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β-Arrestin2 Recruitment and Biased Signaling at the NOP Receptor: Pharmacological Evaluation of a Novel Non-Peptidic Partial Agonist

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To my mother, my family, my friends, and my lab colleagues, whose unwavering support, patience, and encouragement made this journey possible, and to everyone who has been part of my life and helped shape who I am today.

I also dedicate this work to all the women in science—those who came before me, those who inspire me today, and those who will follow in the future. To the women who were not given the opportunity to study but still contributed knowledge, discoveries, and innovations that continue to shape our world every day. To the scientists whose dedication and curiosity inspire me to pursue my own path in science, and to the future generations of women who will expand the boundaries of knowledge and change the world in ways we cannot yet imagine.

May we continue to make space for one another in science, today and in the years to come, and may the odds be ever in our favor.

Annem, bu tezi en çok sana ithaf ediyorum. Başka bir alanda eğitim almış olsan da, bu tezi bilmediğin bir dilde yazıyor olsam da, tez yazma sürecimde beni en iyi anlayan ve en çok destekleyen kişi sen oldun. Her gün sabırla şikâyetlerimi dinlediğin, bana hep inandığın ve her zaman arkamda durduğun için sana kelimelerle anlatılamayacak kadar minnettarım. Bana verdiğin sevgi ve destek, bugün olduğum insana ve bulunduğum yere ulaşmamı sağladı. Hayatımda sahip olduğum en büyük şans ve en değerli hediyesin.

Her şey için teşekkür ederim.

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1. Abstract:

The nociceptin/orphanin FQ peptide (NOP) receptor, the fourth member of the opioid receptor family, regulates physiological processes such as pain modulation, emotional behavior, substance abuse, and vascular function. The receptor's ability to activate specific intracellular pathways through selective coupling to G proteins (guanine nucleotide-binding proteins) or β-arrestins underlies biased agonism in opioid pharmacology. This study aimed to pharmacologically characterize a novel, non-peptidic, selective NOP receptor partial agonist, with a focus on β-arrestin2 recruitment. Our experiments involving HEK293 (human embryonic kidney 293) and CHO (Chinese hamster ovary) cell lines permanently expressing the NOP receptor and β-arrestin2 constructs demonstrated ligand-dependent variations in efficacy and kinetics of recruitment for five different ligands. Bioluminescence resonance energy transfer (BRET) and calcium mobilization assays identified functionally selective signaling patterns, confirming distinct differences in β-arrestin2 interaction and receptor signaling profiles among the ligands. These findings provide evidence for biased agonism at the NOP receptor. Overall, this study enhances the understanding of NOP receptor pharmacology and supports the development of novel NOP-targeted compounds with improved therapeutic potential. Such advances could lead to more effective treatments with fewer side effects for conditions like pain and anxiety, highlighting a promising direction for future therapeutic interventions.

Keywords:

NOP receptor

β-arrestin2

G protein

opioid receptor

biased agonism

BRET

2. Introduction:

2.1 Opioid Receptor Family: Overview

Opioid receptors are members of the G protein–coupled receptor (GPCR) superfamily and mediate the physiological effects of both endogenous opioid peptides and clinically administered opioid drugs[1]. The opioid receptor family comprises four principal subtypes: the μ-opioid receptor (MOP), κ-opioid receptor (KOP), δ-opioid receptor (DOP), and the more recently identified nociceptin/orphanin FQ peptide (NOP) receptor[2]. These subtypes are preferentially activated by endorphins, dynorphins, enkephalins, and nociceptin/orphanin FQ (N/OFQ), respectively[2,3]. Although some secondary sources describe an additional "zeta (ζ)" or "ZOP" receptor—now termed the opioid growth factor receptor (OGFr)—the International Union of Basic and Clinical Pharmacology (IUPHAR) formally recognizes only MOP, DOP, KOP, and NOP as canonical opioid receptors[2,3]. Understanding the distinctions among these subtypes is fundamental to clinical pharmacology, guiding the development of targeted therapies for pain management, addiction, and neuropsychiatric disorders.

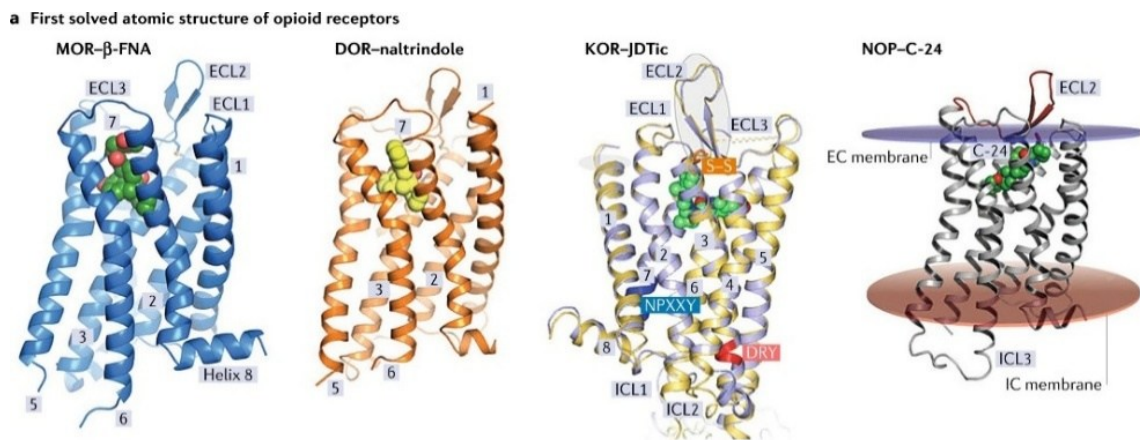


Figure 1 Structural organization and signaling pathways of opioid receptors. Adapted from Darcq & Kieffer (2018).

The four opioid receptor subtypes share substantial sequence and structural homology and primarily couple to inhibitory Gi/o proteins[1]. Upon agonist binding, opioid receptors undergo conformational changes that initiate intracellular signaling cascades[3,4]. Activation of Gi/o proteins inhibits adenylyl cyclase, reducing cyclic AMP (cAMP) production and consequently decreasing protein kinase A (PKA) activity[3,4]. These signaling events also suppress voltage-gated calcium channel activity and enhance potassium channel conductance, collectively reducing neuronal excitability and neurotransmitter release[3,4]. The resulting physiological effects include analgesia, modulation of

reward pathways, regulation of emotional states, and control of stress responses, illustrating the direct link between opioid receptor signaling and systemic physiological outcomes[4]. Despite their therapeutic value, classical opioid drugs are limited by adverse effects such as tolerance, dependence, and respiratory depression[4]. These challenges highlight the need for alternative approaches to pain management. The discovery of the NOP receptor has broadened the landscape of opioid pharmacology and fostered interest in developing novel analgesics that may provide effective pain relief with improved safety profiles[1].

2.2 The NOP Receptor: Distinctive Features and Therapeutic Interest

The nociceptin/orphanin FQ peptide (NOP) receptor, previously known as the opioid receptor-like 1 (ORL1) receptor, is the most recently identified member of the opioid receptor family[1]. Although it shares substantial structural homology with the classical μ-, δ-, and κ-opioid receptors, the NOP receptor exhibits distinct pharmacological properties[1]. Its endogenous ligand, nociceptin/orphanin FQ (N/OFQ), is derived from a precursor protein distinct from those of classical opioid peptides, underscoring the receptor's unique evolutionary and functional position within the opioid system[1,5]. Notably, classical opioid antagonists such as naloxone do not block the NOP receptor, highlighting a key divergence from other opioid receptor subtypes[1,6]. Additional distinguishing features of the NOP receptor include minimal involvement in classical opioid-induced reward or euphoria and bidirectional modulation of pain, producing pronociceptive or antinociceptive effects depending on physiological context[1,5]. Unlike activation of classical opioid receptors, NOP receptor activation does not produce common opioid-associated adverse effects such as respiratory depression or strong reinforcing properties[1,5]. Rather, the NOP receptor regulates a broad spectrum of physiological and behavioral processes[1,7].

NOP receptor signaling influences pain transmission, stress and anxiety regulation, learning and memory, reward processing, and cardiovascular function[1,7]. Its role in pain modulation is particularly complex: depending on anatomical location, NOP activation can produce analgesic (antinociceptive) effects—as observed in the spinal cord—or hyperalgesic (pronociceptive) effects at supraspinal sites[1,5]. These bidirectional effects have been demonstrated in numerous preclinical studies[1,5]. Beyond nociception, NOP signaling modulates emotional and motivational states, supporting its potential relevance for mood and addiction disorders[1,7]. Because NOP agonists generally lack reinforcing properties and exhibit a reduced risk of respiratory depression compared to classical opioids, they are

being actively investigated as potentially safer therapeutic alternatives for pain management, as well as for the treatment of psychiatric and neurological disorders[1,5,6]. Given its broad functional profile, the NOP receptor has emerged as a promising therapeutic target, with potential applications in treating chronic pain, anxiety, depression, addiction, Parkinson's disease, and overactive bladder[5,6]. Several selective NOP agonists are currently undergoing preclinical and clinical development[5,6].

2.3 GPCR signaling: Overview

G protein–coupled receptors (GPCRs), including the NOP receptor, represent a large and versatile family of cell-surface receptors that detect extracellular signals and initiate intracellular signaling cascades. These receptors share a conserved architecture of seven transmembrane α-helices, allowing ligand binding at the cell surface to be translated into diverse intracellular responses[8]. Heterotrimeric G proteins constitute the principal signaling partners of GPCRs and mediate cellular responses to a wide range of molecules, including hormones, neurotransmitters, peptides, odorants, and tastants[3,8]. These G proteins consist of α, β, and γ subunits, and their activation represents the classical and most extensively studied pathway downstream of GPCR stimulation[3,8]. Gα subunits are classified into four major families—Gαs, Gαi/o, Gαq/11, and Gα12/13—each coupling to distinct downstream effectors and producing divergent cellular responses[8,9]. The NOP receptor, like other opioid receptors, primarily couples to the inhibitory Gαi/o family[1].

Upon agonist binding, GPCRs undergo conformational changes that activate their associated heterotrimeric G proteins[3,8]. This activation promotes the exchange of GDP for GTP on the Gα subunit, leading to dissociation of Gα–GTP from the Gβγ dimer[3,4,8]. Both components then interact with downstream effector proteins[3,8]. For example, Gαi inhibits adenylyl cyclase, thereby reducing cyclic AMP (cAMP) levels and subsequent protein kinase A (PKA) activity, ultimately decreasing phosphorylation of targets involved in neuronal excitability[3,4,8]. In parallel, Gβγ subunits modulate ion channels by enhancing potassium efflux and inhibiting calcium influx, thereby reducing neurotransmitter release and neuronal firing[3,4]. Through this diversification, GPCRs can precisely regulate cellular responses to numerous physiological stimuli[3,8]. The signaling outcomes are highly context-dependent, varying with cell type, receptor expression levels, and the specific complement of available effector proteins[3,4].

Beyond these canonical G protein–dependent pathways, GPCRs also engage alternative signaling mechanisms mediated by β-arrestins[9]. Initially characterized for their roles in receptor desensitization

and internalization, β-arrestins are now recognized as multifunctional scaffolding proteins capable of initiating distinct intracellular signaling cascades, including the MAPK/ERK and Akt pathways[4,9]. This dual capacity for G protein–dependent and β-arrestin–dependent signaling highlights the versatility of GPCR signaling and enables ligand-specific signaling patterns that contribute to phenomena such as functional selectivity or biased agonism[9–11].

2.4 Comparison of G Protein and β-Arrestin Signaling

Following agonist activation, GPCRs can initiate signaling through two principal pathways: G proteins and β-arrestins[8,11]. G protein–mediated signaling is typically rapid and transient, producing immediate cellular responses such as changes in ion channel activity, cAMP levels, or kinase activation[8]. In contrast, β-arrestin–mediated signaling develops more slowly and is often more sustained[9,11]. β-arrestins function as multifunctional scaffolding proteins that recruit and organize kinases, including MAPK/ERK, resulting in signaling outcomes distinct from those generated by G proteins[9,11]. The balance between these pathways determines the intensity, duration, and qualitative nature of GPCR-mediated responses, a feature particularly relevant for opioid receptors[10,11]. β-arrestin recruitment has been proposed to contribute to certain adverse effects associated with opioid therapy, motivating the development of ligands that preferentially activate G protein signaling while minimizing β-arrestin engagement[10,12,13].

The mechanistic divergence between G protein and β-arrestin signaling begins with receptor phosphorylation[9,11]. After agonist binding, GPCRs rapidly activate G protein–mediated pathways, producing immediate cellular responses such as modulation of ion channels and changes in cAMP levels, allowing cells to adapt quickly to external stimuli[8]. Mechanistically, this is followed by receptor phosphorylation: GPCR kinases (GRKs) phosphorylate specific intracellular residues on the activated receptor, creating binding sites for β-arrestins[9,11]. β-arrestin recruitment subsequently prevents additional G protein coupling, thereby desensitizing the receptor and terminating further G protein signaling[9,11]. Unlike the short-lived nature of G protein signaling, the association of β-arrestin with the receptor often lasts longer[9]. This enables β-arrestins, acting as multifunctional scaffolding proteins, to assemble signaling complexes that activate pathways such as MAPK/ERK and Akt[9,11]. In addition, β-arrestin binding facilitates receptor internalization via clathrin-coated pits, contributing to receptor trafficking, recycling, or degradation, and supporting longer-term cellular adaptation[9]. Thus, while G protein signaling provides rapid, short-lived responses, β-arrestin–mediated signaling is more

regulatory and prolonged, resulting in qualitatively distinct cellular outcomes[11]. Importantly, this divergence in signaling kinetics and outcomes forms the mechanistic basis of biased agonism, a phenomenon in which certain ligands preferentially activate either G protein or β-arrestin–dominated pathways, offering opportunities for more selective and therapeutically targeted drug effects[10,13].

Among the β-arrestin isoforms, β-arrestin2 is especially significant for opioid receptor regulation, including both the μ-opioid (MOP) and nociceptin/orphanin FQ peptide (NOP) receptors[10,13]. While both β-arrestin1 and β-arrestin2 can mediate receptor desensitization and internalization, β-arrestin2 is often more abundantly expressed in relevant tissues and has been shown to exert a stronger influence on receptor trafficking and associated downstream signaling[9,13]. Recruitment of β-arrestin2 to a phosphorylated receptor not only terminates further G protein signaling but also promotes receptor internalization via clathrin-coated pits and initiates distinct downstream signaling cascades[9,11]. This isoform-specific interaction can differentially affect the magnitude and duration of receptor signaling and plays a key role in determining the balance between therapeutic effects and side effects for opioid ligands[10,13]. Consequently, understanding β-arrestin2 recruitment and function at the NOP receptor is central to the evaluation of biased agonism and the pharmacological properties of novel ligands, such as those investigated in this thesis[1,6,10].

2.5 Biased Agonism: G Protein vs. β-Arrestin2 Signaling at the NOP Receptor

Biased agonism, also known as functional selectivity, refers to the capacity of different ligands acting at the same G protein-coupled receptor (GPCR) to preferentially activate specific intracellular signaling pathways over others[10,11]. Historically, GPCR activation was considered a uniform process in which agonists elicited similar downstream signaling patterns, differing only in potency or efficacy[11]. Accumulating evidence has since demonstrated that GPCRs can adopt multiple active conformational states and that ligand structure plays a critical role in stabilizing these distinct conformations[8,11]. When a ligand binds to a GPCR, it induces specific conformational changes that determine which intracellular signaling proteins—primarily G proteins or β-arrestins— can effectively couple to the receptor[8,11]. Different ligands stabilize distinct receptor conformations that favor coupling to one signaling partner over another, resulting in pathway bias in which the relative activation of G protein versus β-arrestin signaling differs substantially between ligands at the same receptor[10,11]. Quantifying biased agonism requires comparing the relative efficacies of ligands across

multiple signaling pathways, typically by calculating bias factors that normalize potency and efficacy within each pathway relative to a reference ligand[10,11].

Within the opioid receptor family, the concept of biased agonism has received significant attention due to its potential to dissociate therapeutic effects from adverse outcomes[10,13]. At the μ-opioid (MOP) receptor, the first opioid receptor to be discovered and the primary receptor for endogenous opioids, ligands that preferentially activate G protein signaling while limiting β-arrestin2 recruitment have been shown to produce analgesia with reduced side effects, such as respiratory depression and tolerance development[10,13]. A well-known example is TRV130 (oliceridine), a G protein–biased MOP agonist, which demonstrated reduced respiratory depression compared with conventional opioids in clinical studies[12]. These findings have reshaped opioid drug discovery strategies and underscored the therapeutic relevance of pathway-selective signaling, extending interest in biased agonism beyond the MOP receptor to other members of the opioid receptor family, like the NOP receptor[6,10,13].

At the nociceptin/orphanin FQ peptide (NOP) receptor, biased agonism is increasingly recognized as an important determinant of ligand pharmacology, although it remains less comprehensively characterized than at classical opioid receptors[1,6]. Structural differences among NOP ligands can stabilize distinct receptor conformations, leading to preferential activation of either G protein– or β-arrestin2–mediated signaling pathways[8,10]. Given the role of β-arrestin2 in receptor desensitization, internalization, and the initiation of independent signaling cascades, differential recruitment of β-arrestin2 may profoundly influence signaling duration, receptor regulation, and downstream physiological effects, including modulation of pain, stress, and other NOP-mediated responses[9,13]. Accordingly, determining whether novel NOP receptor agonists preferentially engage G protein or β-arrestin2 signaling pathways provides critical mechanistic insights that can guide lead optimization and predict in vivo pharmacological profiles[6,10]. This conceptual framework serves as the basis for evaluating biased agonism and the pharmacological properties of the novel ligands investigated in this thesis.

2.6 Knowledge Gap

Although interest in NOP receptor signaling has increased in recent years, most pharmacological studies have concentrated on G protein-mediated pathways, particularly Gi/o coupling, adenylyl cyclase inhibition, and ion channel modulation[1,5]. These studies have clarified fundamental mechanisms of NOP receptor activation but provide only a partial perspective on the receptor's

signaling capabilities. In contrast, β-arrestin2-mediated signaling at the NOP receptor remains comparatively understudied, despite its critical role in receptor desensitization, internalization, and the initiation of G protein-independent signaling cascades[1,9]. This knowledge gap limits the comprehensive characterization of NOP receptor ligand pharmacology and hinders the evaluation of biased agonism at this receptor.

This knowledge gap is particularly pronounced for recently developed non-peptidic NOP receptor ligands, including partial agonists designed to optimize pharmacokinetic properties and therapeutic efficacy[1,5]. While these compounds are routinely characterized for G protein-dependent signaling using assays such as cAMP inhibition and GTPγS binding, their capacity to recruit β-arrestin2 remains largely undefined[1]. Given the central role of β-arrestin2 in receptor regulation and signaling bias, this lack of data limits the ability to fully evaluate the functional selectivity and therapeutic potential of novel NOP receptor agonists.

2.7 Aim of the Study

Given the limited data on β-arrestin2 signaling at the NOP receptor, the aim of this thesis was to pharmacologically characterize five non-peptidic selective NOP receptor partial agonists, with a particular focus on their ability to recruit β-arrestin2. To this end, β-arrestin2 recruitment was quantified using bioluminescence resonance energy transfer (BRET) assays to assess broader functional responses. In addition, a calcium mobilization assay employing chimeric G proteins was used to evaluate ligand selectivity across the four opioid receptors. By integrating these experimental approaches, this study aimed to define ligand-dependent signaling profiles and evaluate potential biased agonism at the NOP receptor.

3. Materials & Methods

3.1 Drugs and Reagents

The pharmacological characterization of NOP receptor signaling was performed using the ligands and assay reagents described below.

3.1.1 Solutions and Buffers

The following solutions were prepared and used in the experimental procedures.

I. Hanks' Balanced Salt Solution (HBSS): HBSS was prepared containing KCl (5.4 mM), KH_2PO_4 (0.44 mM), NaCl (137 mM), $NaHCO_3$ (4.2 mM), $Na_2HPO_4 \cdot 7H_2O$ (0.25 mM), $CaCl_2 \cdot 2H_2O$ (1.3 mM), $MgSO_4 \cdot 7H_2O$ (1 mM), and glucose (5 mM) in distilled water, and adjusted to pH 7.4.

II. Assay Buffer: For BRET assay, the buffer consisted of HBSS supplemented with 20 mM HEPES (2-[4-(2-hydroxyethyl)-1-piperazinyl]ethanesulfonic acid), adjusted to pH 7.4.

Calcium Mobilization Assay Solutions: For calcium mobilization experiments employing chimeric Gq proteins, the following solutions were prepared:

III. Fluo-4 Loading Solution: HBSS supplemented with 20 mM HEPES (pH 7.4, without BSA), Pluronic F-127 (0.02%), probenecid (2.5 mM), and Fluo-4 AM (3 µM).
The probenecid stock solution was prepared by dissolving 80 mg probenecid in a mixture of 500 µL HBSS/HEPES buffer and 500 µL NaOH. The stock was subsequently diluted in assay buffer to achieve the desired final concentration in the loading solution. All dye-containing solutions were prepared fresh on the day of the experiment and protected from light.

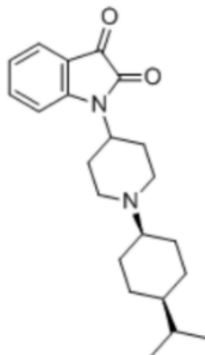
IV. Fluorescence Reduction (Brilliant Black) Solution: HBSS supplemented with 20 mM HEPES, probenecid (2.5 mM), and Brilliant Black (500 µM).

3.1.2 Compounds and Reference Ligands

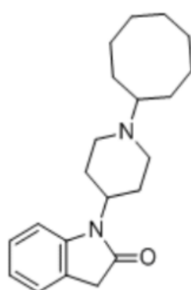
The endogenous NOP receptor agonist nociceptin/orphanin FQ (N/OFQ) was used as the reference full agonist in all concentration–response experiments. For receptor selectivity studies, the following reference agonists were employed: N/OFQ (NOP receptor), Dermorphin (MOP receptor), Dynorphin A (KOP receptor), and DPDPE (DOP receptor). All peptide ligands were purchased from MedChemExpress.

The novel non-peptidic NOP receptor ligands evaluated in this study were AT-090, AT-200, AT-336, AT-403, and AT-523. All compounds were developed by Astraea Therapeutics and supplied for in vitro pharmacological evaluation at >98% purity. Where available, structural information was as follows:

I. AT-090: 1-(1-((1S,4S)-4-isopropylcyclohexyl)piperidin-4-yl)indoline-2,3-dione



II. AT-200 (formerly SR14150): 1-(1-cyclooctylpiperidin-4-yl)indolin-2-one



III. AT-403: 2-(1-(1-((1S,4S)-4-isopropylcyclohexyl)piperidin-4-yl)-2-oxoindolin-3-yl)-N-methylacetamide

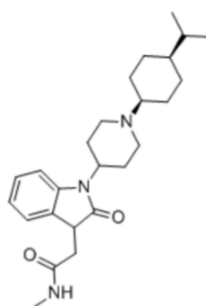


Figure 2 Chemical structures of NOP receptor ligands used in this study: (A) AT-090, (B) AT-200 (formerly SR14150), and (C) AT-403.

Detailed structural information for AT-336 and AT-523 was not publicly available at the time of writing.

3.1.3 Preparation and Storage of Ligands

Stock solutions of all non-peptidic ligands were prepared in dimethyl sulfoxide (DMSO) at a concentration of 10 mM and stored at −80 °C until use. On the day of each experiment, serial dilutions were prepared in the appropriate assay buffer to generate concentration–response curves. The final concentration of DMSO in assay wells did not exceed 0.1% (v/v), and corresponding vehicle controls were included in all experiments.

Nociceptin/orphanin FQ (N/OFQ) and peptide standards (Dermorphin, Dynorphin A, and DPDPE) were dissolved in sterile distilled water, aliquoted, and stored at −80 °C to prevent repeated freeze–thaw cycles.

3.2 Cell Culture

The following cell lines were used in this study:

i. HEK293: Human embryonic kidney 293 cells are a widely used heterologous expression system for G protein–coupled receptor (GPCR) signaling studies. They exhibit high transfection efficiency, robust growth characteristics, and low endogenous opioid receptor expression, making them particularly suitable for controlled pharmacological characterization of transfected receptors and BRET-based interaction assays.

HEK293 cells were cultured in Dulbecco's Modified Eagle Medium (DMEM, high glucose, with L-glutamine) supplemented with 10% fetal bovine serum (FBS), 1 mM sodium pyruvate, 100 IU/mL penicillin, 100 µg/mL streptomycin, 400 µg/mL Geneticin (G418), and 200 µg/mL Hygromycin B.

ii. CHO: Chinese hamster ovary cells are widely used in receptor pharmacology and functional signaling assays due to their stable growth characteristics, high adaptability, and low endogenous opioid receptor expression. They are particularly suitable for calcium mobilization assays and for evaluating receptor activation and ligand selectivity using engineered signaling systems, including chimeric G proteins.
CHO cells expressing opioid receptors were cultured in DMEM and Ham's F-12 (1:1) supplemented with 2 mM L-glutamine, 10% fetal bovine serum (FBS), 100 IU/mL penicillin, 100 µg/mL streptomycin, and 200 µg/mL Geneticin (G418).

For CHO cells co-expressing chimeric G proteins, the culture medium additionally contained 100 µg/mL hygromycin B. The hygromycin selection marker was required because the retroviral vector used to introduce the chimeric G protein carries a hygromycin resistance gene, allowing stable selection and maintenance of cells expressing the engineered G-protein construct.

All cells were cultured in sterile T25 and T75 tissue culture flasks at 37 °C in a humidified incubator with 5% CO_2. Culture medium was replaced every 2–3 days, and cells were passaged at approximately 80–90% confluency.

3.3 Cell Culture Maintenance and Experimental Preparation

3.3.1 Routine Cell Passaging

Cells were routinely passaged upon reaching approximately 80–90% confluency. All procedures were performed under sterile conditions in a biological safety cabinet. The culture medium was aspirated, and cells were washed once with 5 mL sterile phosphate-buffered saline (PBS). Cells were then incubated with Trypsin–EDTA (1X); typical volumes were 2 mL for T25 flasks and 5 mL for T75

flasks. Flasks were incubated at 37 °C for approximately 5 minutes, until cell detachment was observed. The flask was gently shaken in a horizontal motion to detach the cells when necessary.

Trypsin activity was neutralized by adding three volumes of complete culture medium. The resulting cell suspension was transferred to a Falcon tube and centrifuged at 1250–1350 rpm (≈300 × g) for 5 minutes. The supernatant was discarded, and the cell pellet was resuspended in fresh complete medium.

Cells were redistributed into new sterile T25 or T75 culture flasks, with final medium volumes adjusted to 13 mL for T25 flasks and 25 mL for T75 flasks. Cell morphology and confluency were routinely assessed using light microscopy and an automated cell counter (Corning® Cell Counter, CytoSMART). All flasks were labeled with the cell line name, date, passage number, confluency estimate, and observed cell condition.

CHO cells attached rapidly but required adequate enzymatic incubation for efficient detachment.

3.3.2 Mechanical Cell Detachment for Membrane Preparations

For membrane-based procedures, cells were mechanically detached in phosphate-buffered saline (PBS) to avoid enzymatic treatment. After aspirating the culture medium, 5 mL sterile PBS was added to the flask, and the flask was gently shaken in a horizontal motion to detach the cells. The cell suspension was collected, and the procedure was repeated with an additional 5 mL PBS to maximize cell recovery. The combined cell suspensions were centrifuged at 2500 rpm for 5 minutes, and the resulting cell pellet was used for subsequent membrane preparation steps.

3.3.3 Cryopreservation

Cells were cryopreserved at appropriate passage numbers to maintain stable receptor expression and experimental consistency. For freezing, the culture medium was aspirated, and cells were washed once with 5 mL sterile PBS. Cells were then incubated with Trypsin–EDTA (1×) at 37 °C for approximately 5 minutes until detachment was observed. Trypsin activity was neutralized by adding three volumes of complete culture medium.

The cell suspension was transferred to a Falcon tube and centrifuged at 1250–1350 rpm (≈300 × g) for 5 minutes. The supernatant was discarded, and the cell pellet was resuspended in freezing medium.

Cell density was determined using an automated cell counter (Corning® Cell Counter, CytoSMART) prior to freezing. Cells were resuspended at a final density of 5 × 10^6 cells/mL and aliquoted into sterile cryovials at volumes of 1–2 mL per vial.

The freezing medium consisted of fetal bovine serum (FBS) supplemented with 10% (v/v) dimethyl sulfoxide (DMSO), freshly prepared prior to use. Alternatively, Bambanker™ freezing medium was used according to the manufacturer's instructions. When FBS/DMSO freezing medium was used, cryovials were placed in a controlled-rate freezing container to allow gradual cooling at −80 °C prior to transfer to liquid nitrogen for long-term storage. When Bambanker™ was used, cryovials were directly transferred to −80 °C before long-term storage.

3.3.4 Cell Seeding for Experimental Assays

For calcium mobilization assays performed in black 384-well plates, approximately 4 × 10^4 cells were seeded in 50 µL per well. For a full 384-well plate, a total volume of 20–21 mL of cell suspension was prepared to ensure uniform dispensing across all wells. Following seeding, plates were incubated overnight at 37 °C in a humidified 5% CO_2 incubator prior to experimentation. Where required, specific columns were intentionally left unseeded to accommodate experimental controls.

3.4 Bioluminescence Resonance Energy Transfer (BRET) Assay

Bioluminescence Resonance Energy Transfer (BRET) experiments were performed to evaluate NOP receptor–mediated signaling in living cells. White 96-well Lumitrac plates were coated with poly-D-lysine (PDL) prior to cell seeding. After incubation, excess PDL was removed, and the wells were washed with sterile buffer before use. Human embryonic kidney (HEK293) cells stably expressing NOP receptor BRET constructs were used. Two cell lines were utilized: one co-expressing NOP–Renilla luciferase (RLuc) and Gβ1–GFP, and another co-expressing NOP–RLuc and β-arrestin2–GFP. Cells were seeded in white 96-well plates at a density of 50,000 cells per well and incubated overnight at 37 °C in a humidified atmosphere containing 5% CO_2 to allow cell attachment and recovery.

On the day of the experiment, the culture medium was removed and replaced with assay buffer containing Hanks' Balanced Salt Solution (HBSS) supplemented with 20 mM HEPES (pH 7.4). Where indicated, bovine serum albumin (BSA, 0.1–0.5% w/v) was included to minimize nonspecific ligand adsorption. Test ligands were prepared freshly in assay buffer at the desired concentrations

immediately prior to use. BRET measurements were initiated by adding the luciferase substrate Prolume Purple (methoxy e-coelenterazine, Me-O-e-CTZ) to each well at a final concentration of 50 μM. The substrate was stored at −80 °C, protected from light, and prepared fresh prior to use. Following substrate equilibration, ligand solutions were added, and luminescence was recorded using a Victor Nivo multilabel plate reader (PerkinElmer).

Donor and acceptor emissions were recorded at 405 nm and 515 nm, respectively. BRET ratios were calculated as the ratio of acceptor emission (515 nm) to donor emission (405 nm) after subtraction of background signals measured in control wells (last row H, containing assay buffer without ligand), which were measured simultaneously with experimental wells and used for background correction. Concentration–response curves were generated following baseline correction and normalization, and data were analyzed as described in Section 3.6. Experiments were performed in five independent biological replicates (n = 5).

3.5 Calcium Mobilization Assay

Intracellular calcium mobilization was assessed as a functional readout of NOP receptor activation using a fluorescence-based calcium assay. These experiments were performed using CHO cells expressing chimeric G proteins, which enable receptors that normally couple to Gi/o proteins, such as the NOP receptor, to activate the PLC–IP_3–Ca^{2+} signaling pathway and generate measurable intracellular calcium responses. This strategy allows receptors that do not normally trigger calcium signaling to be monitored using fluorescence-based calcium assays.

Cells were seeded in black 384-well plates at a density of 25,000 cells per well in 50 μL of complete culture medium and incubated overnight at 37 °C in a humidified atmosphere containing 5% CO_2 to allow cell attachment. On the day of the experiment, cells were incubated with a loading solution containing the calcium-sensitive fluorescent dye Fluo-4 AM (Invitrogen) in assay buffer (HBSS supplemented with 20 mM HEPES, pH 7.4). Probenecid was included to prevent dye leakage. Approximately 30–40 μL of dye solution was added per well, and plates were incubated for 60 minutes at 37 °C to allow intracellular dye loading.

Following incubation, the loading solution was removed, and cells were washed with assay buffer containing Brilliant Black and probenecid to reduce background fluorescence. Wells were left with 50 μL of assay buffer prior to measurement. Test compounds were prepared in a separate 384-well

compound plate, where serial 1:10 dilutions were performed directly in the plate to generate concentration–response curves. Compounds were prepared at five-fold higher concentrations relative to the final assay concentration. During the experiment, 12.5 µL of compound solution was automatically injected into each well containing 50 µL of buffer using the FlexStation 3 microplate reader (Molecular Devices), allowing kinetic fluorescence measurements of agonist-induced calcium responses.

Changes in fluorescence intensity were expressed as relative fluorescence units (RFU). Data were baseline-corrected and normalized prior to generation of concentration–response curves, and analyzed as described in Section 3.6. Experiments were performed in three independent biological replicates (n = 3).

3.6 Data Analysis and Statistics

Data analysis was performed using GraphPad Prism (version 9.5) together with instrument-specific acquisition software.

For BRET assays, donor and acceptor luminescence signals were background-corrected using control wells containing assay buffer without ligand. BRET ratios were calculated as the ratio of acceptor to donor emission and normalized to baseline values prior to ligand addition. Concentration–response curves were generated by plotting normalized BRET signals against the logarithm of ligand concentration.

For calcium mobilization assays, fluorescence signals were recorded as relative fluorescence units (RFU). Raw fluorescence traces were baseline-corrected using the signal measured immediately prior to compound injection. The maximal fluorescence response following ligand stimulation was used for concentration–response analysis.

Dose–response curves were fitted using nonlinear regression with a four-parameter logistic model to determine pharmacological parameters including pEC_{50} and Emax values. Bias factors were calculated by comparing signaling responses across pathways relative to the endogenous NOP receptor ligand nociceptin/orphanin FQ (N/OFQ). Data are presented as mean ± standard error of the mean (SEM) from independent biological replicates. Experiments were performed as described in the respective assay sections and included independent biological replicates.

4. Results

4.1 Bioluminescence Resonance Energy Transfer (BRET) Assay

BRET experiments were performed to characterize the signaling properties of novel non-peptidic NOP receptor ligands by measuring receptor interactions with either $G\beta_1$ or β-arrestin2. Concentration–response curves were generated for each ligand in both signaling pathways, and pharmacological parameters including pEC_{50} and Emax values were determined. Concentration–response curves for NOP–G protein interaction and β-arrestin2 recruitment are shown in Figure 3 and 4, respectively.

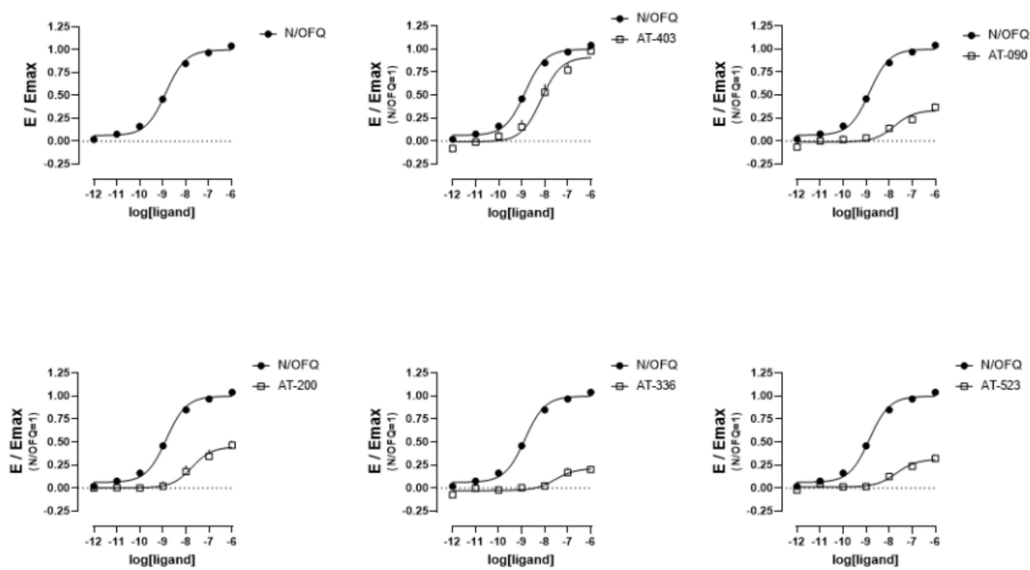


Figure 3 Concentration–response curves for NOP receptor–mediated $G\beta_1$ protein interaction measured using the BRET assay. Responses induced by N/OFQ and the tested ligands (AT-403, AT-090, AT-200, AT-336, and AT-523) are normalized to the maximal response of N/OFQ (Emax = 1).

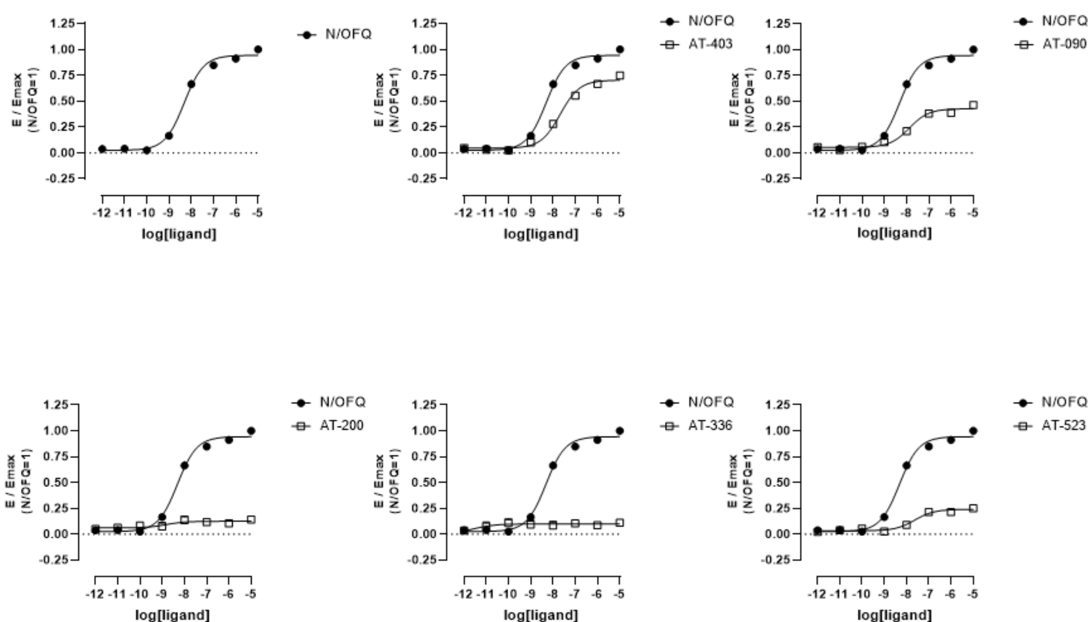


Figure 4 Concentration–response curves for β-arrestin2 recruitment at the NOP receptor measured using the BRET assay. N/OFQ served as the reference agonist; AT-200 and AT-336 did not produce detectable β-arrestin2 recruitment.

The endogenous NOP receptor agonist nociceptin/orphanin FQ (N/OFQ) served as the reference ligand and produced robust activation of both pathways. N/OFQ exhibited high potency in the G protein assay (pEC_{50} = 8.85) and in the β-arrestin2 recruitment assay (pEC_{50} = 8.31), with maximal responses normalized to 1.00 in both systems.

Among the tested compounds, AT-403 demonstrated the highest potency after N/OFQ. In the G protein assay, AT-403 displayed a pEC_{50} of 8.17 with near-maximal efficacy (Emax = 0.91 ± 0.05). In the β-arrestin2 assay, AT-403 exhibited slightly lower potency (pEC_{50} = 7.69) and reduced efficacy (Emax = 0.71 ± 0.02).

AT-090 demonstrated moderate potency in both pathways. In the G protein assay, AT-090 displayed a pEC_{50} of 7.63 with partial efficacy (Emax = 0.35 ± 0.03), whereas in the β-arrestin2 assay it showed slightly higher potency (pEC_{50} = 8.05) with an Emax of 0.43 ± 0.04.

AT-200 and AT-336 produced measurable responses only in the G protein assay. AT-200 displayed a pEC_{50} of 7.59 with partial efficacy (Emax = 0.48 ± 0.04), and AT-336 exhibited a pEC_{50} of 7.40 with lower efficacy (Emax = 0.23 ± 0.05). Neither compound elicited detectable β-arrestin2 recruitment under the tested conditions.

AT-523 displayed comparable potencies in both signaling pathways, with pEC_{50} values of 7.67 in the G protein assay and 7.62 in the β-arrestin2 assay. However, maximal responses were relatively low in both systems (Emax ≈ 0.30), indicating partial agonist activity. Bias factors were calculated by comparing pathway responses relative to N/OFQ, as summarized in Table 1.

	NOP - $G\beta_1$		NOP – β-Arrestin 2			
COMPOUNDS	pEC_{50} ($CL_{95\%}$)	E_{max} ± S.E.M.	pEC_{50} ($CL_{95\%}$)	E_{max} ± S.E.M.	pA_2 ($CL_{95\%}$)	Bias factor
N/OFQ	**8.85 (8.67-9.03)**	**1.00**	**8.31 (8.15-8.46)**	**1.00**		**0.00**
AT-403	8.17 (7.74-8.59)	0.91 ± 0.05	7.69 (7.45-7.93)	0.71 ± 0.02		0.47 (-0.12 - 1.07)
AT-090	7.63 (7.04-8.22)	0.35 ± 0.03	8.05 (7.35-8.75)	0.43 ± 0.04		-0.82 (-1.81-0.17)
AT-200	7.59 (6.78-8.40)	0.48 ± 0.04	inactive		6.50 (6.22-6.78)	-
AT-336	7.40 (6.93-7.87)	0.23 ± 0.05	inactive		7.25 (6.88-7.62)	-
AT-523	7.67 (6.93-8.41)	0.30 ± 0.06	7.62 (7.23-8.02)	0.25 ± 0.02		-0.38 (-1.72 - 0.96)

Table 1 Pharmacological parameters of NOP receptor ligands determined in BRET assays measuring $G\beta_1$ interaction and β-arrestin2 recruitment. Values represent pEC_{50}, Emax ± S.E.M., and calculated bias factors relative to N/OFQ.

AT-403 showed a modest preference for G protein signaling, whereas AT-090 and AT-523 displayed higher activity in the β-arrestin2 assay. AT-200 and AT-336 exhibited functional selectivity for G protein signaling due to the absence of detectable β-arrestin2 recruitment.

Overall, the investigated compounds displayed diverse pharmacological profiles at the NOP receptor, ranging from balanced agonism to pathway-selective signaling.

4.2 Calcium Mobilization Assay

Intracellular calcium mobilization assays were performed to evaluate functional activation of opioid receptors and to assess the selectivity of the investigated NOP receptor ligands. Experiments were conducted using CHO cells expressing chimeric G proteins, which enable Gi/o-coupled receptors to activate calcium signaling pathways. Fluorescence responses were recorded as relative fluorescence units (RFU) following ligand stimulation.

Activation of the NOP receptor resulted in concentration-dependent increases in intracellular calcium levels. The endogenous ligand, nociceptin/orphanin FQ (N/OFQ), served as the reference agonist and produced robust calcium responses (Figure 5).

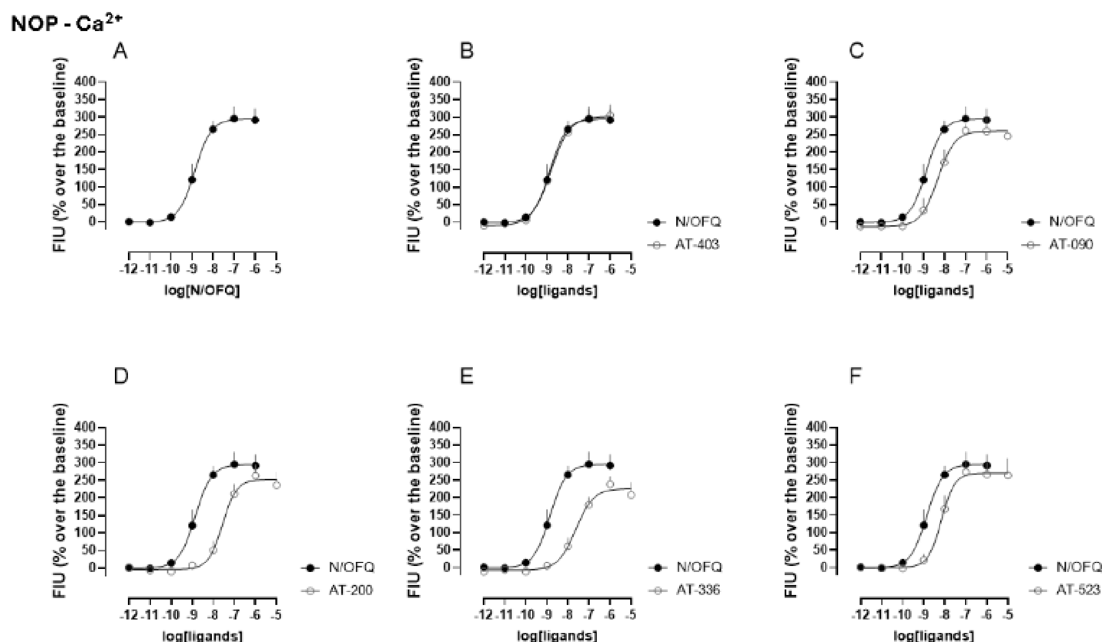


Figure 5 Concentration–response curves for intracellular calcium mobilization following NOP receptor activation in CHO cells expressing chimeric G proteins. Responses are expressed as relative fluorescence units (RFU) relative to baseline.

Among the tested compounds, AT-403 produced calcium responses comparable to those of N/OFQ, indicating strong agonist activity at the NOP receptor. AT-090 and AT-523 also elicited clear concentration-dependent responses, although with slightly reduced maximal effects compared to the endogenous ligand. In contrast, AT-200 and AT-336 induced weaker calcium responses and displayed reduced maximal efficacy (Emax) compared with the endogenous ligand N/OFQ. This reduced maximal response relative to the reference agonist is consistent with their classification as partial agonists at the NOP receptor.

To evaluate receptor selectivity, the compounds were additionally tested against the κ-opioid (KOP), μ-opioid (MOP), and δ-opioid (DOP) receptors using the same calcium mobilization assay. The reference agonists dynorphin A, dermorphin, and DPDPE produced strong activation of the KOP, MOP, and DOP receptors, respectively, whereas the tested NOP ligands displayed minimal activity at these receptors (Figures 6 and 7).

kappa - Ca^{2+}

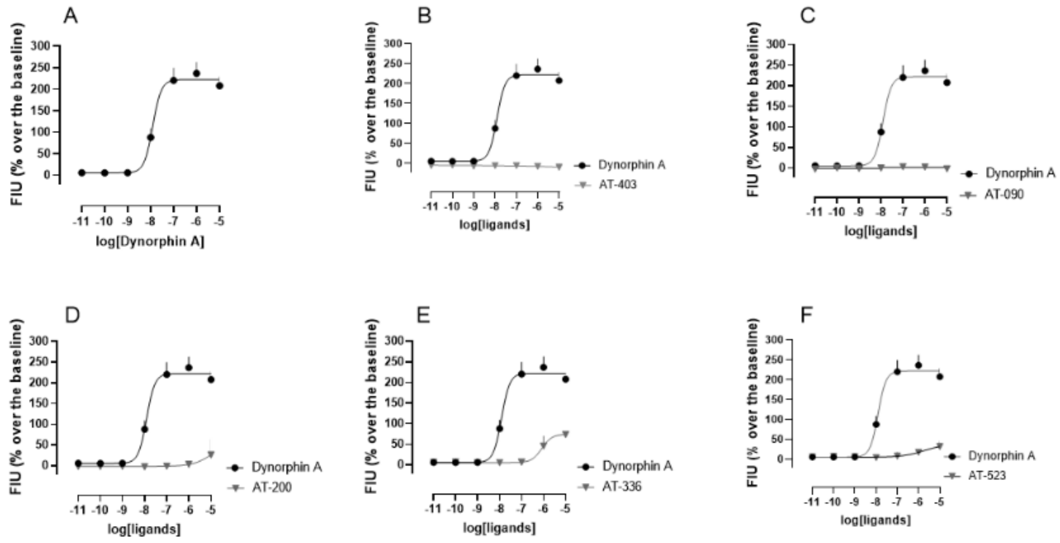
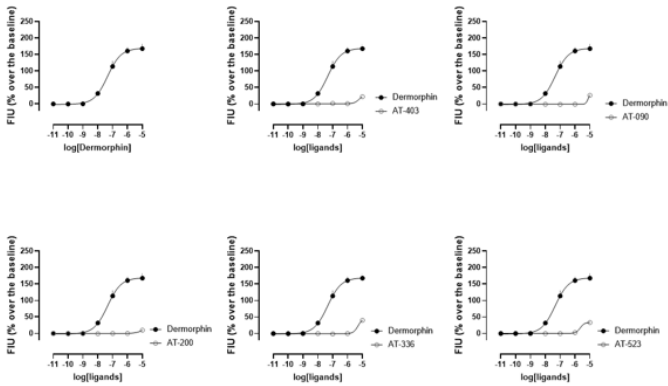


Figure 6 Calcium mobilization responses at the κ-opioid receptor (KOP). Dynorphin A served as the reference agonist and was compared with the investigated NOP ligands.

mu - Ca^{2+}



delta - Ca^{2+}

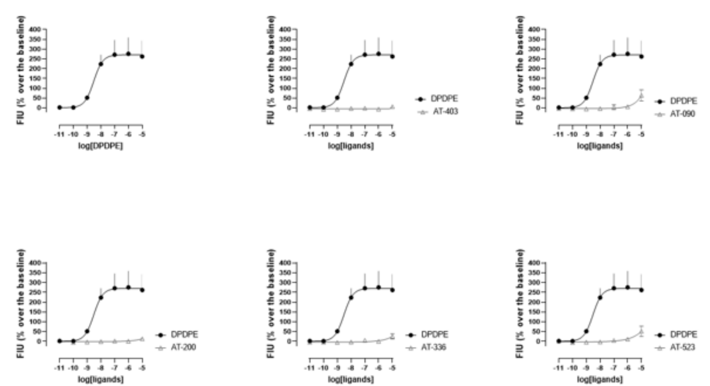


Figure 7 Left Panel: Calcium mobilization responses at the μ-opioid receptor (MOP). Dermorphin was used as the reference agonist and compared with the investigated NOP ligands. Right Panel: Calcium mobilization responses at the δ-opioid receptor (DOP). DPDPE served as the reference agonist and was compared with the investigated NOP ligands.

Overall, the investigated compounds demonstrated strong selectivity for the NOP receptor compared to other opioid receptor subtypes. Quantitative analysis confirmed this selectivity, with AT-403 and AT-090 showing particularly high selectivity for the NOP receptor Table 2.

	NOP – Ca2+		kappa-Ca2+		mu-Ca2+		delta-Ca2+		
COMPOUNDS	**pEC_{50} ($CL_{95\%}$)**	**E_{max} ± S.E.M.**	**pEC_{50} ($CL_{95\%}$)**	**E_{max} ± S.E.M.**	**pEC_{50} ($CL_{95\%}$)**	**E_{max} ± S.E.M.**	**pEC_{50} ($CL_{95\%}$)**	**E_{max} ± S.E.M.**	**Selectivity (over OP)**
Standards	**8.86 (8.50-9.20)**	**303 ± 33**	**7.91 (7.60-8.08)**	**230 ± 23**	**8.58 (8.05-9.02)**	**168 ± 12**	**8.52 (7.05-10.00)**	**276 ± 79**	
AT-403	8.82 (8.45-9.15)	308 ± 30	<5		<5		<5		>20000
AT-090	8.32 (7.94-8.74)	283 ± 40	<5		<6		<5		>6000
AT-200	7.57 (8.50-9.20)	275 ± 37	<5		<5		<5		>1000
AT-336	7.63 (8.50-9.21)	245 ± 30	~6	~74	<5		<5		≥ 35
AT-523	8.16 (8.51-7.90)	282 ± 33	<5		<6		< 6		> 300

Table 2 Pharmacological parameters obtained from calcium mobilization assays at NOP, κ-, μ-, and δ-opioid receptors. Selectivity values represent preference for the NOP receptor relative to other opioid receptor subtypes. Reference agonists: N/OFQ, Dynorphin A, Dermorphin, and DPDPE.

5. Discussion and Conclusions

The present study aimed to pharmacologically characterize a series of novel non-peptidic ligands targeting the nociceptin/orphanin FQ (NOP) receptor using complementary in vitro signaling assays. The compounds AT-090, AT-200, AT-336, AT-403, and AT-523 were evaluated using bioluminescence resonance energy transfer (BRET) and calcium mobilization assays to assess receptor activation, signaling bias, and receptor selectivity.

BRET experiments revealed that the investigated compounds display distinct signaling profiles at the NOP receptor. The endogenous ligand nociceptin/orphanin FQ (N/OFQ) produced strong activation of both G protein signaling and β-arrestin2 recruitment, confirming its role as a balanced reference agonist. Among the tested ligands, AT-403 exhibited the highest potency and efficacy after N/OFQ in both signaling pathways. As AT-403 is a previously reported NOP receptor agonist, it was used as a reference compound for comparison with the newly synthesized ligands.

The remaining compounds showed varying degrees of efficacy and pathway preference. AT-090 demonstrated moderate potency and partial efficacy in both signaling pathways, with slightly higher activity in the β-arrestin2 recruitment assay. In contrast, AT-200 and AT-336 displayed measurable activity only in the G protein assay and were inactive in the β-arrestin2 recruitment assay, indicating functional selectivity for G protein signaling. AT-523 produced relatively low maximal responses in both pathways, consistent with partial agonist behavior. Collectively, these results indicate that the investigated ligands exhibit diverse pharmacological profiles at the NOP receptor, ranging from balanced activation to pathway-selective signaling.

Calcium mobilization assays confirmed the functional activity of the ligands at the NOP receptor and further enabled the evaluation of receptor selectivity across the opioid receptor family. All tested compounds induced concentration-dependent calcium responses at the NOP receptor, although with varying potencies and maximal effects. Consistent with the BRET results, AT-403 produced responses comparable to those of N/OFQ, whereas AT-090 and AT-523 displayed slightly reduced maximal responses. AT-200 and AT-336 showed lower efficacy, supporting their classification as partial agonists.

Importantly, selectivity experiments demonstrated minimal activation of κ-, μ-, and δ-opioid receptors by the investigated compounds. In contrast, the reference agonists dynorphin A, dermorphin, and DPDPE produced strong responses at the κ-, μ-, and δ-opioid receptors, respectively. Quantitative

analysis confirmed that the tested ligands display strong selectivity for the NOP receptor over the other opioid receptor subtypes.

In summary, this study demonstrates that the investigated non-peptidic ligands act as selective NOP receptor agonists with distinct signaling profiles. While none of the newly evaluated compounds surpassed the potency or efficacy of the reference agonist AT-403, several compounds exhibited partial agonist activity and pathway-dependent signaling behavior. Such pharmacological diversity may be relevant for the development of NOP receptor-targeted ligands, as ligand-dependent signaling bias has been proposed to influence therapeutic efficacy and side-effect profiles. Future studies will be required to further investigate the pharmacological properties and potential therapeutic relevance of these ligands in more complex biological systems.

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