH

Boc-Lys(Box)-OMe (190 mg, 0.50 mmol) was dissolved in a mixture of THF/H_2O (7:1, total 3.2 mL). Lithium hydroxide monohydrate (148 mg, 3.52 mmol, 7 equiv.) was added and continuously stirred for 16 h at room temperature. The reaction mixture was concentrated by evaporation and resuspended in H_2O (10 mL). The aqueous phase was washed with CH_2Cl_2 (2

x 5 mL) and carefully acidified to pH = 3, as indicated by pH paper, with 1N HCl. The aqueous layer was extracted with CH_2Cl_2 (4 x 10 mL). The combined organic layers were collected and washed with brine (1 x 20 mL) and dried over $MgSO_4$, filtered and concentrated to obtain the corresponding carboxylic acid as a pink solid in 74% yield (135 mg). The building block was used in Boc-based SPPS without further purification. Yield: 74% (135 mg); HPLC: t_R = 11.8 min; HRMS (ES^+): found 364.1867, calculated 364.1867 $[M+H]^+$.

Boc-Lys(Bth)-OH

Boc-Lys(Bth)-OMe (198 mg, 0.50 mmol) was dissolved in a mixture of THF/H_2O (7:1, total 3.2 mL). Lithium hydroxide monohydrate (148 mg, 3.52 mmol, 7 equiv.) was added and continuously stirred for 16 h at room temperature. The reaction mixture was concentrated by evaporation and resuspended in H_2O (10 mL). The aqueous phase was washed with CH_2Cl_2 (2 x 5 mL) and carefully acidified to pH = 3, as indicated by pH paper, with 1N HCl. The aqueous layer was extracted with CH_2Cl_2 (4 x 10 mL). The combined organic layers were collected and washed with brine (1 x 20 mL) and dried over $MgSO_4$, filtered and concentrated to obtain the corresponding carboxylic acid as a pink solid in 72% yield. The building block was used in Boc-based SPPS without further purification. Yield: 72% (13 8 mg); HPLC: t_R = 12.1 min; HRMS (ES^+): found 380.1617, calculated 380.1638 $[M+H]^+$.

Chiral derivatization of Boc-Lys(Bim,PMB)-OH, Boc-Lys(Box)-OH and Boc-Lys(Bth)-OH with Marfey's reagent (FDAA)

The chiral derivatization was performed starting from the *N*-Boc-deprotected substrates. Compounds (10 mg) are treated with a cocktail of TFA/CH_2Cl_2 (1:1) for 1 h and concentrated. The crude was redissolved in H_2O with a minimal amount of AcN and lyophilized to obtain

the deprotected mimetics as white to off-white solids in quantitative yields. The samples were analyzed via LC/MS and used without further purification.

Subsequently, the enantiomeric purity was checked via chiral derivatization with Marfey's reagent (FDAA). For this, a stock solution of 20.0 mM FDAA in acetone was prepared (5.44 mg FDAA/1 mL acetone). The analyte (1 mg) was dissolved in 1 mL 1 M $NaHCO_3$. Two equiv. of the stock solution were added to 100 µL of the analyte solution and the mixture was incubated at 40°C overnight. After quenching with 100 µL of a 1 M HCl solution, the sample was diluted to 1 mL with water and analyzed by LC-MS. Integration of the peak area (340 nm) gave an estimate of the enantiomeric excess. The derivatized product was obtained as a single peak, indicating that no epimerization took place during the synthesis.

Further analysis of the enantiomeric excess was performed by coupling of Boc-Lys(Bim-PMB)-OH to HCl.H- Ala-OMe by use of EDC/HOAt (1.6 equiv.) to yield the dipeptide. Analysis via HPLC showed only one single peak and NMR did not show any trace of the other diastereomer.

Synthesis of 4-amino-tetrahydro-aminobenzazepinon (Aba)-NH

N-Boc-ortho-aminomethyl-L-Phe (507 mg, 1.72 mmol, 1 equiv.), was submitted to an intramolecular cyclization [1]. The product was dissolved in 172 mL of CH_2Cl_2 (10 mM) and EDC.HCl (495 mg, 2.58 mmol, 1.5 equiv.), Et_3N (601 µL, 4.31 mmol, 2.5 equiv.) and HOBt.H_2O (396 mg, 2.58 mmol, 1.5 equiv.) were added. The reaction was stirred for 16 h and then the organic phase was washed with 20% citric acid and sat. $NaHCO_3$-solution. The solvent was dried with $MgSO_4$ and evaporated. The product was purified by flash chromatography using ethyl acetate/petroleum ether (4/6) to give a white solid in 19% yield. Yield: 19% (92.4 mg); Formula: $C_{15}H_{20}N_2O_3$; MW = 276.15g/mol; TLC Rf = 0.34 (EtOAc/petroleum ether4/6); HPLC t_R = 13.9 min; MS (ES^+) : 277 $[M + H]^+$, 299 $[M + Na]^+$,

177 [M- Boc]$^{+}$; Melting interval 149.0-150.5°C; 1H-NMR ($CDCl_3$, 500MHz) in ppm δ: 1.46 (9H, s, Boc), 2.97 (1H, dd, ^2J=16.7Hz, ^3J=13.0Hz, H_δ), 3.41 (1H, dd, ^2J= 16.9 Hz, ^3J= 3.2 Hz, $H_{\delta'}$) 3.96 (1H, dd, ^2J= 16.7 Hz, ^3J= 7.1 Hz, H_δ), 4.85 (1H, dd, ^2J= 16.5 Hz, ^3J= 3.8 Hz, $H_{\delta'}$), 5.86 (1H, d, ^2J= 5.8 Hz, NH), 7.01 (1H, d, ^3J= 7.5 Hz, CH arom.), 7.10 (2H, m, CH arom.), 7.19 (1H, m, CH arom.); 13C-NMR ($CDCl_3$, 125 MHz) in ppm δ: 28.6 (Boc), 37.2 (η-CH_2), 46.0 (η-CH_2), 49.4 (η-CH), 79.9 (Cq Boc), 126.5 (CH arom.), 128.0 (CH arom.), 128.4 (CH arom.), 131.3 (CH arom.), 134.2 (Cq arom.), 135.7 (Cq arom.), 155.4 (C=O Boc), 174.4 (C=O Aba).

The product could be deprotected with 50% TFA in CH_2Cl_2 for 2 h and directly coupled to the dipeptide.

References

[1] Van Rompaey K, Van den Eynde I, de Kimpe N, Tourwe D. A versatile synthesis of 2-substituted 4-amino-1,2,4,5-tetrahydro-2-benzazepine-3-ones. Tetrahedron letters 2003;59(24):4421-4432.

Table SDC2: Binding affinity constant (K_i) values of KGFF compounds for human MOPr, NPFF1R and NPFF2R.

	Ki ± SEM (nM)		
compound	**MOPr**	**NPFF1R**	**NPFF2R**
DAMGO	14.6 ± 3.8	nd	nd
RFRP3	nd	0.053 ± 0.003	nd
NPFF	nd	nd	0.28 ± 0.16
KGOP01	0.12 ± 0.02	4,600 ± 1,300	4,500 ± 1,800
KGFF01	0.59 ± 0.21	57 ± 10	1.2 ± 0.5
KGFF02	0.59 ± 0.15	2,540 ± 460	251 ± 25
KGFF03	0.24 ± 0.03	2.7 ± 0.3	0.077 ± 0.01
KGFF04	0.67 ± 0.29	780 ± 90	45 ± 8
KGFF05	3.2 ± 1.1	3,100 ± 1,100	360 ± 50
KGFF06	46 ± 22	147 ± 20	15.9 ± 1.8
KGFF07	0.29 ± 0.13	11.8 ± 3.6	84 ± 26
KGFF08	0.94 ± 0.19	136 ± 26	3.9 ± 0.1
KGFF09	2.43 ± 0.18	83 ± 21	3.2 ± 0.7
KGFF10	68 ± 48	>10,000	2,100 ± 600
KGFF11	>10,000	2,720 ± 760	406 ± 95
KGFF12	0.17 ± 0.05	2,490 ± 1,170	375 ± 108
KGFF13	0.11 ± 0.05	147 ± 16	206 ± 47
KGFF14	1.6 ± 0.3	4.54 ± 0.01	4.15 ± 1.35
KGFF15	1.8 ± 0.9	88 ± 8	9.6 ± 0.6
KGFF16	2.6 ± 1.3	80 ± 13	10.4 ± 2.3

Data are mean ± SEM of at least 2 independent experiments performed in duplicate. K_i values were determined from competition binding curves using [^{3}H]-diprenorphine for MOPr, and [^{125}I]-1-DMe-NPFF for NPFF1R and NPFF2R.

Table SDC3: Agonist activity constant (EC_{50} and E_{max}) values of KGFF compounds for human MOPr, NPFF1R and NPFF2R.

compound	MOPr (cAMP)		MOPr (β-arrestin-2)		NPFF1R (GTPγS)		NPFF2R (GTPγS)	
	EC_{50} (nM)	**E_{max} (%)**	**EC_{50} (nM)**	**E_{max} (%)**	**EC_{50} (nM)**	**E_{max} (%)**	**EC_{50} (nM)**	**E_{max} (%)**
DAMGO	80.4 ± 23.2	100 ± 4	240 ± 65	100 ± 2	nd		nd	nd
RFRP3	nd				10.1 ± 2.8	100 ± 10	nd	nd
NPFF	nd				nd	nd	18.2 ± 6.4	100 ± 4
KGOP01	0.204 ± 0.05	89 ± 2	1.6 ± 0.1	103 ± 9	> 10,000	nd	> 10,000	nd
KGFF01	2.4 ± 1.0	101 ± 7			155 ± 25	44 ± 8	128 ± 23	95 ± 6
KGFF02	1.5 ± 0.7	89 ± 2			nd	nd	nd	nd
KGFF03	12.0 ± 2	92 ± 1	33.9 ± 8.8	41 ± 11	84.8 ± 22.2	88 ± 7	11 ± 2	111 ± 17
KGFF04	9.5 ± 1.5	70 ± 4			>10,000	25 ± 4	963 ± 168	67 ± 3
KGFF05	59.3 ± 24.5	94 ± 2			nd	nd	nd	nd
KGFF06	2,300 ± 300	nd			nd	nd	nd	nd
KGFF07	1.7 ± 0.4	102 ± 2			87 ± 7	84 ± 8	1,930 ± 66	87 ±2
KGFF08	9.9 ± 2.0	102 ± 3			>10,000	23± 1	574 ± 129	97 ±2
KGFF09	18.2 ± 6.1	85 ± 2	56.4 ± 26.5	42 ± 2	176 ± 66	10 ± 2	157 ± 49	38 ± 14
KGFF10	598 ± 35	91 ± 10			nd	nd	nd	nd
KGFF11	>10,000	nd			nd	nd	nd	nd
KGFF12	2.7 ± 1.5	103 ± 4			>10,000	13 ± 3	>10,000	32 ± 17
KGFF13	1.0 ± 0.4	97 ± 2			>10,000	39 ± 2	>10,000	68 ± 8
KGFF14	5.9 ± 2.5	99 ± 4			76 ± 26	74 ± 1	89 ± 7	73 ± 18
KGFF15	4.1 ± 1.7	92 ± 13			1,020 ± 610	51 ± 5	930 ± 210	105 ± 15
KGFF16	6.2 ± 2.0	93 ± 6			273 ± 115	51 ± 7	526 ± 12	88 ± 12

Efficacy (E_{max}) is expressed as the percentage relative to the reference compound (DAMGO, RFRP3 and NPFF for MOPr, NPFF1R and NPFF2R, respectively). Values are mean ± SEM of at least 2 independent experiments performed in duplicate. nd, not determined.

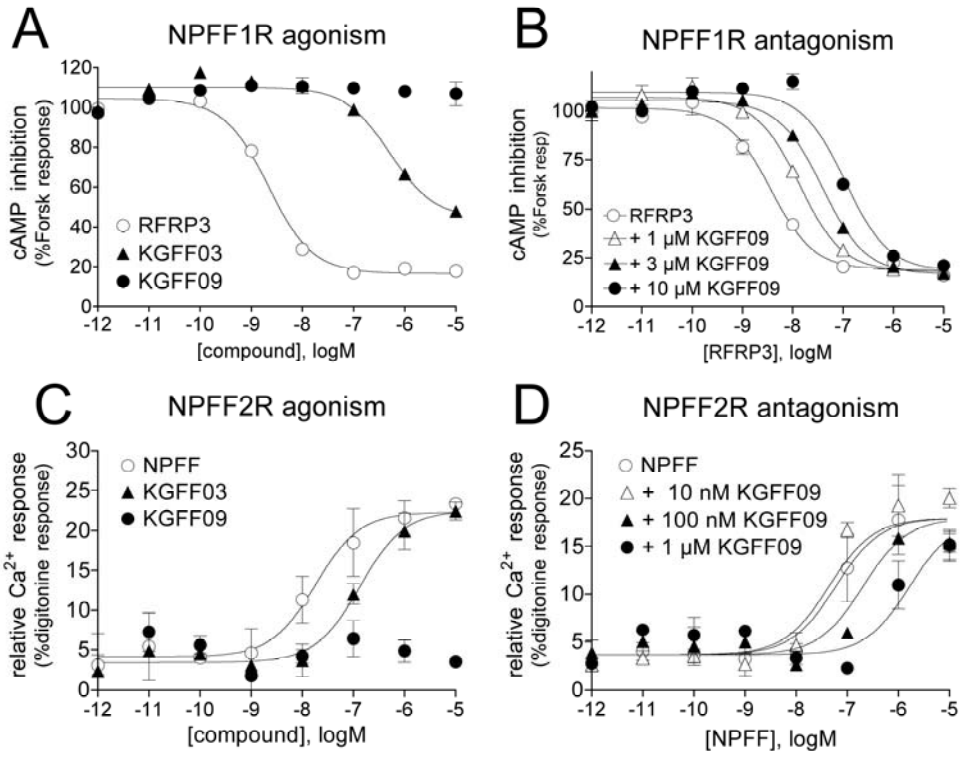


Figure SDC 4: in vitro characterization of KGFF03 and KGFF09 on NPFFRs.

Agonist (**A**) or antagonist (**B**) activity of KGFF03 and KGFF09 measured by inhibition of forskoline-induced cAMP accumulation (**A**, **B**) in HEK293-Glo20F cells stably expressing NPFF1R. Data are expressed as percentage of maximal cAMP levels. Agonist (**C**) or antagonist (**D**) activity of KGFF03 and KGFF09 measured by Ca^{2+} release (**C**, **D**) in HEK293-Glo20F cells stably expressing NPFF2R. Data are expressed as percentage of maximal digitonine-induced Ca^{2+} response, relative to basal. Data are mean ± SEM of at least 2 independent experiments performed in duplicate.

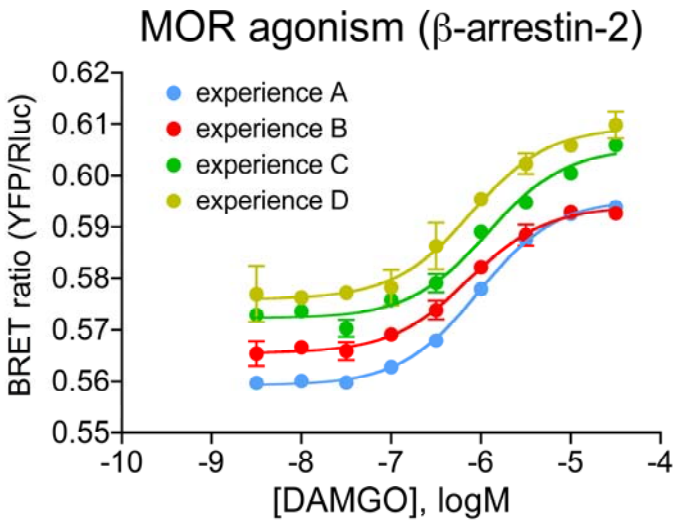


Figure SDC 5: Robustness of β-arrestin-2 recruitment to MOR induced by DAMGO. eYFP-labelled β-arrestin-2 translocation to Rluc-hMOR in HEK293 cells. BRET1 ratio corresponds to the YFP signal (band-pass filter 510-560 nm) divided by the Rluc signal (band-pass filter 435-485 nm). Each experiment is represented as mean ± SD and performed in duplicate.

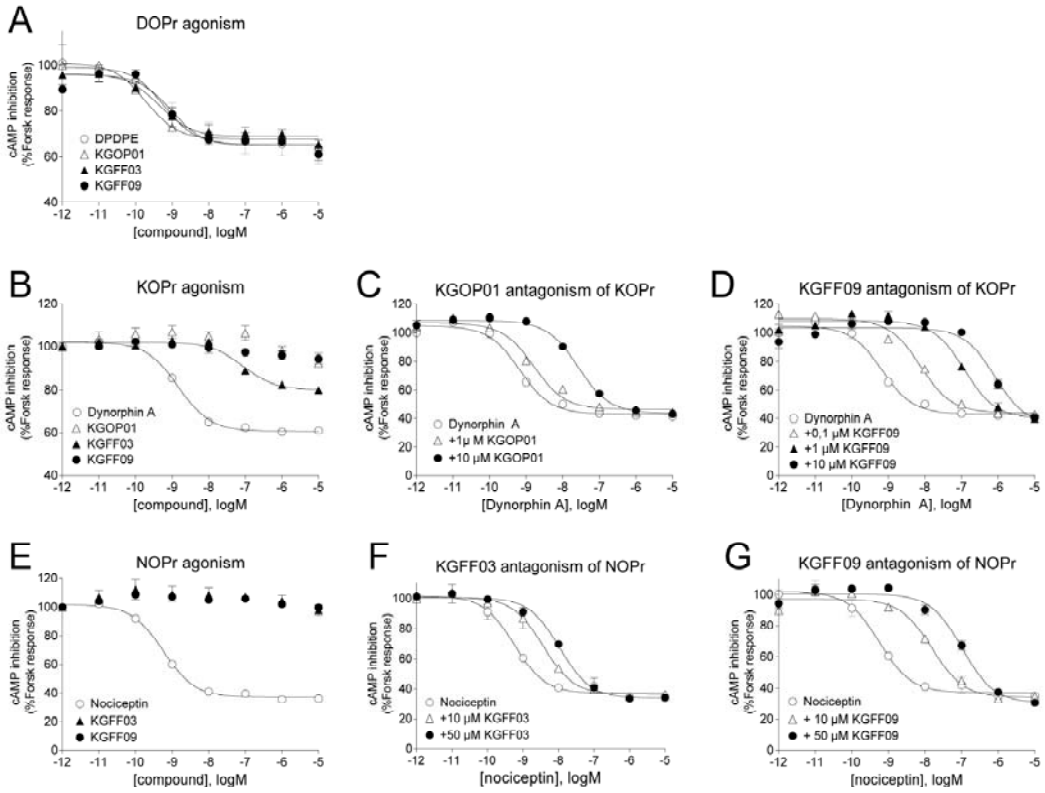


Figure SDC 6: In vitro characterization and selectivity of KGOP01, KGFF03 and KGFF09 for activity at DOPr, DOPr and NOPr.

Agonist (**A**, **B** and **E**) or antagonist (**C**, **D**, **F** and **G**) activity of KGOP01, KGFF03 and KGFF09 measured by inhibition of forskoline-induced cAMP accumulation in HEK293-Glo20F cells stably expressing human DOPr (**A**), KOPr (**B**, **C** and **D**) or NOPr (**E**, **F** and **G**). Data are expressed as percentage of maximal cAMP levels and shown as mean ± SEM of at least 2 independent experiments performed in duplicate.

Table SDC 7: Binding affinity constant (K_i) values of KGFF compounds for GPR10, GPR54 and GPR103.

	K_i ± SEM (nM)		
compound	**GPR10**	**GPR54**	**GPR103**
PrRP20	2.1 ± 0.4	nd	nd
Kp10	nd	0.062 ± 0.009	nd
26RFa	nd	nd	2.04 ± 0.58
KGOP01	> 10,000	> 10,000	> 10,000
KGFF03	> 10,000	4,370 ± 960	> 10,000
KGFF09	> 10,000	2,000 ± 300	1,200 ± 340

Data are mean ± SEM of at least two independent experiments performed in duplicate. K_i values were determined from competition binding curves using [^{3}H]-PrRP-20, [^{125}I]-Kp-10 and [^{125}I]-43RFa for GPR10, GPR54 and GPR103, respectively. nd, not determined.

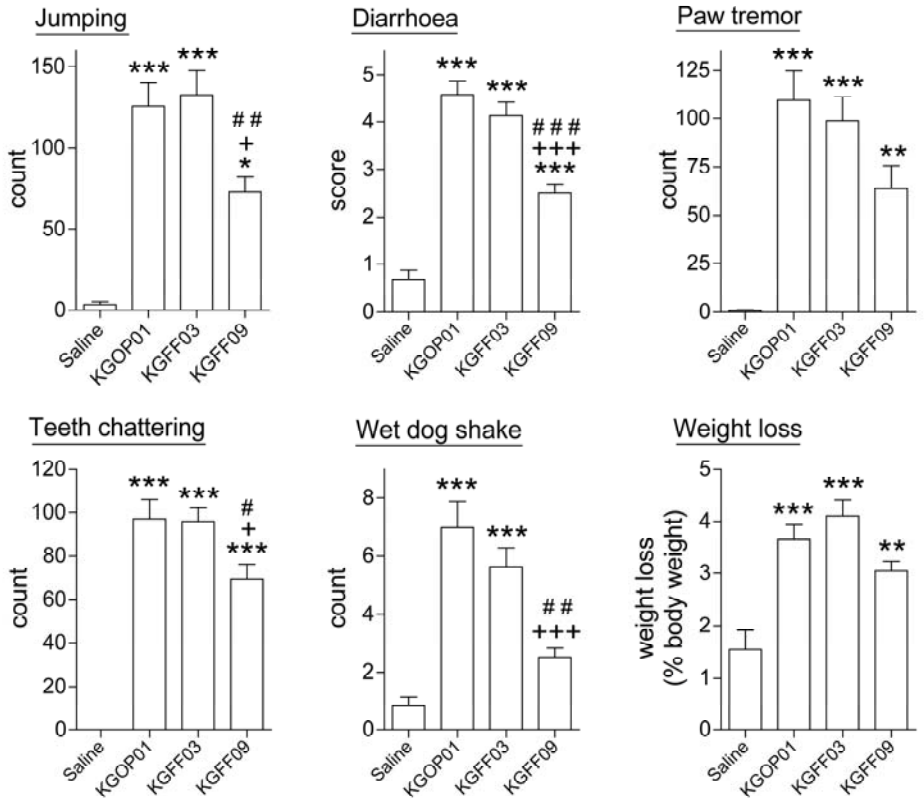


Figure SDC 8: Effect of KGOP01, KGFF03 or KGFF09 on naltrexone-precipitated withdrawal syndrome in C57BL/6 mice after chronic sc. treatment.

The signs of withdrawal were measured over 30 min immediately after naltrexone sc. injection. Mice were treated with KGOP01 (1.8 µmol/kg, sc.), KGFF03 (1.2 µmol/kg, sc.), KGFF09 (7.4 µmol/kg, sc.) or saline (control) twice daily over a 7-days period. Data are mean ± SEM, n = 6-8. One way ANOVA with Bonferroni's post hoc test *p<0.05, **p<0.01, ***p<0.001 as compared to saline, +p<0.05, +++p<0.001 as compared to KGOP01 and #p<0.05, ##p<0.01, ###p<0.001 as compared to KGFF03.

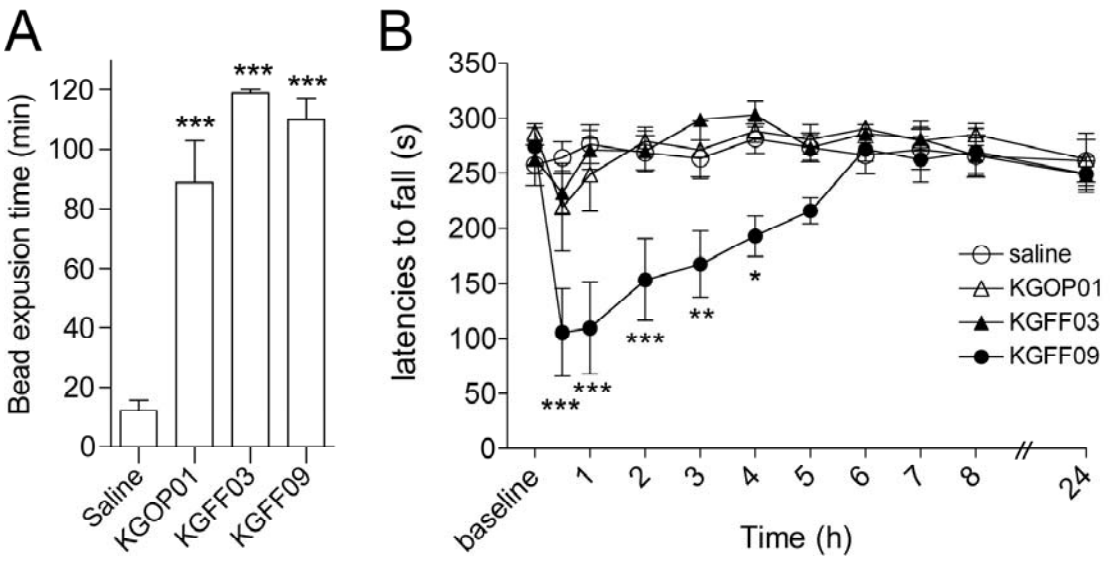


Figure SDC 9: Effect of KGOP01, KGFF03 or KGFF09 on gastro-intestinal motility and motor coordination.

(A) The gastrointestinal motility was evaluated in CD1 mice by measuring the colonic bead expulsion time over 120 min, 30 min after administration of KGOP01 (1.8 µmol/kg, sc.), KGFF03 (1.2 µmol/kg, sc.), KGFF09 (7.4 µmol/kg, sc.) or saline (control) injection. Data are mean ± SEM, n = 5-6. One-way ANOVA with Bonferroni's post hoc test ***p<0.001 compared to saline. **(B)** Motor coordination was evaluated in CD1 mice by measuring the time spent on the rotarod before and after KGOP01 (1.8 µmol/kg, sc.), KGFF03 (1.2 µmol/kg, sc.), KGFF09 (7.4 µmol/kg, sc.) or saline (control). Data are mean ± SEM, n = 5-7. Two-way ANOVA with Bonferroni's post hoc test *p<0.05, **p<0.01, ***p<0.001 compared to saline.

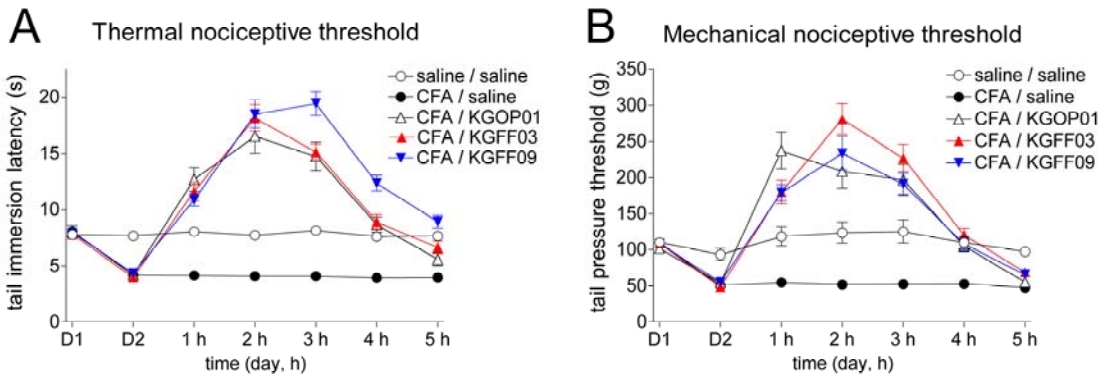


Figure SDC 10: Acute antinociceptive effect of KGOP01, KGFF03 and KGFF09 in the CFA-induced inflammatory pain model.

Time-dependent antinociceptive effect of KGOP01, KGFF03 and KGFF09 sc. administration in C57BL/6 mice in the tail immersion test **(A)** and in the tail pressure test **(B)**. KGOP01 (1.8 µmol/kg/d, sc.), KGFF03 (1.2 µmol/kg/d, sc.), KGFF09 (7.4 µmol/kg/d, sc.) or saline (control) were administered 24 h after CFA (or saline) injection in the tail. Data are mean ± SEM, n = 9-10.