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In vitro functional characterization of novel nociceptin/orphanin FQ receptor agonists in recombinant and native preparations☆

Federica Ferrari[a], **Maria Camilla Cerlesi**[a], **Davide Malfacini**[a], **Laila Asth**[b], **Elaine C. Gavioli**[b], **Blair V. Journigan**[c], **Uma Gayathri Kamakolanu**[c], **Michael E. Meyer**[c], **Dennis Yasuda**[c], **Willma E. Polgar**[d], **Anna Rizzi**[a], **Remo Guerrini**[e], **Chiara Ruzza**[a], **Nurulain T. Zaveri**[c], and **Girolamo Calo**[a,*]

[a]Section of Pharmacology, Department of Medical Sciences, and National Institute of Neurosciences, University of Ferrara, Italy

[b]Behavioral Pharmacology Laboratory, Department of Biophysics and Pharmacology, Federal University of Rio Grande do Norte, Natal, RN, Brazil

[c]Astraea Therapeutics, LLC. 320 Logue Avenue, Mountain View, CA, USA

[d]SRI International, Biosciences Division, 333 Ravenswood Avenue, Menlo Park, CA, USA

[e]Department of Chemical and Pharmaceutical Sciences and LTTA, University of Ferrara, Italy

Abstract

Nociceptin/Orphanin FQ (N/OFQ) regulates several biological functions via selective activation of the N/OFQ receptor (NOP). In this study novel nonpeptide NOP ligands were characterized in vitro in receptor binding and [^{35}S]GTPγS stimulated binding in membranes of cells expressing human NOP and classical opioid receptors, calcium mobilization assay in cells coexpressing the receptors and chimeric G proteins, bioluminescence resonance energy transfer (BRET) based assay for studying NOP receptor interaction with G protein and arrestin, the electrically stimulated mouse vas deferens and the mouse colon bioassays. The action of the AT compounds were compared with standard NOP agonists (N/OFQ and Ro 65-6570) and the NOP selective antagonist SB-612111. AT compounds displayed high NOP affinity and behaved as NOP agonists in all the functional assays consistently showing the following rank order of potency AT-127≥AT-090≥AT-035 > AT-004= AT-001. AT compounds behaved as NOP full agonists in the calcium mobilization and mouse colon assays and as partial agonists in the [^{35}S]GTPγS and BRET assays. Interestingly AT-090 and AT-127, contrary to standard nonpeptide agonists that display G protein biased agonism, behaved as an unbiased agonists. AT-090 and AT-127 displayed higher NOP selectivity than Ro 65-6570 at native mouse receptors. AT-090 and AT-127 might be useful pharmacological tools for investigating the therapeutic potential of NOP partial agonists.

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*Correspondence to: Department of Medical Sciences, Section of Pharmacology, University of Ferrara, via Fossato di Mortara 19, 44121 Ferrara, Italy. g.calo@unife.it (G. Calo).

Keywords

NOP and classical opioid receptors; Bioluminescence resonance energy transfer; Calcium mobilization and mouse colon vas; deferens and assays; Receptor and [^{35}S]GTPγS binding

1. Introduction

Nociceptin/orphanin FQ (N/OFQ) is the endogenous ligand of the N/OFQ peptide receptor (NOP), the fourth member of the opioid receptor family. Despite close similarities in structure and signaling mechanisms, the pharmacological profile of the NOP receptor is distinct from that of classical opioid receptors (Lambert, 2008; Toll et al., 2016). The N/OFQ – NOP receptor system controls several biological functions including pain transmission, emotional states, motor behavior, memory and learning, drug abuse, cough and micturition reflexes and activities of the cardiovascular, respiratory, gastrointestinal and immune systems (Lambert, 2008; Toll et al., 2016). Several NOP receptor ligands have been identified and characterized by academic and industrial laboratories (Mustazza and Bastanzio, 2011; Zaveri, 2016). Selective NOP antagonists have been investigated as antidepressants (Gavioli and Calo, 2013; Post et al., 2016) and for Parkinson's disease treatment (Marti et al., 2013, 2004, 2010) whereas selective nonpeptide NOP agonists have shown significant efficacy for anxiety (Gavioli and Calo, 2006; Shoblock, 2007; Witkin et al., 2014) and pain (Schroder et al., 2014; Toll et al., 2016). Recently, NOP receptor partial agonists have also been developed and investigated in models of anxiety and depression (Asth et al., 2016; Ross et al., 2015).

With our active work in the design and synthesis of novel NOP ligands, we have discovered several peptide (Calo and Guerrini, 2013) and nonpeptide (Zaveri et al., 2013) NOP ligands as useful pharmacological tools. The novel nonpeptide NOP ligands AT-001, AT-004, AT-035, AT-090, and AT-127 were recently identified and their initial characterization in the GTPγS binding assay using the human NOP receptor transfected Chinese hamster ovary (CHO) cells showed that these compounds had partial agonist activity with efficacies ranging from 0.21 to 0.61 (N/OFQ 1.00). Since the maximal effects elicited by partial agonists strongly depend on the efficiency of the stimulus response coupling of the different preparations, the aim of the present study was to characterize the in vitro functional efficacy of these compounds in detail in several other in vitro biochemical assays of downstream signal transduction as well as in ex vivo assays with N/OFQ sensitive rodent tissues. The following in vitro assays were used: receptor binding and [^{35}S]GTPγS binding assay, a calcium mobilization assay performed in cells expressing the human NOP or classical opioid receptors and chimeric G proteins, a bioluminescence resonance energy transfer (BRET) based assay for studying NOP receptor interaction with G protein and arrestin, the electrically stimulated mouse vas deferens and the mouse colon bioassays. The effect of AT compounds were compared to those of standard NOP ligands such as the agonists N/OFQ and Ro 65-6570 and the antagonist SB-612111.

2. Materials and methods

2.1. Drugs and reagents

N/OFQ and Ro 65-6570 were synthesized and purified in the Prof. Guerrini's laboratories (Department of Chemical and Pharmaceutical Sciences, University of Ferrara). SB-612111 was purchased from Tocris (Bristol, UK). AT-001 [(1-(1-((1s,4s)–4-isopropylcyclohexyl)piperidin-4-yl)indoline], AT-004 [1-(1-((1s,4s)–4-isopropylcyclohexyl)piperidin-4-yl)-1H-indole], AT-035 [(1-(1-((1s,4s)–4-isopropylcyclohexyl)piperidin-4-yl)-1H-indol-3-yl)methanamine], AT-090 [1-(1-((1s,4s)–4-iso-propylcyclohexyl)piperidin-4-yl)indoline-2,3-dione], and AT-127 [(Z)–3-(hydroxyimino)–1-(1-((1s,4s)–4-isopropylcyclohexyl)piperidin-4-yl)indolin-2-one] were synthesized at Astraea Therapeutics, Mountain view, CA, USA. Chemical structures of AT compounds are showed in Fig. 1. Stock solution of N/OFQ was made in bidistilled water (1 mM), that of Ro 65-6570 and SB-612111 in DMSO (10 mM) whereas the AT compounds were solubilized in 10% Tween 20 in DMSO (10 mM) and stored at −20 °C.

2.2. Receptor binding

The human NOP, mu, delta and kappa opioid receptors were stably expressed in CHO cells. The cells were grown in Dulbecco's Modified Eagle Medium (DMEM) with 10% fetal bovine serum, in the presence of 0.4 mg/ml G418 and 0.1% penicillin/streptomycin, in 100-mm plastic culture dishes. Binding to cell membranes was conducted in a 96-well format, as described previously (Adapa and Toll, 1997). Cells were removed from the plates by scraping with a rubber policeman, homogenized in Tris buffer using a Polytron homogenizer, then centrifuged once and washed by an additional centrifugation at 27,000g for 15 min. The pellet was resuspended in 50 mM Tris, pH 7.5, and the suspension incubated with [^{3}H]N/OFQ, [^{3}H]DAMGO, [^{3}H]DPDPE, or [^{3}H]U69593, for binding to NOP, mu, delta, or kappa opioid receptors, respectively. The total volume of incubation was 1.0 ml and samples were incubated for 60–120 min at 25 °C. The amount of protein in the binding reaction varied from approximately 15 to 30 μg. The reaction was terminated by filtration using a Tomtec 96 harvester (Orange, CT) with glass fiber filters. Bound radioactivity was counted on a Pharmacia Biotech beta-plate liquid scintillation counter (Piscataway, NJ) and expressed in counts per minute. IC_{50} values were determined using at least six concentrations of each peptide analog, and calculated using Graphpad/Prism (ISI, San Diego, CA). Ki values were determined by the method of Cheng and Prusoff (1973).

2.3. [^{35}S]GTPγS binding assay

CHO cells stably expressing human NOP or mu opioid receptors were grown in Dulbecco's Modified Eagle Medium (DMEM) with 10% fetal bovine serum, in the presence of 0.4 mg/ml G418 and 0.1% penicillin/streptomycin, in 100-mm plastic culture dishes. [^{35}S]GTPγS binding was conducted basically as described by Traynor and Nahorski (1995). Cells were scraped from tissue culture dishes into 20 mM HEPES, 1 mM EDTA, then centrifuged at 500*g* for 10 min. Cells were resuspended in this buffer and homogenized using a Polytron homogenizer. The homogenate was centrifuged at 27,000*g* for 15 min and the pellet resuspended in Buffer A, containing 20 mM HEPES, 10 mM $MgCl_2$, 100 mM NaCl, pH 7.4. The suspension was recentrifuged at 27,000*g* and suspended once more in

Buffer A. The pellet was sometimes frozen at −70 °C prior to use. For the binding assay, membranes (8–15 μg protein) were incubated with [^{35}S]GTPγS (50 pM), GDP (10 μm), and the appropriate compound, in a total volume of 1.0 ml for 60 min at 25 °C. Samples were filtered over glass fiber filters using a Tomtec 96 harvester (Orange, CT). Bound radioactivity was counted on a Pharmacia Biotech beta-plate liquid scintillation counter (Piscataway, NJ) and expressed in counts per minute.

2.4. Calcium mobilization assay

CHO cells stably co-expressing the human NOP, kappa, or mu opioid receptors and the C-terminally modified $G\alpha_{qi5}$ and CHO cells expressing the delta opioid receptor and the $G\alpha_{qG66Di5}$ protein were generated as previously described (Camarda and Calo, 2013; Camarda et al., 2009). Cells were maintained in culture medium consisting of Dulbecco's MEM/HAM'S F-12 (50/50) supplemented with 10% fetal bovine serum, penicillin (100 IU/ml), streptomycin (100 μg/ml), fungizone (1 μg/ml), geneticin (G418; 200 μg/ml) and hygromycin B (100 μg/ml). Cell cultures were kept at 37 °C in 5% CO_2 humidified air. When confluence was reached (3–4 days), cells were sub-cultured as required using trypsin/EDTA and used for testing. Cells were seeded at a density of 50,000 cells/well into 96-well black, clear-bottom plates. After 24 h incubation the cells were loaded with medium supplemented with 2.5 mM probenecid, 3 μm of the calcium sensitive fluorescent dye Fluo-4 AM and 0.01% pluronic acid, for 30 min at 37 °C. Afterwards the loading solution was aspirated and 100 μl/well of assay buffer: Hank's Balanced Salt Solution (HBSS) supplemented with 20 mM HEPES, 2.5 mM probenecid and 500 μm Brilliant Black (Aldrich) was added. Serial dilutions of stock solutions of ligands for experimental use were made in HBSS/HEPES (20 mM) buffer (containing 0.03% of bovine serum albumin, BSA, Sigma Chemical Co., Poole, UK). After placing both plates (cell culture and compound plate) into the FlexStation II (Molecular Device, Union City, CA 94587, US), fluorescence changes were measured. On-line additions were carried out in a volume of 50 μl/well. In antagonism experiments the antagonists was injected in the well 24 min before adding the agonist. To facilitate drug diffusion into the wells in antagonist type experiments, studies were performed at 37 °C and three cycles of mixing (25 μl from each well moved up and down 3 times) were performed immediately after antagonist injection.

2.5. BRET assay

Human Embryonic Kidney (HEK293) cells were grown in Dulbecco's modified Eagle's medium supplemented with 10% (v/v) fetal bovine serum, 100 units/ml penicillin G, and 100 μg/ml streptomycin sulfate in a humidified atmosphere of 5% CO_2 at 37 °C. Cell lines permanently co-expressing the different pairs of fusion proteins (NOP-RLuc/Gβ1-RGFP and NOP-RLuc/β-arrestin 2-RGFP) were prepared using the pantropic retroviral expression system by Clontech as described previously (Malfacini et al., 2015). For G-protein experiments, enriched plasma membrane aliquots from transfected cells were prepared by differential centrifugation. Cells were detached with PBS/EDTA solution (1 mM, pH 7.4 NaOH) and then, after 5 min, subjected to 500g centrifugation and dounce-homogenized (30 strokes) in cold homogenization buffer (TRIS 5 mM, EGTA 1 mM, DTT 1 mM, pH 7.4 HCl) in the presence of sucrose (0.32 M). Three subsequent centrifugations were performed at 1000*g* (4 °C) and the supernatants retained. Two 25,000*g* (4 °C) subsequent

centrifugations (the second in the absence of sucrose) were performed for separating enriched membranes that, after discarding the supernatant, were kept in ultrapure water at −80 °C. The protein concentration in membrane preparations was determined using the QPRO-BCA kit (Cyanagen Srl, Bologna, IT) and the spectrophotometer Beckman DU 520 (Brea, CA, USA).

Luminescence in membranes was recorded in 96-well untreated white opaque microplates, while in whole cells was recorded in 96-well sterile poly-D-lysine-coated white opaque microplates (PerkinElmer, Waltham, MA, USA) using the luminometer Victor 2030 (PerkinElmer, Waltham, MA, USA). For the determination of NOP/G-protein interaction, membranes (3 μg of protein) prepared from cells co-expressing NOP/RLuc and Gβ1/RGFP were added to wells in DPBS. For the determination of NOP/β-arrestin 2 interaction, cells co-expressing NOP/RLuc and β-arrestin 2/RGFP were plated 24 h before the experiment in poly-D-Lysine treated plates (100,000 cells / well). The cells were prepared for the experiment substituting the medium with PBS with $MgCl_2$ (0.5 mM) and $CaCl_2$ (0.9 mM). Coelenterazine at a final concentration of 5 μm was injected 15 min prior reading the cell plate. Different concentrations of ligands in 20 μl of PBS-BSA 0.01% were added and incubated 5 min before reading luminescence. All the experiments were performed at room temperature.

2.6. Bioassays

All experiments involving animals were performed according to the European Communities Council directives (2010/63/EU) and Italian regulations (D.L.26/2014). Protocols were approved by Ethic Committees for Animal Use of the University of Ferrara and by the Italian Ministry of Health (Protocol No. 10086). These studies have been reported according to ARRIVE guidelines (Kilkenny et al., 2010). Male CD-1 mice (12–16 weeks old, 30–35 g, Harlan, Udine, Italy) were used together with NOP(+/+) and NOP(−/−) mice (8–12 weeks old). Details about the generation of mutant mice have been published previously (Bertorelli et al., 2002). NOP(+/+) and NOP(−/−) mice have been recently backcrossed on CD-1 strain in our laboratories. Mice were housed in 425×266×155 mm cages (Tecniplast, VA, Italy), 5 mice/cage, under standard conditions (22 °C, 55% humidity, 12 h light–dark cycle, lights on 7.00 am) with food (4RF, Mucedola, MI, Italy) and water ad libitum. A mouse red house (Tecniplast, VA, Italy) and nesting materials were present in each cage. Each animal was killed with CO_2 before tissues extraction. A total number of 94 mice was used for the present study.

The mouse vas deferens tissues were prepared as previously described (Calo et al., 1996). Tissues were suspended in 5 ml organ baths containing heated Krebs solution (composition in mM: NaCl 118.5, KCl 4.7, KH_2PO_4 1.2, $NaHCO_3$ 25, glucose 10 and $CaCl_2$ 2.5) oxygenated with 95% O_2 and 5% CO_2. The bath temperature was set at 33 °C. Tissues were continuously stimulated through two platinum ring electrodes with supramaximal rectangular pulses of 1 ms duration and 0.05 Hz frequency. A resting tension of 0.3 g was applied to the tissues. The electrically evoked contractions (twitches) were measured isotonically with a strain gauge transducer (Basile 7006, UgoBasile s.r.l., Varese, Italy) and recorded with the PC based acquisition system Power Lab (ADInstrument, USA). Following

an equilibration period of 60 min, the contractions induced by electrical field stimulation were stable. At this time, cumulative concentration-response curves to N/OFQ, Ro 65-6570, and AT compounds were performed (0.5 log unit steps). All the compounds inactive as agonist were tested as antagonists. In antagonism experiments the concentration response curve to N/OFQ has been performed in absence and in presence of a fixed concentration of antagonist, incubated for 15 min.

Segments of mouse colon (approximately 1 cm length) were prepared as previously described (Rizzi et al., 1999) to record isometric smooth muscle contractions. Briefly, the preparations were mounted longitudinally under 1 g tension in an organ bath (5 ml) containing Krebs at 37 °C and continuously gassed with 5% CO_2 and 95% O_2. Tissues were equilibrated for 60 min with washing every 10 min. For recording the maximal contractile response of the tissues 10 μm carbachol was used. The concentration-response curves to N/OFQ and AT-compounds were determined non-cumulatively by adding different concentrations of compound to the bath every 20 min followed by washing. Since its effects were resistant to washing, single concentrations of Ro 65-6570 were tested in each tissue.

2.7. Data analysis and terminology

The pharmacological terminology adopted in this paper is consistent with IUPHAR recommendations (Neubig et al., 2003). All data are expressed as means ± S.E.M of n experiments. For potency values 95% confidence limits are reported. In calcium mobilization experiments, maximum change in fluorescence, expressed as percent over the baseline fluorescence, was used to determine agonist response. For BRET experiments receptor-transducer interactions were calculated as BRET ratio between CPS measured for the RGFP and RLuc light emitted using 460(25) and 510(10) filters (PerkinElmer, Waltham, MA, USA), respectively. Data were expressed as stimulated BRET ratio obtained by subtracting the vehicle value to that measured in the presence of ligand. Agonist intrinsic activity was expressed as fraction of the N/OFQ E_{max}; the maximal effect of N/OFQ has been assayed in all the plates. For the electrically stimulated mouse vas deferens data the effect of agonists was expressed as % of inhibition of the control twitch, while for mouse colon data as percent of the contraction elicited by 10 μm carbachol. Non-linear regression analysis using GraphPad Prism software (5.0) allowed logistic iterative fitting of the resultant responses and the calculation of agonist potencies and maximal effects. Agonists potencies were given as pEC_{50} (the negative logarithm to base 10 of the molar concentration of an agonist that produces 50% of the maximal possible effect). Antagonist potencies were expressed as pK_B, calculated using the following equation: $pK_B=\log(CR-1)–\log[A]$, where CR is the ratio between the EC_{50} (nM) values of the agonist in the presence and absence of antagonist and [A] is the concentration (M) of antagonist used. Data have been statistically analyzed with the Student's t-test; P values less than 0.05 were considered statistically significant. Bias factors were calculated by choosing the standard NOP agonist N/OFQ, as standard unbiased ligand. The concentration response curves of each compound were fitted to the Black-Leff operational model described in Nagi and Pineyro (2016):

$$\text{response}=\frac{[A^n]\tau^n E_m}{[A]^n\tau^n+([A]+K_A)^n}$$

where [A] is the agonist concentration, the maximal response of the system is given by E_m, n is a fitting parameter for the slope, the affinity of the agonist is represented by the equilibrium dissociation constant of the agonist-receptor complex (K_A), and the efficacy of the agonist is defined by τ. τ and K_A are descriptive parameters of intrinsic efficacy and binding affinity and may be directly obtained by fitting experimental data to the operational equation and can be expressed as "transduction coefficients" $\log(\tau/K_A)$. The relative efficiency of agonists producing activation of any pathways can thus be quantified with a "normalized" transduction coefficient, namely $\Delta\log(\tau/K_A)$. Finally, the bias factors were calculated as difference between $\Delta\log(\tau/K_A)$ values for a given agonist between the pathways (G protein and β-arrestin 2):

$$\text{bias factors} = \Delta\log(\tau/K_A)_{\text{G Protein}} - \Delta\log(\tau/K_A)_{\beta-\text{arrestin2}}$$

bias factors are expressed as the mean ± S.E.M. of at least three independent experiments.

3. Results

3.1. Receptor binding assay

The new AT series of ligands showed high binding affinity for the NOP receptor, with K_is ranging from 3 to 10 nM at the recombinant human NOP receptor transfected into CHO cells (Table 1). These NOP ligands showed significantly lower binding affinities for the mu and kappa opioid receptors, showing NOP selectivity ranging from 17 (AT-035) to 61 fold (AT-127). All AT compounds showed poor if any affinity for the delta opioid receptor (data not shown).

3.2. [^{35}S]GTPγS binding assay

Agonist potency and efficacy were measured in the GTPγS binding assay using membranes from CHO cells expressing NOP or mu receptors. Maximal effects were normalized to that elicited by the full agonists N/OFQ and DAMGO for NOP and mu receptors, respectively. All the new AT compounds had intrinsic activities ranging from 0.21 to 0.61 of the response to N/OFQ, thus showing partial agonist activity at NOP. AT-001, AT-004, AT-035 and AT-090 showed partial agonist efficacy at the lower end of the spectrum (0.21–0.36), whereas AT-127 showed a higher level of efficacy, i.e. 61% of N/OFQ response (Table 1). As far as potency is concerned, the EC_{50} values of AT compounds were 5–40 fold higher than their K_i. The rank order of the potency was AT-127> AT-090=AT-001 > AT-035=AT-004. At the mu receptor all AT compounds displayed lower potency and efficacy with the only exception of AT-001 that produced higher maximal effects at mu than at NOP receptor (Table 1).

3.3. Calcium mobilization assay

In CHO cells coexpressing the NOP receptor and the $G\alpha_{qi5}$ chimeric protein, N/OFQ increased intracellular calcium levels in a concentration dependent manner with high potency and maximal effects. Ro 65-6570 mimicked N/OFQ action showing similar maximal effects but 10-fold lower potency (Fig. 2, panel A). AT compounds stimulated

calcium mobilization showing maximal effects slightly lower than that of N/OFQ (α range 0.73–0.82) and the following rank order of potency AT-127=AT-090 > AT-035 > AT-001 >AT-004 (Fig. 2, panel B). The effects of standard agonists and the most potent AT compounds was challenged with the NOP antagonist SB-612111 in order to investigate the involvement of the NOP receptor in their action. As shown in Fig. 3 panel A, 100 nM SB-612111 was able to shift the concentration response curve of N/OFQ to the right, without modifying the agonist maximal effect. A similar effect was obtained for agonist response curves for Ro 65-6570, AT-090, and AT-127 in presence of the antagonist (Fig. 3 panels B, C and D). SB-612111 pK_B values calculated from these experiments were in the range 8.49–8.72. The selectivity of the AT compounds over classical opioid receptors was assessed using as standard opioid receptor agonists dermorphin, DPDPE, and dynorphin A. The results of these experiments are summarized in Table 2. N/OFQ, dermorphin, DPDPE, and dynorphin A behaved as selective agonists for the NOP, mu, delta, and kappa receptor, respectively. Ro 65-6570 displayed relatively high (at least 100 fold) selectivity for the NOP receptor. AT compounds showed moderate (AT-004 and AT-035) to high (AT-090 and AT-127) NOP selectivity with the only exception of AT-001 that displayed similar potency at NOP and kappa receptors.

3.4. BRET assay

Membrane extracts taken from HEK293 cells stably coexpressing the NOP/RLuc and the Gβ1/RGFP fusoproteins were used to evaluate NOP/G-protein interaction. N/OFQ promoted NOP/G-protein interaction in a concentration-dependent manner with high potency (pEC_{50} 8.52 (8.46–8.58)) and maximal effect of 0.34 ± 0.02 stimulated BRET ratio. The intrinsic activities of the compounds under study were computed as fraction of N/OFQ maximal-stimulated BRET ratio (N/OFQ=1.00). Ro 65-6570 mimicked N/OFQ action showing similar maximal effects but 4-fold lower potency (pEC_{50} 7.90 (7.42–8.39); Fig. 4, panel A). For assessing whether compounds affect luciferase activity, AT compounds were assayed at 1 and 10 μm employing cell membranes obtained from HEK293 expressing NOP/RLuc and Gβ1/RGFP. AT-001, AT-004 and AT-035, at 10 μm but not at 1 μm, inhibited the light emitted by RLuc. On this basis the concentration response curves to these compounds were carried using 1 μm as maximal concentration. AT-090 and AT-127 stimulated NOP/G-protein interaction in a concentration-dependent manner showing lower potency and efficacy compared with N/OFQ. Potency values of 7.19 (7.12–7.26) and 6.81 (6.53–7.09) with maximal effects of 0.49 ±0.08 and 0.69 ± 0.09 were calculated for AT-090 and AT-127, respectively. AT-001, AT-004 and AT-035 were only poorly active in promoting NOP/G-protein interaction, generating incomplete concentration response curves (Fig. 4, panel B).

Whole HEK293 cells stably expressing the NOP/RLuc and the β-arrestin 2/RGFP fusoproteins were used to evaluate the NOP/arrestin interactions. N/OFQ stimulated the interaction of the NOP receptor with β-arrestin 2 in a concentration dependent manner with high potency (pEC_{50} 8.00 (7.91–8.09)) and maximal effects corresponding to 0.11 ± 0.01 stimulated BRET ratio. Ro 65-6570 mimicked N/OFQ action displaying similar maximal effect but 50-fold lower potency (Fig. 4, panel C). AT-090 and AT-127 mimicked the stimulatory effect of N/OFQ showing lower potency and efficacy (pEC_{50} 6.96 (6.11–7.81) and maximal effects of 0.57 ± 0.08 for AT-090 and pEC_{50} 6.38 (5.81– 6.95) and maximal

effects of 0.60±0.13 for AT-127). The other compounds were nearly inactive in stimulating NOP/β-arrestin 2 interaction (Fig. 4, panel D).

Fig. 5 illustrates the comparison of the concentration response curves of standard agonists and the two best AT compounds on NOP/G protein and NOP/arrestin interaction. N/OFQ displayed slight lower potency at arrestin compared to G protein. Similar results were obtained with AT-127. Ro 65-6570 was approximately 40 fold more potent in the G protein assay. Of note AT-090 displayed the same potency in the two assays. To obtain a quantitative measure of biased agonism, the bias factor values were calculated for Ro 65-6570 and the two active AT compounds using N/OFQ as reference agonist. Ro 65-6570 displayed a 10 fold biased behavior towards G protein (1.00 ± 0.20). AT-090 and AT-127 showed bias factor values of −0.78 ± 0.27 and 0.27 ± 0.25, respectively.

3.5. Mouse vas deferens

N/OFQ inhibited the electrically induced contractions of mouse vas deferens tissue in a concentration-dependent manner with a potency value of 7.27 (7.21–7.33) and maximal effect of 81 ± 1% of the control twitch. Ro 65-6570 mimicked the inhibitory effect of N/OFQ but showing lower potency (pEC_{50} 6.53 (6.34–6.72)) and higher maximal effects (Fig. 6, panel A). In this preparation AT-004 and AT-035 were inactive. AT-001 at micromolar concentrations slightly increased the twitch response to electrical stimulation. These compounds were then tested as antagonists at 1 μm against N/OFQ. The peptide elicited superimposable concentration response curve in the presence and in the absence of these molecules (data not shown). On the contrary, AT-090 and AT-127 mimicked the inhibitory effect of N/OFQ but with lower potency (pEC_{50} 6.77 (6.55–6.99) and 6.50 (6.26–6.74), respectively) and higher maximal effects ($\alpha > 1.0$) (Fig. 6, panel B). Of note, the kinetics of action of NOP agonists were very different (Fig. 7). The action of N/OFQ was very rapid and immediately reversible after washing, on the contrary the effects elicited by Ro 65-6570 were very slow to develop and virtually resistant to wash. AT-090 and AT-127 displayed intermediate kinetics of action.

To investigate the receptor(s) involved in the action of AT-090 and AT-127, both antagonism and knockout studies were performed. SB-612111 0.1 μm shifted to right the concentration response curve to N/OFQ without changing its maximal effects. A pK_B of 8.69 (7.85–9.53) was derived from this experiment (Fig. 8, panel A). At the same concentration, the antagonist was not able to shift to right the concentration response curve to Ro 65-6570 (Fig. 8, panel B). SB-612111 0.1 μm shifted to right the concentration response curve to AT-090 and AT-127 but, compared to N/OFQ, with lower potency (pK_B of 7.41 (6.96–7.86) for AT-090 and of 7.95 (7.12–8.78) for AT-127, Fig. 8, panels C and D). N/OFQ inhibited the electrically induced contractions of tissues taken from NOP(+/+) mice (pEC_{50} 7.48 (7.32–7.64), E_{max} =87 ±3%) but not from NOP(−/−) mice (Fig. 9, panel A). The delta opioid receptor agonist DPDPE elicited similar inhibitory effects in tissues taken from NOP(+/+) and NOP(−/−) mice (data not shown). Ro 65-6570 mimicked the inhibitory effects of N/OFQ in both tissues, with a potency value 2-fold lower in NOP(−/−) than NOP(+/+) mice (Fig. 9, panel B). AT-090 and AT-127 inhibited the electrically induced contractions of the

mouse vas deferens both in NOP(+/+) and NOP(−/−) mice, being approximately 10 fold less potent in NOP(−/−) tissues (Fig. 9, panel C and D).

3.6. Mouse colon

N/OFQ induced a concentration-dependent contraction of the mouse colon with potency of 8.51 (8.43–8.58) and an E_{max}=45 ± 5% of the contraction induced by 10 μm carbachol (Fig. 10, panel A). The contractile effect elicited by Ro 65-6570 could not be repeated in the same tissue and therefore its concentration response curve was generated using different tissues and only three concentrations i.e. 10, 100, and 1000 nM. As shown in (Fig. 10, panel A) Ro 65-6570 elicited maximal effect similar to N/OFQ being however 30-fold less potent. AT-090 and AT-127 induced concentration dependent contractions of the mouse colon tissues with potency and efficacy similar to N/OFQ. A potency value of 8.49 (8.42–8.54) and E_{max} =38± 4% was calculated for AT-090 and a potency value of 8.23 (7.85–8.61) and E_{max} =42±6% was obtained for AT-127. In the same preparation AT-001 and AT-035 were only poorly active, while AT-004 was inactive up to 10 μm (Fig. 10, panel B). Of note, the kinetic of action of these NOP ligands were different. As shown in Fig. 11, all compounds showed a rapid contractile effect, but after washing the tissues N/OFQ-induced contraction rapidly disappeared, while the contraction evoked by Ro 65-6570 and the two AT compounds were less sensitive to washing.

To test the selectivity of action of AT-090 and AT-127 some experiments with tissues extracted from NOP(+/+) and NOP(−/−) mice were performed. N/OFQ 0.1 μm induced a contractile response in mouse colon from NOP(+/+) mice but not in mC from NOP(−/−) mice. The mu opioid agonist endomorphin-1 1 μm contracted both tissues. The effects elicited by Ro 65-6570 1 μm was similar in tissues from NOP(+/+) and NOP(−/−) mice. Similarly to N/OFQ, both AT-090 0.1 μm and AT-127 0.1 μm were able to induce mouse colon contraction in NOP(+/+) but not in NOP(−/−) mice (Fig. 12).

4. Discussion

In the present study, detailed in vitro pharmacological characterization of novel NOP partial agonists ligands has been presented. The novel compounds were evaluated at human recombinant receptors in receptor binding, stimulated GTPγS binding, calcium mobilization, and BRET assays. Moreover the novel compounds were tested in isolated mouse tissues to assess their pharmacological actions at native receptors. Results demonstrated that AT-090 and AT-127 behave as potent NOP receptor partial agonists in all functional assays. Moreover these compounds displayed a level of NOP selectivity higher than the standard nonpeptide NOP agonist Ro 65-6570. Thus, AT-090 and AT-127 might be useful pharmacological tools for investigating the therapeutic potential of NOP partial agonists.

Receptor bindings studies demonstrated that AT compounds behave as high affinity NOP ligands showing a moderate selectivity for NOP over classical opioid receptors. GTPγS binding studies demonstrated that AT compounds act as NOP partial agonists with AT-127 showing the higher potency and efficacy. These results were substantially confirmed in calcium mobilization studies performed on cells expressing human NOP or classical opioid

receptors and chimeric G proteins. This assay has been previously validated for NOP (Camarda et al., 2009) and later for classical opioid receptors (Camarda and Calo, 2013) and then used for investigating several NOP ligands (Toll et al., 2016). N/OFQ and Ro 65-6570 behaved as full agonists with the nonpeptide ligand being, in line with previous findings (Hashiba et al., 2001), 10-fold less potent. The stimulatory effects of these control agonists were mimicked by the AT compounds that showed an order of potency, i.e. AT-127≥AT-090≥AT-035>AT-001≥AT-004 in line with receptor affinity and GTPγS functional potency. All AT compounds displayed reduced efficacy (α 0.73–0.82) although these differences did not reach statistical significance; it is worthy of mention that the calcium mobilization assay tends to overestimate ligand efficacy as previously demonstrated with other NOP partial agonists (Camarda et al., 2009). This is probably due to signal amplification phenomena that make high the efficiency of the stimulus / response coupling in this assay. SB-612111 displayed similar pA_2 values versus standard agonists as well as versus AT compounds (range 8.49–8.72) and surmountable antagonist behavior, in line with its described competitive nature (Spagnolo et al., 2007; Zaratin et al., 2004). These results strongly suggest that AT compounds bind the same NOP site recognized by N/OFQ and SB-612111 and recently described at atomic level (Miller et al., 2015).

As far as selectivity over opioid receptors is concerned, N/OFQ displayed extraordinary high selectivity while Ro 65-6570 showed approximately 100-fold selectivity for NOP. AT-090 and AT-127 displayed similar selectivity as Ro 65-6570 whereas the other AT compounds showed reduced selectivity with AT-001 displaying similar potency at NOP and kappa receptors.

AT compounds were also assessed in a BRET based assay that measures receptor interaction with G protein and β-arrestin 2. This assay was first set up and validated for opioid receptors (Molinari et al., 2010) and recently extended to the investigation of NOP (Malfacini et al., 2015). In the NOP/G protein interaction, Ro 65-6570 behaved as a full agonist showing slightly lower potency compared to the natural peptide. AT-090 and AT-127 mimicked the stimulatory effects of standard agonists but with lower potency and efficacy while the other AT compounds displayed very low potency and their concentration response curves could not be completed. The rank order of potency in this assay is similar to what observed in the other assays. Of note, AT-090 and AT-127 clearly behaved as partial agonists confirming the GTPγS binding results. In fact a high determination coefficient has been previously calculated between the two assays using a large panel of NOP ligands (Malfacini et al., 2015).

In the NOP/β-arrestin 2 assay, Ro 65-6570 behaved as a full agonist showing, however, a large loss of potency compared to the natural peptide. AT-090 and AT-127 mimicked the stimulatory effects of standard agonists showing relatively high potency but lower efficacy. The other AT compounds were virtually inactive. Comparing the ligand efficacy at G protein and β-arrestin 2 suggests that Ro 65-6570 behaved as a G protein-biased agonist. This seems to be the rule for NOP (Malfacini et al., 2015; Chang et al., 2015b, 2015a) and opioid (Molinari et al., 2010) nonpeptide agonists. AT-090 and AT-127 seem to be the exception to this rule. In fact these compounds displayed similar ability to promote NOP/G protein and NOP/arrestin interactions, behaving as the unbiased standard agonist N/OFQ. Further

investigations are needed to identify NOP receptor agonists biased toward β-arrestin. The availability of unbiased, G protein and β-arrestin biased agonists would be of paramount value for investigating functional selectivity in vivo and assessing the therapeutic potential of biased agonists in the NOP receptor field.

In the mouse vas deferens, N/OFQ showed values of potency and maximal effects in line with previous studies (Calo et al., 1996; Berzetei-Gurske et al., 1996). Ro 65-6570, AT-090 and AT-127 mimicked the action of the peptide, showing lower potency but higher maximal effects thus suggesting possible off-target effects. This was confirmed in receptor antagonist and knockout studies. In fact, SB-612111 antagonized N/OFQ action with a pA_2 value in line with previous results (Spagnolo et al., 2007) while it showed lower pA_2 values against AT compounds and was almost inactive against Ro 65-6570. These results demonstrate that the biological action of N/OFQ in this preparation is solely due to NOP receptor activation while that of Ro 65-6570 mainly derives from the interaction with an unknown inhibitory site. The same can be said for AT-090 and AT-127 however the amount of effect deriving from NOP receptor activation by these molecules is larger than that of Ro 65-6570. This interpretation is corroborated by findings in tissues from knockout animals. In fact, effect of N/OFQ is completely lost whereas Ro 65-6570 displayed a only slightly reduced potency in NOP(−/−) tissues. AT-090 and AT-127 were almost 10-fold less potent in NOP(−/−) than NOP(+/+) tissues. Collectively, receptor antagonists and knockout studies demonstrate that in the mouse vas deferens the effects of AT compounds and particularly of Ro 65-6570 result from their interaction with the NOP receptor and possibly with an additional inhibitory site. Similar results were obtained previously when investigating the effects of a related NOP agonist Ro 64-6198 in the mouse vas deferens. Notably, the inhibitory effects elicited by Ro 64-6198 were resistant to NOP antagonists ([$Nphe^1$]N/OFQ(1–13)NH_2 and J-113397). Interestingly the concentration response curve for Ro 64-6198 was not affected even in the presence of naloxone or of a cocktail of naloxone and NOP antagonist (Rizzi et al., 2001), thus suggesting that classical opioid receptors are likely not involved in the action of this ligand. These findings, however, do not underscore the usefulness of these tools for investigating in vivo the biological actions controlled by the N/OFQ-NOP system. In fact several in vivo actions of Ro 64-6198 and Ro 65-6570 were sensitive to NOP antagonists and no longer evident in NOP(−/−) animals (reviewed in Toll et al. (2016)). In addition we recently demonstrated that the anxiolytic-like effect of AT-090 in the mouse elevated plus maze can be detected in NOP(+/+) but not NOP(−/−) mice (Asth et al., 2016).

In line with previous findings in the mouse colon (Rizzi et al., 1999), N/OFQ produced concentration-dependent contractions with high potency. Ro 65-6570 mimicked the N/OFQ effect being 30-fold less potent while AT-090 and AT-127 displayed similar maximal effects and potency as N/OFQ. Interestingly AT compounds behaved as full agonists in this preparation. This is not unexpected since in this preparation, similar to the calcium mobilization assay, the efficiency of the stimulus / response coupling is high as suggested by the full agonist behavior of known NOP partial agonists (Rizzi et al., 1999). To investigate the receptor involved in the action of AT compounds in this preparation, knockout studies were performed. In line with previous studies (Di Giannuario et al., 2001) the action of N/OFQ was no longer evident in NOP(−/−) tissues while that elicited by the mu-selective agonist endomorphin-1 was unaffected or even increased. The effect of 1 μm Ro 65-6570

was only slightly and nonsignificantly reduced in NOP(−/−) tissue while that elicited by 0.1 μm of AT compounds was virtually abolished. Thus these results together with those obtained in the mouse vas deferens suggest that the NOP selectivity of the AT compounds is greater than that of Ro 65-6570.

Collectively the analysis of present results suggests that AT compounds behave as NOP partial agonists with the following rank order of potency: AT-090≥AT-127 >AT-035≥AT-004=AT-001. AT-090 and AT-127 displayed high NOP potency similar to that of the standard agonist Ro 65-6570. The rank order of the potency of the functional responses among the various assays appears to be similar. The maximal effects elicited by these partial agonists varies depending on the efficiency of the stimulus/response coupling of the preparation used. AT-090 and AT-127 displayed similar selectivity over classical opioid receptors as Ro 65-6570 in recombinant receptor studies; however at native mouse receptors they displayed higher NOP selectivity than Ro 65-6570. Finally, the NOP partial agonists AT-090 and AT-127 are particularly interesting due to their ability stimulate NOP interaction with both G protein and arrestin with the same potency and efficacy. This is different from other nonpeptide NOP agonists previously examined, which in general tend to show at least some degree of bias toward G protein.

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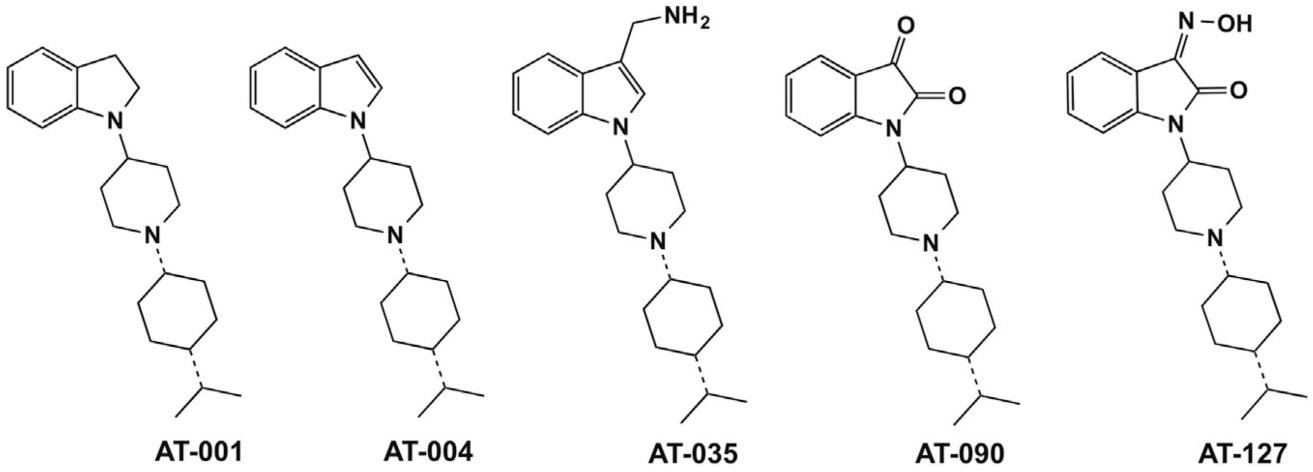

Fig. 1.
Chemical structures of AT-001, AT-004, AT-035, AT-090 and AT-127.

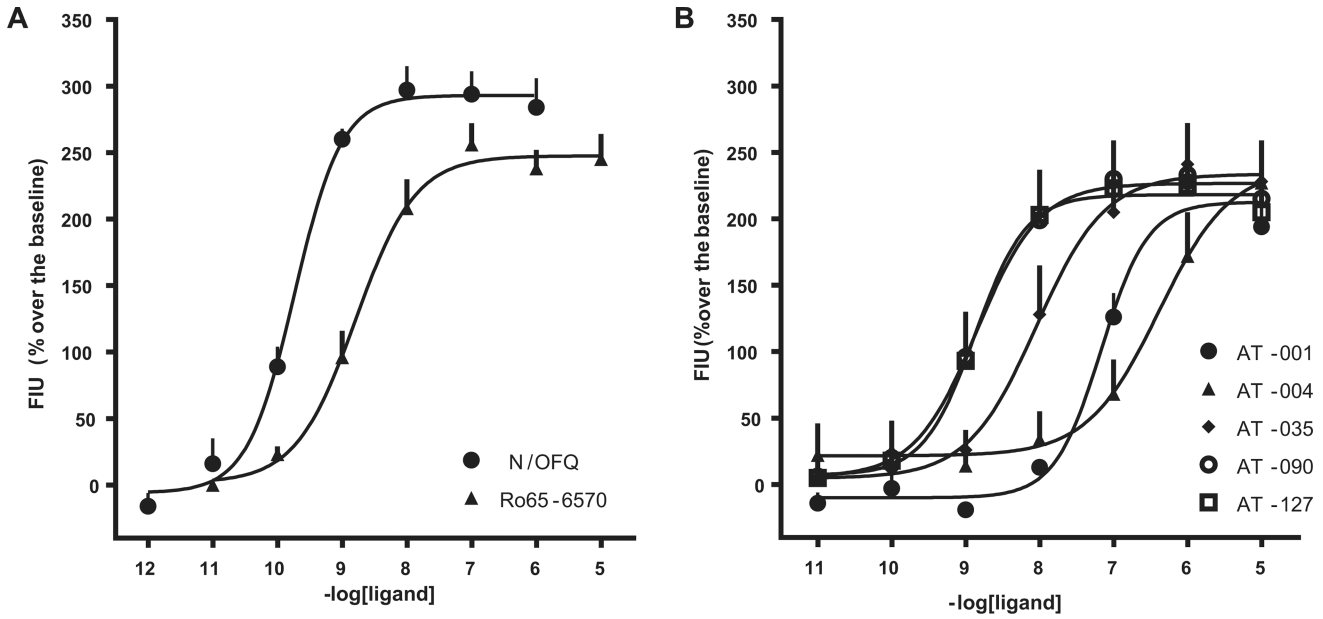

Fig. 2.

Calcium mobilization assay performed in CHO cells expressing the NOP receptor and the chimeric Ga_qi_5 protein. Concentration response curve to N/OFQ and Ro 65-6570 (panel A) and to AT compounds (panel B). Data are expressed as mean ± S.E.M. of four separate experiments made in duplicate.

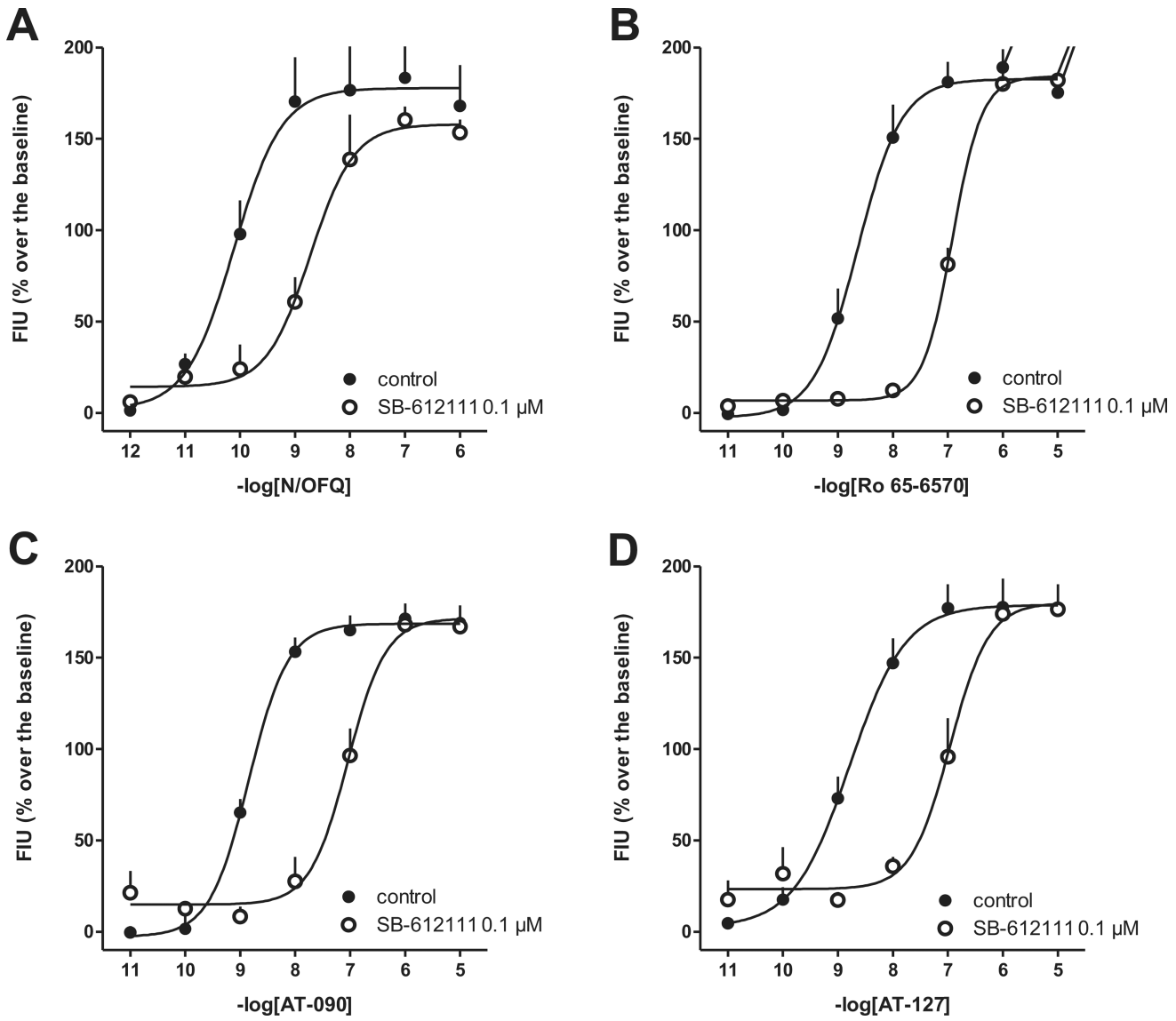

Fig. 3.

Calcium mobilization assay performed in CHO cells expressing the NOP receptor and the chimeric $G\alpha_{qi5}$ protein. Concentration response curve to N/OFQ (panel A), Ro 65-6570 (panel B), AT-090 (panel C) and AT-127 (panel D) in absence and in presence of SB-612111 0.1 μm. The following values of pK_B were calculated for SB-612111: 8.49 (7.80–9.18) vs N/OFQ, 8.72 (8.20–9.24) vs Ro 65-6570, 8.65 (8.22–9.08) vs AT-090, 8.59 (7.53–9.65) vs AT-127. Data are expressed as mean ± S.E.M. of four separate experiments made in duplicate.

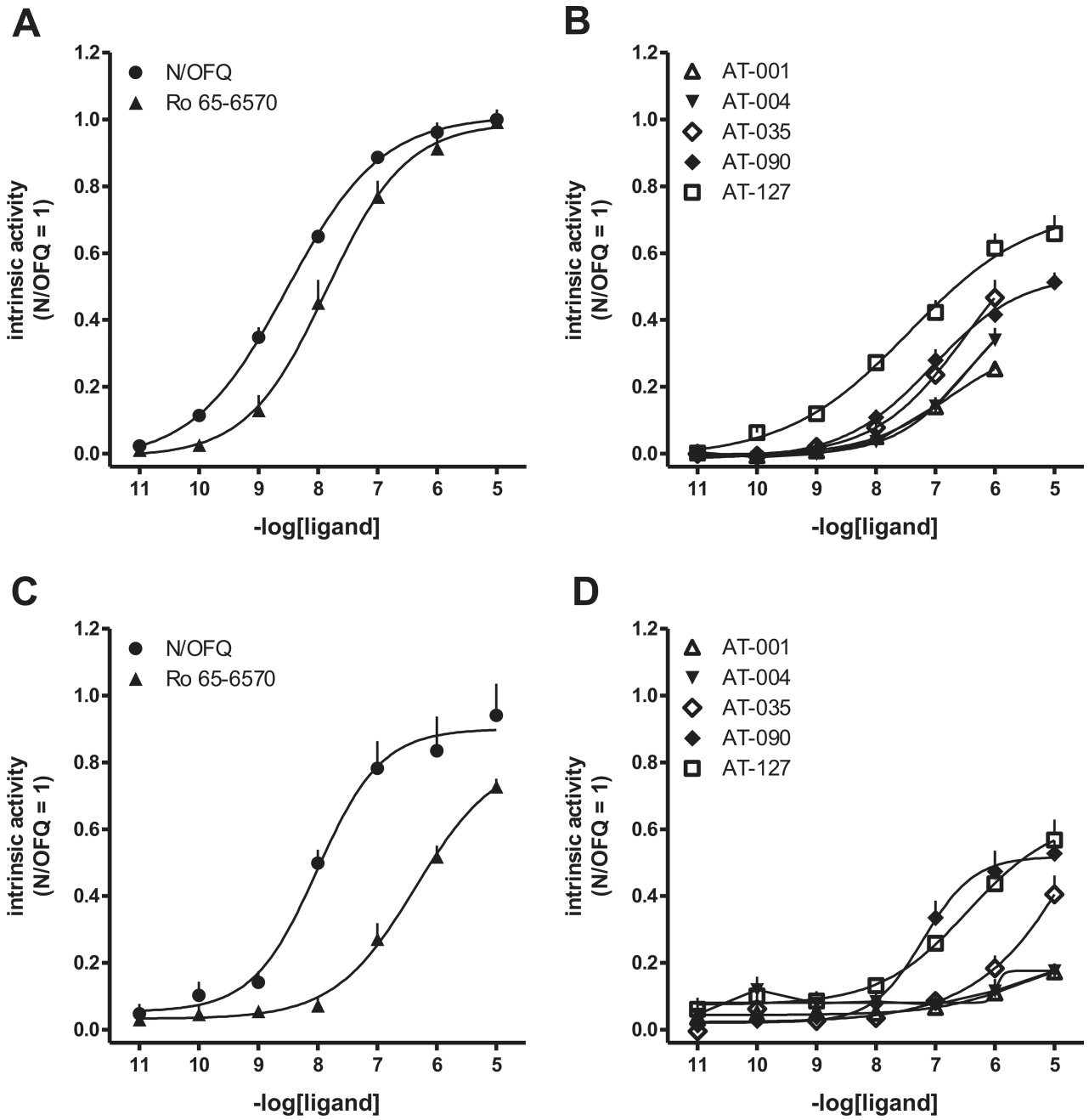

Fig. 4.

BRET assay. Concentration response curve to N/OFQ and Ro 65-6570 (panel A) and to AT compounds (panel B) in NOP/G protein interaction. Concentration response curve to N/OFQ and Ro 65-6570 (panel C) and to AT compounds (panel D) in NOP/β-arrestin 2 interaction. Data are the mean ± S.E.M. of at least three experiments.

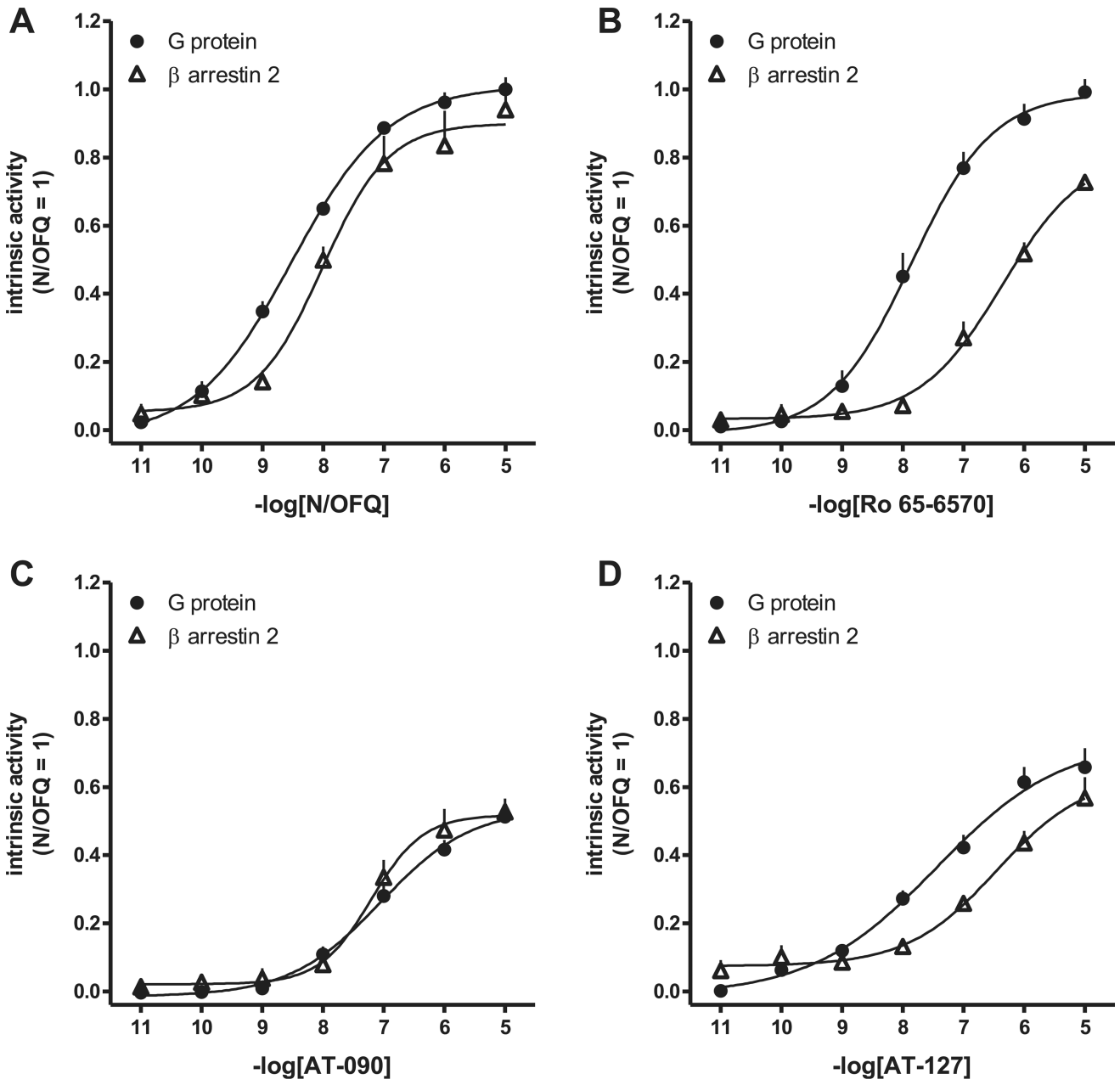

Fig. 5.

NOP/G protein and NOP/β-arrestin 2 interactions. Concentration response curves to N/OFQ (panel A), Ro 65-6570 (panel B), AT-090 (panel C), and AT-127 (panel D). Data are the mean ± S.E.M. of at least three experiments.

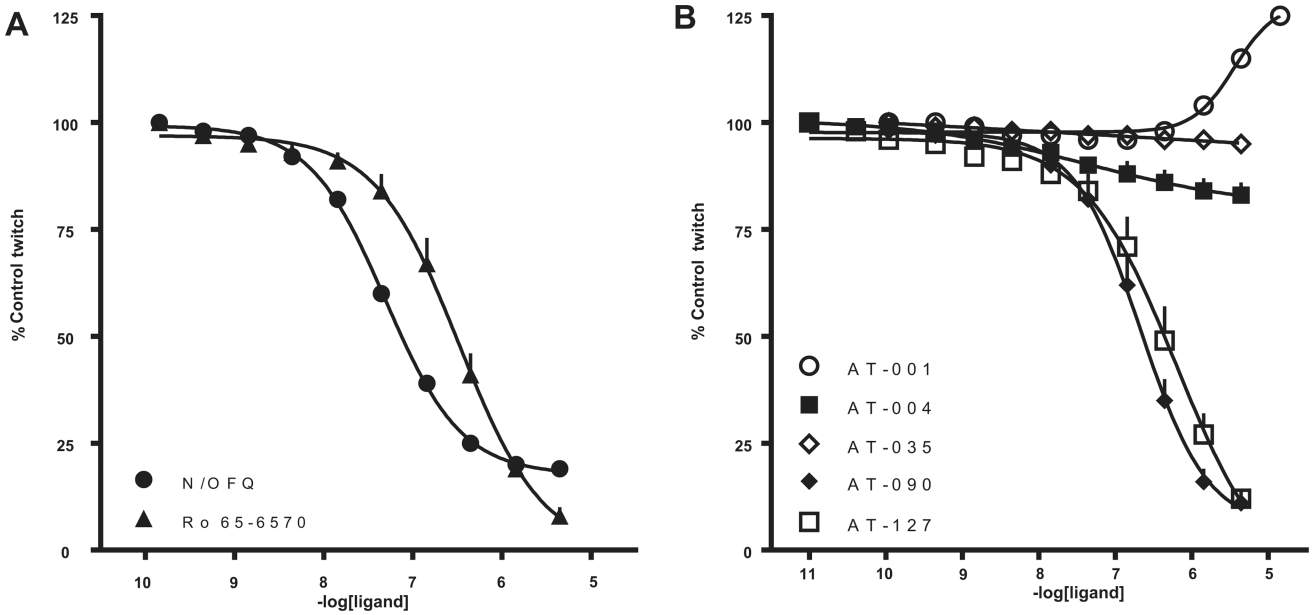

Fig. 6.

Mouse vas deferens bioassay. Concentration response curves to N/OFQ and Ro 65-6570 (panel A) and to AT compounds (panel B). Data are the mean ± S.E.M. of at least three experiments.

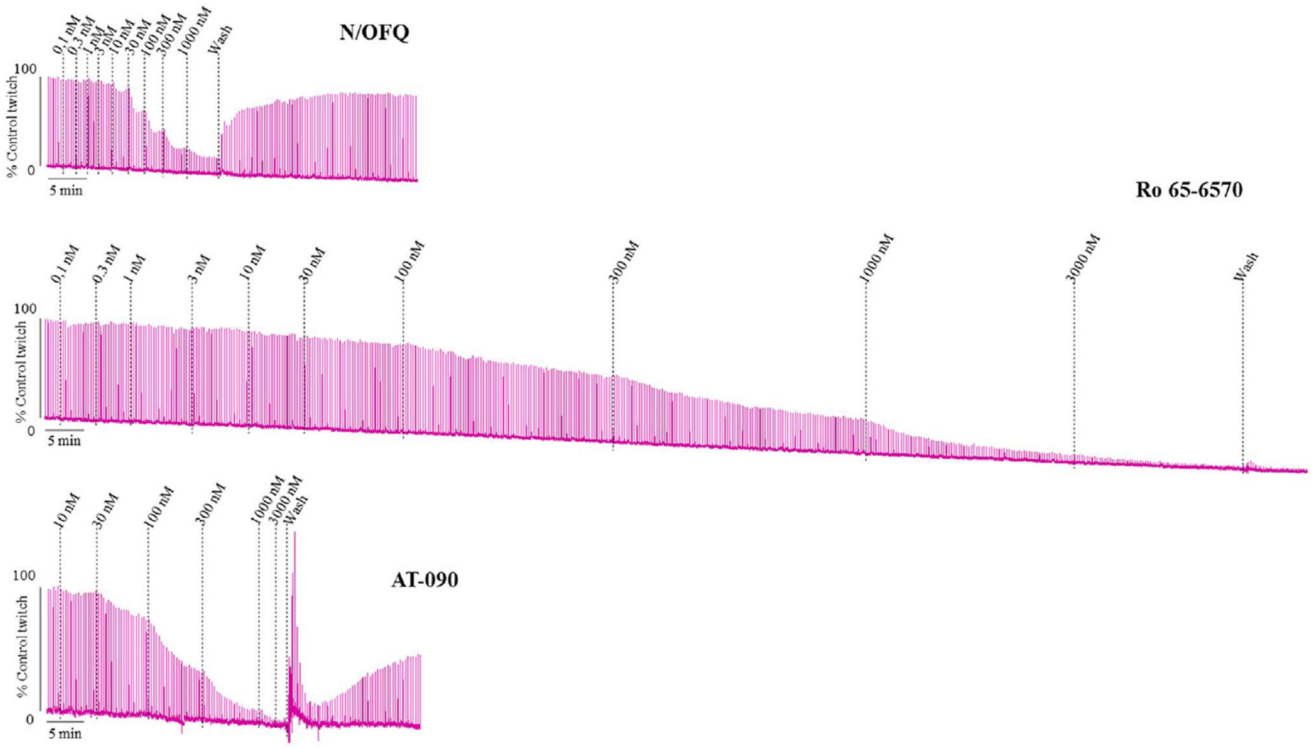

Fig. 7.

Representative tracings of tissue contraction in response to N/OFQ, Ro 65-6570 and AT-090 in the electrically mouse vas deferens.

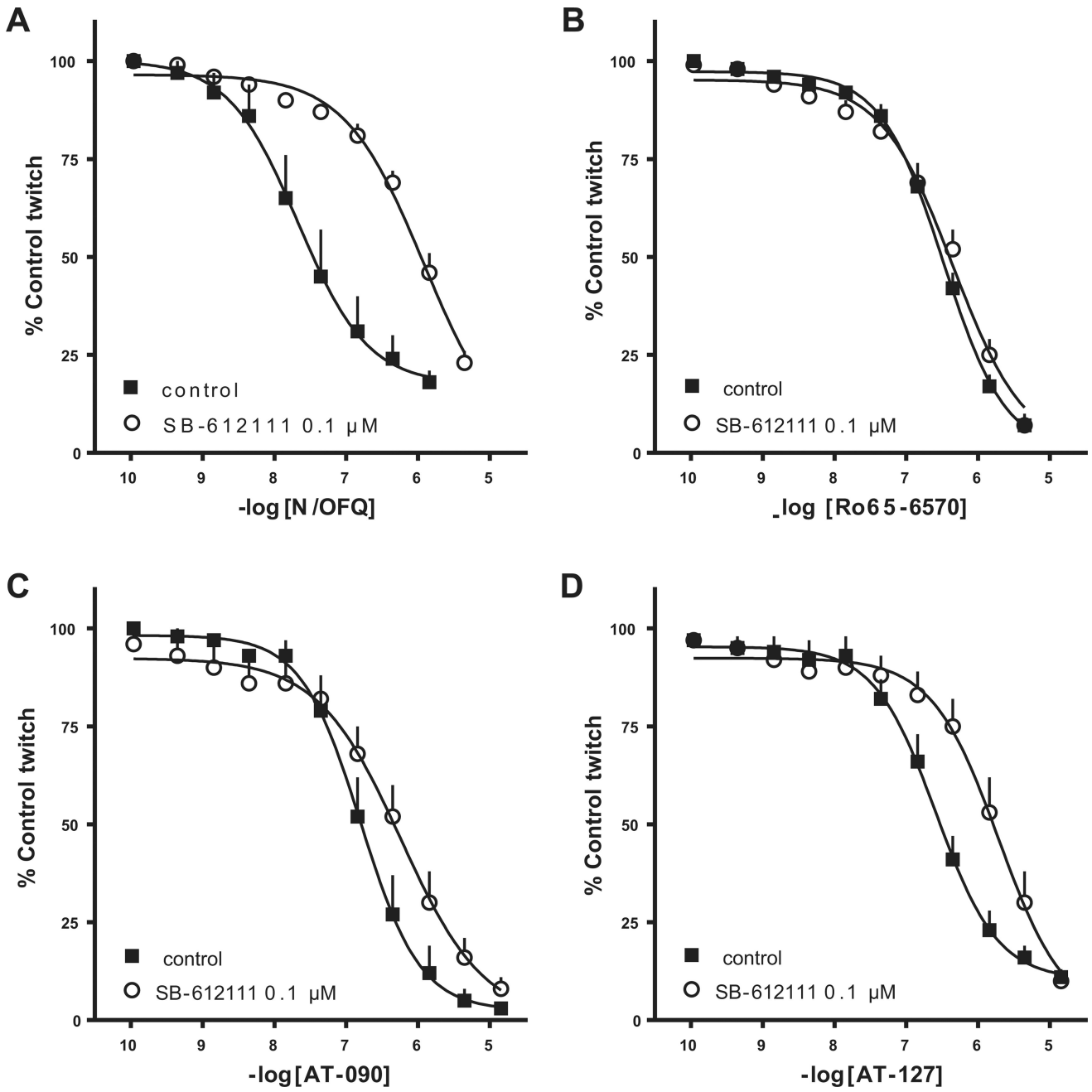

Fig. 8.

Mouse vas deferens bioassay. Concentration response curve to N/OFQ (panel A), Ro 65-6570 (panel B), AT-090 (panel C) and AT-127 (panel D) in absence and in presence of SB-612111 (0.1 µm). Data are the mean ± S.E.M. of at least three experiments.

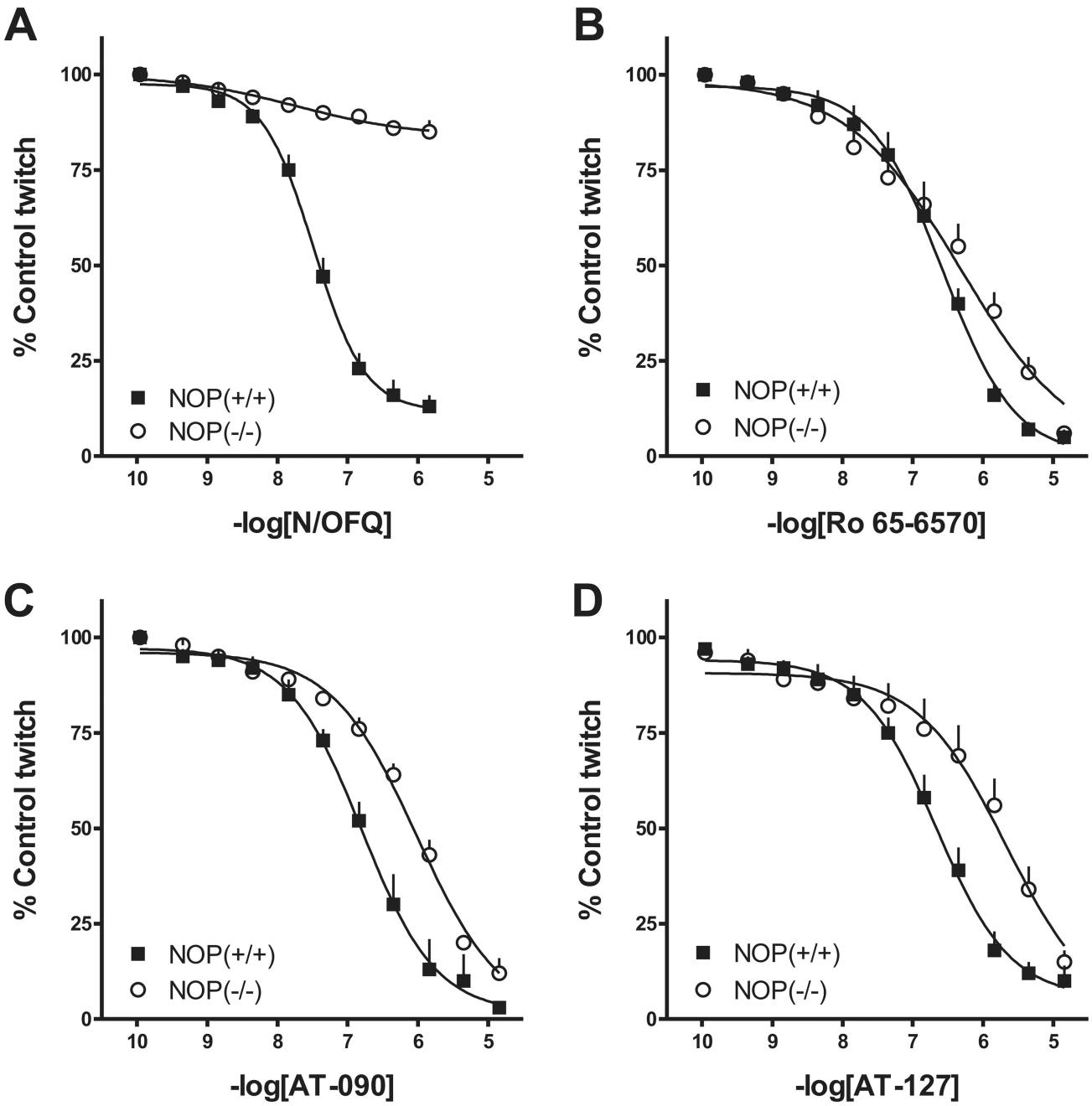

Fig. 9.

Mouse vas deferens bioassay. Concentration response curve to N/OFQ (panel A), Ro 65-6570 (panel B), AT-090 (panel C) and AT-127 (panel D) in NOP(+/+) and NOP(−/−) tissues. Data are the mean ± S.E.M. of at least five experiments.

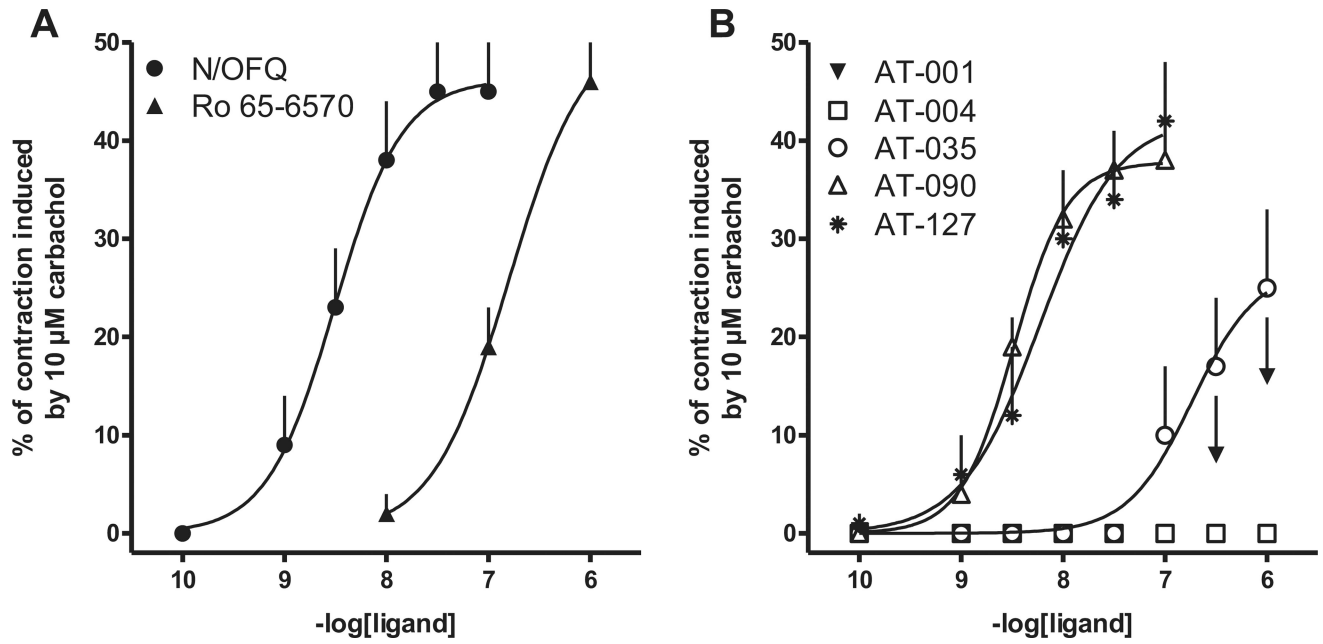

Fig. 10.

Mouse colon bioassay. Concentration response curve to N/OFQ and Ro 65-6570 (panel A) and to AT compounds (panel B). Data are the mean ± S.E.M. of at least three experiments.

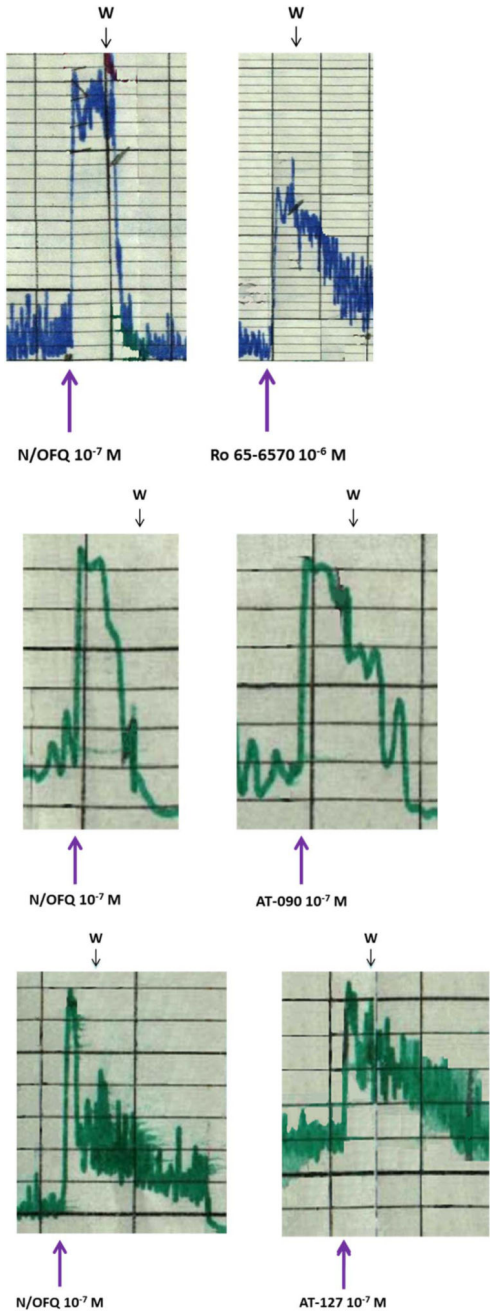

Fig. 11.
Typical tracings of N/OFQ, Ro 65-6570, AT-090 and AT-127 in the mouse colon bioassay.

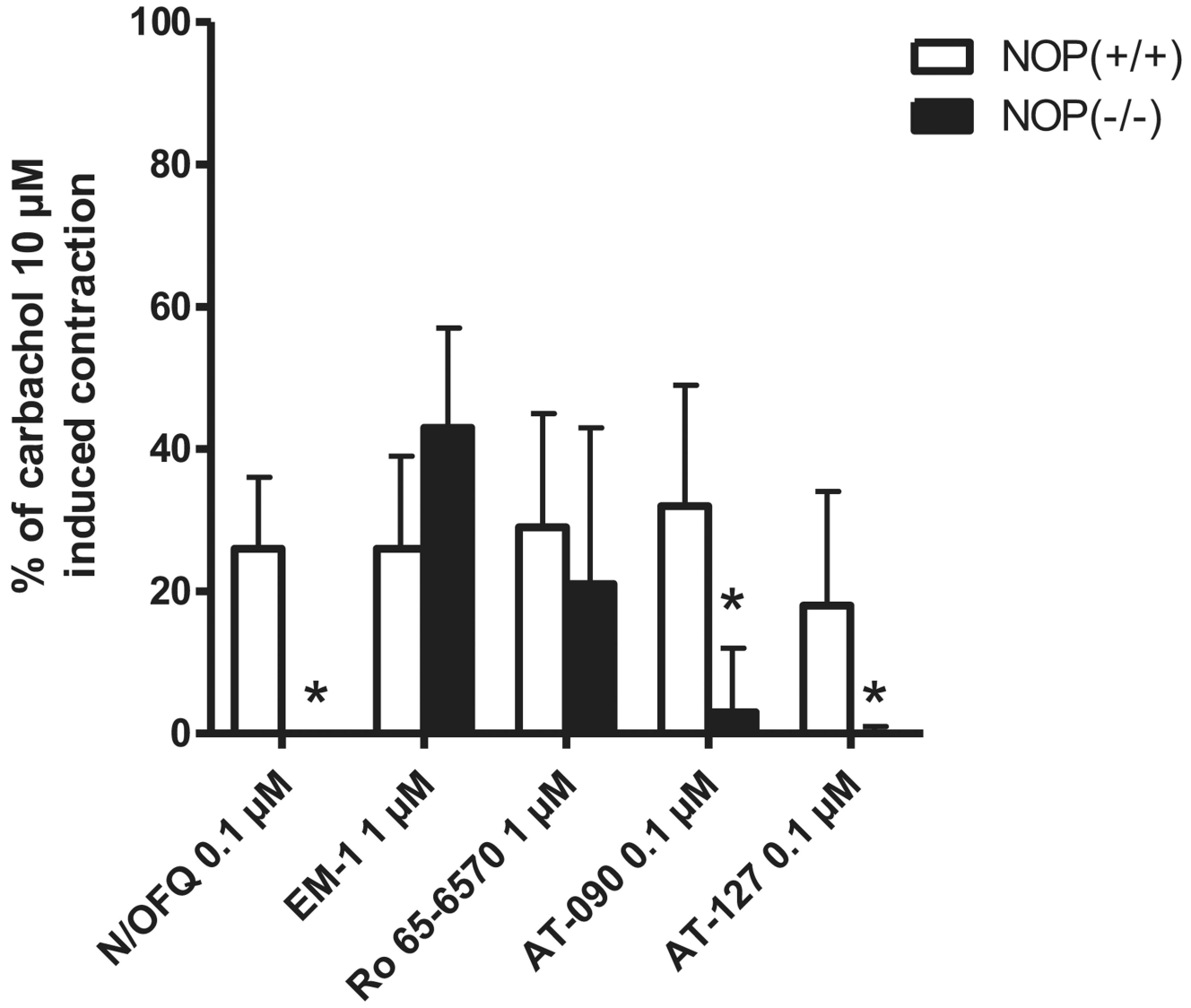

Fig. 12.

Mouse colon bioassay. Effects of N/OFQ, EM-1, Ro 65-6570, AT-090 and AT-127 in NOP(+/+) and NOP(−/−) tissues. Data are the mean±S.E.M. of six separate experiments. *P < 0.05 vs NOP(+/+) Student *t*-test.

Table 1

Receptor binding and stimulated [^{35}S]GTPγS binding of AT compounds in membranes of CHO cells stably expressing the human recombinant receptors.

	Receptor Binding K_i (nM)			**[^{35}S]GTPγS NOP**		**[^{35}S]GTPγS mu**	
	NOP	**Mu**	**kappa**	**EC_{50} (nM)**	**α**	**EC_{50} (nM)**	**α**
AT-001	10.3 ± 0.03	604.35 ± 5.02	562 ±150	54.5 ± 17.55	0.33 ± 0.03	1781± 337	0.48 ± 0.10
AT-004	9.80 ± 0.86	375.5 ± 36.5	1593.6 ± 57	188.2 ± 32.8	0.25 ± 0.09	231.7± 10	0.16 ± 0.08
AT-035	3.27 ± 0.3	65.29 ± 2.42	1736.8 ± 172	121.2 ± 51.7	0.36 ± 0.06	410 ±105	0.11 ± 0.03
AT-090	5.61 ± 1.72	95.35 ± 3.46	232.9 ± 18.1	50.1 ± 6.4	0.21 ± 0.06	FLAT	0.06 ± 0.03
AT-127	1.18 ± 0.2	71.65 ± 27.8	149.2 ± 18.3	15.5 ± 3.1	0.61 ± 0.02	59.2 ± 3.0	0.37 ± 0.02

K_i values for standard NOP (N/OFQ), mu (DAMGO), and kappa (U69593) ligands were 0.12, 2.96, and 1.05, respectively.

EC_{50} values of standard agonists N/OFQ at NOP and DAMGO at mu receptor were 3.6 and 32.6, respectively.

Table 2

Calcium mobilization assay performed in CHO cells expressing NOP or classical opioid receptor and chimeric G proteins. Potencies and efficacy of standard agonists and AT compounds.

	NOP		mu		Kappa		Delta	
	pEC_{50} ($CL_{95\%}$)	**α ± S.E.M.**	**pEC_{50} ($CL_{95\%}$)**	**α ± S.E.M.**	**pEC_{50} ($CL_{95\%}$)**	**α ± S.E.M.**	**pEC_{50} ($CL_{95\%}$)**	**α ± S.E.M.**
N/OFQ	9.77 (9.52–10.02)	1.00	Inactive		Inactive		Inactive	
Ro 65-6570	8.75 (8.15–9.40)	0.95 ± 0.05	<6		Crc incomplete		Inactive	
Dermorphin	Inactive		8.31 (7.90–8.72)	1.00	Inactive		5.98 (5.76–6.10)	0.8 ± 0.07
DPDPE	Inactive		Inactive		Inactive		8.23 (7.72–8.74)	1.00
Dynorphin A	Inactive		6.49 (5.96–7.02)	0.73 ±0.11	9.26 (8.69–9.83)	1.00	6.40 (5.87–6.93)	0.75 ± 0.13
AT-001	7.13 (6.79–7.47)	0.73 ± 0.03	Inactive		7.12 (6.42–7.84)	0.95 ± 0.01	Inactive	
AT-004	6.41 (5.87–6.95)	0.82 ± 0.07	Inactive		Inactive		Inactive	
AT-035	7.96 (7.36–8.56)	0.82 ± 0.08	Crc incomplete		Inactive		Inactive	
AT-090	8.84 (7.99–9.69)	0.80 ± 0.11	Crc incomplete		Crc incomplete		Inactive	
AT-127	8.85 (8.23–9.47)	0.79 ± 0.12	Crc incomplete		Inactive		Crc incomplete	

Inactive: inactive up to 1 μm; crc incomplete: weak stimulatory effect at 1–10 μM. Data are the mean of three separate experiments performed in duplicate.