Supporting Information

Dual alleviation of acute and neuropathic pain by fused opioid agonist-neurokinin 1 antagonist peptidomimetics

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Synthesis

Material and methods

Thin-layer chromatography (TLC) was performed on glass plates precoated with silica gel $60F_{254}$ (Merck, Darmstadt, Germany) using the mentioned solvent systems. Mass Spectrometry (MS) was done on a Micromass Q-Tof Micro spectrometer with electrospray ionization (ESI). Data collection and spectrum analysis was done with Masslynx software. Analytical RP-HPLC was performed using an Agilent 1100 Series system (Waldbronn, Germany) with a SUPELCO Discovery BIO Wide Pore® (Bellefonte, PA, USA) RP C-18 column (15 cm x 2.1 mm, 3 □m) using UV detection at 215 nm. The mobile phase (water/acetonitrile) contained 0.1% TFA. The gradient consisted of a 20 min run from 3 to 97% acetonitrile at a flow rate of 0.3 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 μm) with the same conditions as the analytical RP-HPLC but with a flow rate of 20 ml/min. After purification, the purity of all compounds was evaluated as being more than 95% by analytical RP-HPLC. All fractions were lyophilised using a Flexy-Dry lyophilizer (FTS Systems, Warminster, PA).

General Peptide Synthesis

All peptides were synthesized manually by Fmoc-based solid phase peptide synthesis (SPPS) on 2-chlorotritylchloride resin (0.1 mmol scale). The first amino acid was loaded onto the resin by use of 2 eq. of Fmoc-protected amino acid with 4 eq. DIPEA in CH_2Cl_2 for 2 hours. The remaining chlorines were substituted by treatment of the resin with a mixture of MeOH/CH_2Cl_2/DIPEA (2:17:1) during 5 minutes and repeated 4 times. The coupling reactions were performed with 3 eq of amino acid, 3 eq of TBTU and 9 eq of DIPEA (2.79 mmol, 461 μL) in DMF, for 1.5 h. Fmoc deprotections were realized by means of 20% 4-Me-piperidine in DMF (5 + 15 min). Boc-Dmt-OH was used as last amino acids in sequence. The coupling of this amino acid was performed using 3 eq of DIC and 3 eq of HOBt as coupling reagents in order to avoid side reactions that could occur when TBTU/DIPEA mixture is used. After every reaction step, the resin was washed with DMF (3x 1 min), iPrOH (3x 1 min) and CH_2Cl_2. The fully protected peptide was cleaved from the resin with 1% TFA in DMF for 30 minutes. The filtrate was evaporated in vacuo and the precipitated peptide was then dissolved in acetonitrile/H_2O and lyophilised to get the compounds as a powder.

Peptide Synthesis including Aba scaffold[1]

Reductive amination. The reductive amination was executed after Fmoc deprotection of the first amino acid. The resin was first swollen in 0.5% AcOH in TMOF/CH_2Cl_2 for 30 minutes and filtered. Two equivalents of Phth-*ortho*-formyl phenylalanine[2] were dissolved in the same mixture and 4 eq. of $NaBH_3CN$ was dissolved in a minimum volume of DMF. Both solutions were added to the resin and the reaction vessel was shaken for 30 minutes. The course of the reaction was monitored after this time by the Kaiser test. When the test was positive, the reaction was left for another 30 minutes. The monitoring is repeated until the reaction remains complete, which is indicated by a light red colour of the Kaiser test due to the presence of the secondary amine.

Cyclisation towards the Aba building block. An excess of 3 eq. TBTU is added to the resin and shaken for 3 hours. Phthaloyl deprotection. The resin was treated with 6 eq of hydrazine monohydrate in DMF for 18 hours.

*Coupling of NK1 antagonists **4**-**6** to the peptide acids and final deprotection*

The crude protected peptide was dissolved in a minimal amount of CH_2Cl_2. 1.5 eq. of DIC, 1.5 eq. of HOBt (for couplig of amines **4** and **6**), or 1.5 eq. of HOAt (for the coupling of compound **5**) are added. The reaction mixture was stirred overnight at room temperature. At completion, the solvent was evaporated and the residue was purified by flash chromatography, using a mixture of DCM/MeOH as eluent, in order to separate the protected peptidomimetic from the unreacted amines **4**-**6**. The purified protected product were treated with TFA/TES/H_2O (95:2.5:2.5) for 3 hours, after which the solvent was removed *in vacuo*.

Purification

The crude peptides were dissolved in H_2O and acetonitrile was added until complete dissolving was observed. The solution was injected on a Gilson preparative RP-HPLC. Fractions were collected and combined and lyophilised. The peptides were obtained as white powders with a purity of > 95% as determined by analytical HPLC. The structures were confirmed by high-resolution electrospray mass spectrometry.

Peptide characterization

7 white powder, yield: 42%. HPLC (standard gradient): t_{ret} = 13.52 min. ESI-HRMS: m/z = 1108.5572 [M+H$^+$] (calculated for $C_{56}H_{71}H^+F_6N_{11}O_6$: 1108.5566)

8 white powder, yield: 40%. HPLC (standard gradient): t_{ret} = 13.96 min. ESI-HRMS: m/z = 1033.5162 [M+H$^+$] (calculated for $C_{53}H_{66}H^+F_6N_{10}O_6$: 1053.5144)

9 white powder, yield: 45%. HPLC (standard gradient): t_{ret} = 14.22 min. ESI-HRMS: m/z = 1066.5235 [M+H$^+$] (calculated for $C_{54}H_{67}H^+F_6N_{10}O_6$: 1066.5227)

10 white powder, yield: 39 %. HPLC (standard gradient): t_{ret} = 9.08 min. ESI-HRMS [M+H$^+$] : m/z = 654.4091 (calculated for $C_{33}H_{51}H^+N_9O_5$: 654.4086)

11 white powder, yield: 25 %. HPLC (standard gradient): t_{ret} = 15.67 min. ESI-HRMS [M+H$^+$]: m/z = 1081.5439 (calculated for $C_{55}H_{70}H^+F_6N_{10}O_6$: 1081.5457)

12 white powder, yield: 39%. HPLC (standard gradient): t_{ret} = 13.46 min. ESI-HRMS: m/z = 1063.4919 [M+H$^+$] (calculated for $C_{54}H_{64}H^+F_6N_{10}O_6$: 1063.4988)

13 white powder, yield: 23%. HPLC (standard gradient): t_{ret} = 15.27 min. ESI-HRMS: m/z = 1008.4180 [M+H$^+$] (calculated for $C_{51}H_{59}H^+F_6N_9O_6$: 1008.4565)

14 white powder, yield: 34%. HPLC (standard gradient): t_{ret} = 15.73 min. ESI-HRMS: m/z = 1022.4739 [M+H$^+$] (calculated for $C_{52}H_{61}H^+F_6N_9O_6$: 1022.4722)

In vitro assays

Functional NK1R Assay[3].

Cell Line and Cell Culture Conditions.

The Chinese hamster ovary K1 (CHO-K1) cell line, stably expressing human NK1 receptor (hereafter referred to as CHO-NK1 cells), was transfected with an apoaequorin expression vector (pER2) using Fugene6 (Roche Applied Science). The cell line and expression vector were obtained from Euroscreen (Belgium). The CHO-NK1 cells were cultured in sterile DMEM/HAM's F12 medium (Sigma) supplemented with 10% fetal bovine serum, 100 IU/mL penicillin, 100 μg/mL streptomycin, and 400 μg/mL G 418 (Geneticin, Gibco) at 37 °C with 5% CO2 and were trypsinized every 3 days.

Aequorin Charging Protocol.

Transfected cells in the midlog phase were detached by changing the growth medium for PBS buffer supplemented with 5 mM EDTA (pH 8). The cells were spun down and incubated for 4 h at a concentration of 5 x 106 cells/mL in DMEM-F12 medium without phenol red (Gibco) supplemented with 0.1% BSA (BSA medium) and 5 μM coelenterazine h (Molecular Probes). After coelenterazine loading, the cells were diluted 10-fold in the same medium and incubated

for an additional period of 30 min. The cells were mildly shaken during the incubation periods.

Aequorin Luminescence Assay.

A dilution series of peptide agonist (SP was purchased from Sigma) ranging from 10-11 to 10-4 M was distributed in a white 96-well plate. For investigating antagonism, the synthetic compounds were added to these wells to obtain the desired concentrations (ranging from 10-8 to 10-4 M). One negative control sample (BSA medium only) was included in each row of the 96-well plate. The plate was loaded in a "Multimode Reader Mithras, LB940" (Berthold). The wells were screened one by one, and each measurement started at the moment of injection of 50 μL of the coelenterazine-loaded cell suspension, containing 2.5 x104 cells. Light emission was measured every second for 30 s after which 50 μL of 10 nM ATP solution (positive control) was injected. Each measurement was carried out in duplicate. Light emission was recorded for an additional period of 10 s per well, and the data were presented in relative light units (RLU).

Data Analysis.

Luminescence data (peak integration) were calculated using MikroWin 2000 software (Berthold), which was linked to the Microsoft Excel program. All statistical and curve-fitting analyses were performed using Prism 4.0 (GraphPad) software. Data are expressed in percentage (% RLU) of the maximal luminescence that was detected with 10-4 M SP (without antagonist). The competitive nature of antagonism was evaluated using the Schild plot method.[4] All antagonists analyzed inthis study provided linear regression plots and were considered competitive. The pA2 values were calculated using Schild's equation.[5]

hNK1/CHO Cell Membrane Preparation and Radioligand Binding Assay

Recombinant hNK1/CHO cells were grown to confluency in 37 °C, 95% air and 5% CO2, humidified atmosphere, in a Forma Scientific (Thermo Forma, OH) incubator in Ham's F12 medium supplemented with 10% fetal bovine serum, 100 U/mL penicillin,100 μg/mL streptomycin, and 500 μg/mL geneticin. The confluent cell monolayers were then washed with Ca^{2+}, Mg^{2+} deficient phosphate-buffered saline (PD buffer) and harvested in the same buffer containing 0.02% EDTA. After centrifugation at 2700 rpm for 12 min, the cells were homogenized in ice-cold 10 mM Tris-HCl and 1 mM EDTA, pH 7.4, buffer. A crude membrane fraction was collected by centrifugation at 18000 rpm for 12 min at 4 °C, the pellet was suspended in 50 mM Tris-Mg buffer, and the protein concentration of the membrane preparation was determined by using Bradford assay. Six different concentrations of the test compound were each incubated, in duplicates, with 20 μg of membrane homogenate, and 0.4

nM [3H]SP (135 Ci/mmol, Perkin-Elmer, United States) in 1 mL final volume of assay buffer (50mMTris, pH 7.4, containing 5mMMgCl2, 50 μg/mL bacitracin, 30 μM bestatin, 10 μM captopril, and 100 μM phenylmethylsulfonylfluoride) SP at 10 μM was used to define the nonspecific binding. The samples were incubated in a shaking water bath at 25 °C for 20 min. The [3H]SP concentration and the incubation time were selected based on the studies of Yamamoto et al.[6] The reaction was terminated by rapid filtration through Whatman grade GF/B filter paper (Gaithersburg, MD) presoaked in 1% polyethyleneimine, washed four times each with 2 mL of cold saline, and the filter bound radioactivity was determined by liquid scintillation counting (Beckman LS5000 TD). The media and chemicals listed above were purchased from Sigma (Sigma-Aldrich, St. Louis, MO) unless otherwise stated.

Data Analysis.

Analysis of data collected from three independent experiments performed in duplicates is done using GraphPad Prizm 4 software (GraphPad, San Diego, CA). Log IC50 values for each test compound were determined from nonlinear regression. The inhibition constant (Ki) was calculated from the antilogarithmic IC50 value by the Cheng and Prusoff equation.[7,8]

Functional GPI and Mouse Vas Deferens (MVD) Assays.

The GPI[8] and MVD[9] bioassays were carried out as described in detail elsewhere.[10,11] A dose-response curve was determined with [Leu^5]enkephalin as standard for each ileum and vas preparation, and IC_{50} values of the compounds being tested were normalized according to a published procedure.[12]

Opioid Receptor Binding Assays.

Opioid receptor binding studies were performed as described in detail elsewhere[13]. Binding affinities for μ and δ opioid receptors were determined by displacing, respectively, [^{3}H]DAMGO (Multiple Peptide Systems, San Diego, CA) and [^{3}H]DSLET (Multiple Peptide Systems) from rat brain membrane binding sites, and κ opioid receptor (KOR) binding affinities were measured by displacement of [^{3}H]U69,593 (Amersham) from guinea pig brain membrane binding sites. Incubations were performed for 2 h at 0 °C with [^{3}H]DAMGO, [^{3}H]DSLET, and [^{3}H]U69,593 at respective concentrations of 0.72, 0.78, and 0.80 nM. IC_{50} values were determined form log-dose displacement curves, and Ki values were calculated from the IC_{50} values by means of the equation of Cheng and Prusoff,[7] using values of 1.3, 2.6, and 2.9 nM for the dissociation constants of [^{3}H]DAMGO, [^{3}H]DSLET, and [^{3}H]U69,593, respectively.

In vivo analgesic tests in rats

Animals

Male Wistar rats (300-350 g) obtained from Charles River Breeding Laboratories, Germany, were housed one animal per cage lined with sawdust under a standard 12/12 h light/dark cycle (lights on at 6:00 am) with food and water available ad libitum. All experiments were performed according to the recommendations of IASP, the NIH Guide for Care and Use of Laboratory Animals, and were approved by the local Bioethics Committee (Krakow, Poland).

Implantation of intrathecal cannulas

The rats were chronically implanted with i.t. catheters according to Yaksh and Rudy[14] under pentobarbital anesthesia (60 mg/kg; i.p.). The rats were placed on a stereotaxic table (David Kopf), and a sterile catheter (PE 10, INTRAMEDIC, Clay Adams, Becton Dickinson and Company, Rutherford, NJ, USA.), flushed with sterile water prior to insertion, was carefully introduced through the atlanto-occipital membrane to the subarachnoid space at the rostral level of the spinal cord lumbar enlargement (L4-L6).

Neuropathic pain model

A chronic constriction injury (CCI) was produced according to Bennett and Xie.[15] The right sciatic nerve was exposed under sodium pentobarbital anesthesia (60 mg/kg; i.p.). Four ligatures (4/0 silk) were made around the nerve distal to the sciatic notch with 1 mm spacing until a brief twitch in the respective hind limb was observed. After CCI, all animals developed allodynia and hyperalgesia. Testing procedures were conducted 7-14 days after surgery.

Drugs and administration procedure

Two novel designed compounds containing both opioid receptor agonist and neurokinin1 receptor antagonist pharmacophores (hybrids), two compounds containing only respective opioid or neurokinin1 pharmacophores (parents) and morphine hydrochloride (Kutno, Poland) were used in the study. Drugs were dissolved in sterile water for injection (Aqua pro injection, Polpharma, Poland) and administered intrathecally (i.t.) in 5 µl dose volume, followed by an injection of 10 ml of distilled water to flush the catheter. In naïve animals, the compounds were administered in cumulative doses (0.01, 0.1, 1 and 10 µg) starting from the lowest one, with at least one hour interval between doses. Groups of 7 – 12 animals were used. The tests were performed 30' after each dose. In CCI animals, compounds were administered i.t. using the same paradigm. Groups of 4 – 8 animals were used. For tolerance studies, daily a dose 1µg was administered for 6 consecutive days, and tests were performed (30' after administration) on a daily basis, starting from day 3rd. On day 7th, after tolerance development proved by behavioral tests, morphine-tolerated animals obtained either **12** or **13**,

and **12**-, **13**- and **5**-tolerated animals obtained morphine. Control groups obtained vehicle (Aqua pro injection) in the same paradigm. Inter-group differences were analysed by ANOVA Bonferroni's Multiple Comparison Test; *p<0.05, **p<0.01, ***p<0.001 versus verhicle treated animals.

Behavioral tests

Tail flick test

The pain threshold to a thermal stimulus in naïve animals was assessed using tail- flick latency evoked by noxious thermal stimulation as determined with a tail-flick analgesic meter (Analgesia Meter; Ugo Basile, Comerio, Italy) as described previously.[16] The tail-flick test consisted of focusing a beam of light on the dorsal tail surface approximately 2 cm from the tip of the tail. The intensity of the light was adjusted so that the baseline tail-flick latencies were 1.4 s. The cut-off time for the tail-flick reaction was set to 9 s.

Von Frey test

Allodynia was measured after application of a non-noxious touch stimulus using an automatic von Frey apparatus (Dynamic Plantar Aesthesiometer Cat. no. 37400, Ugo Basile, Italy). Animals were placed in plastic cages with wire mesh floors 5 min before the experiment. The von Frey filament was applied to the midplantar surface of the hind paw, and measurements were taken automatically, as described previously.[17] We tested the ipsilateral paw twice in 3-min intervals, and the mean value was calculated. The strengths of the von Frey stimuli used in our experiments ranged from 0.5 to 26 g. There was almost no response in naïve animals to the highest strengths (26 g). Therefore, a cut-off was drawn at this value.

Cold plate test

Hyperalgesia was measured after the application of a noxious (low temperature) stimulus using the cold plate test (Cold/Hot Plate Analgesia Meter no. 05044, Columbus Instruments, USA) as described previously.[17] The temperature of the cold plate was maintained at 5 °C, and the cut-off latency was 30 s. The animals were placed on the cold plate, and the time until a lifting of the hind paw occurred was recorded. The reaction of the first hind paw to be lifted was measured in the naïve rat group. The ipsilateral paw reacted the first in rats subjected to nerve injury.

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