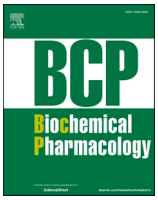
Biochemical Pharmacology 238 (2025) 116972

Contents lists available at ScienceDirect

Biochemical Pharmacology

journal homepage: www.elsevier.com/locate/biochempharm



In vitro pharmacological evaluation of the novel NOP receptor partial agonist sunobinop☆

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ARTICLE INFO

Keywords:
Sunobinop
Sleep modulation
NOP receptor
In vitro pharmacology
GPCRs

ABSTRACT

Sleep-related disorders affect a significant portion of the global population. The nociceptin/orphanin FQ (N/OFQ) peptide (NOP) receptor has become a promising candidate for the development of innovative drugs to treat sleep disorders. In this study, we conducted an in-depth pharmacological characterization of sunobinop, a selective NOP receptor partial agonist already under clinical evaluation for treating multiple conditions including insomnia, using a wide range of in vitro and ex vivo methodologies. Sunobinop exhibited partial agonist activity in calcium mobilization, NOP – G protein interaction, label-free bioimpedance, and cAMP inhibition assays. Notably, it demonstrated competitive antagonism in the NOP – β-Arrestin 2 recruitment and ex vivo electrically stimulated mouse Vas Deferens, assays characterized by low amplification. The findings reported here confirm and extend the characterization of sunobinop as a tool for studying NOP receptor pharmacology providing new insights into its mode of action, relevant to its clinical evaluation.

1. Introduction

Sleep disorders – such as insomnia, sleep apnea, restless leg syndrome, and narcolepsy – impact millions of people globally. For instance, chronic insomnia alone affects approximately 10–15 % of the population [1]. Poor sleep quality is linked to a range of adverse outcomes, including increased risks of cardiovascular disease, metabolic disorders (e.g., diabetes), immune dysfunction, and mental health issues (e.g., depression and anxiety) [2]. The primary treatment for many sleep disorders, especially insomnia, often includes medications such as benzodiazepines and Z-drugs (altering Gamma-Aminobutyric Acid Type A (GABA-A) function). These medications can cause dependency, tolerance, and side effects such as daytime drowsiness, cognitive impairment, respiratory depression especially in combination with alcohol, and increased risk of falls in older adults. Sleep is regulated by multiple interconnected neural and molecular pathways; for instance, agonists for the melatonin receptors [3], histamine [4] and orexin receptor antagonists [5,6] are now marketed drugs. Recent evidence suggests that nociceptin/orphanin FQ (N/OFQ) peptide (NOP) receptor activation influences sleep-wake cycles in both animals and humans

Abbreviations: BRET, Bioluminescence Resonance Energy Transfer; cAMP, Cyclic Adenosine Monophosphate; CHO, Chinese Hamster Ovary; CI, Cell Index; DMEM, Dulbecco's Modified Eagle Medium; FSK, Forskolin; GABA-A, Gamma-Aminobutyric Acid Type A; GPCRs, G Protein-Coupled Receptors; HAM's F12, Ham's F12 Nutrient Mix; HEK293, Human Embryonic Kidney 293; HEPES, N-2-Hydroxyethylpiperazine-N'-2-ethanesulfonic acid; HBSS, Hank's Balanced Salt Solution; IBMX, 3-Isobutyl-1-Methylxanthine; mVD, Mouse Vas Deferens; NOP, Nociceptin/Orphanin FQ Peptide; N/OFQ, Nociceptin/Orphanin FQ; RLuc, Renilla Luciferase; RGFP, Renilla Green Fluorescent Protein; [^{35}S]GTPγS, Sulfur-35 Labeled Guanosine 5′-O-(γ-thio)-Triphosphate; REM, Rapid Eye Movement; NREM, Non-Rapid Eye Movement.

☆ This article is part of a special issue entitled: 'GPCRs in Neuropharmacology' published in Biochemical Pharmacology.

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https://doi.org/10.1016/j.bcp.2025.116972
Received 28 March 2025; Received in revised form 16 April 2025; Accepted 30 April 2025
Available online 9 May 2025

[7,8]. The NOP receptor [9,10] is the non-opioid member of the opioid receptor subfamily. NOP receptor is structurally related to classical opioid receptors [11–13], but it does not bind traditional opioid ligands. N/OFQ is a 17-amino acid neuropeptide with very high selectivity for the NOP receptor that does not bind classical opioid receptors. Precedent studies have shown that the NOP receptor is involved in diverse physiological processes, including pain regulation, emotional behavior, stress response, learning and memory, and autonomic control [14]. Differently from classical opioid receptors, NOP activation does not produce respiratory depression or reinforcing effects. NOP receptor ligands [15] have been tested in clinical studies for treating pain (Cebranopadol [16–21]), cough (SCH486757 [21]), and depression and alcohol dependance (LY2940094 [22–24]). Remarkably, N/OFQ was reported to induce sedation and loss of the righting reflex (i.c.v. administration) in rodents by its very discovery [10], observation later confirmed with the non-peptide selective agonists Ro 64–6198 [25], and Ro 65–6570 [26]. It is, however, only recently that the role of the NOP receptor in sleep was reported [7]. Morairty et al., employed rats, mice, and non-human primates and tested the NOP selective agonist Ro 64–6198 on the sleep/wake cycle. Results obtained indicated a dose-dependent increase in non rapid eye movement (NREM) and decrease in rapid eye movement (REM) sleep across species. Importantly, animals (rats) were still arousable upon stimulation even at the highest tested dose. Moreover, the recently reported NOP selective ligand sunobinop (4-[(1*R*,5*S*)-9-[(1*S*,5*S*)-3-bicyclo[3.3.1]nonanyl]-9-azabicyclo[3.3.1]nonan-3-yl]-3-oxoquinoxaline-2-carboxylic acid;4-methylbenzenesulfonic acid) demonstrated potential in managing sleep-related disorders in preclinical as well as clinical studies [8,27,28]. Sunobinop was described as a potent and selective NOP receptor partial agonist and led to a robust decrease in wakefulness and increase in NREM sleep, with concomitant variable decrease in REM sleep in wild type but not NOP knock-out rats. Importantly no statistically significant changes in learning and memory, reward, respiration, and intestinal transit were observed in treated animals. Interestingly enough, the NOP selective antagonist LY2940094 has been reported to evoke in rats effects on NREM sleep which are opposite to those of Ro 64–6198 and sunobinop [22]. Data from a small study of insomnia disorder patients treated with sunobinop showed better sleep efficiency compared to placebo, with increased perceived sleep quality and no relevant side-effects [8]. Altogether, these results validate activation of the NOP receptor as a novel and differentiated option to treat sleep disorders.

Previously reported datasets for sunobinop show high affinity (3.3 nM), and selectivity for the NOP receptor in the radioactive receptor binding assay; the compound displayed as well high potency, selectivity and partial agonist behavior at the NOP receptor when assayed by the stimulation of [^{35}S]GTPγS binding [8]. Here, with the aim of performing a thorough in vitro pharmacological characterization of sunobinop, we employed a wide array of in vitro and ex vivo approaches [29] including calcium-mobilization [30], cAMP levels inhibition [31], receptor-transducer interaction [32], label-free bioimpedance [33], and the electrically stimulated mouse Vas Deferens (mVD) [34,35] assays. All experiments with sunobinop were pursued in parallel with the endogenous neuropeptide N/OFQ and the peptide partial agonist UFP-113 [36].

2. Materials and Methods

2.1. Drugs and reagents

N/OFQ, UFP-113, Dermorphin, and Dynorphin A were synthesized in house following the procedures previously described in detail [36,37]. DPDPE was purchased from Tocris (UK). Sunobinop tosylate was provided by Imbrium Therapeutics L. P.. Stock solutions of ligands were prepared in DMSO (10 mM) and kept at −20 °C. Cell culture and media were purchased from Euroclone (IT). Coelenterazine and Luciferine were from NanoLight Technology (CA, US). Fluo-4 AM was from Thermofisher (MA, US). Forskolin (FSK) and other reagents were from Merck (DE) if not stated elsewhere.

2.2. Calcium mobilization

Chinese Hamster Ovary (CHO) cells stably co-expressing the human NOP, mu, or kappa opioid receptors and the $G\alpha_{qi5}$ protein or the human delta and the $G\alpha_{qG66Di5}$ protein were used in this assay [38,39]. Cells were cultured in Dulbecco's Modified Eagle Medium (DMEM)/ Ham's F12 Nutrient Mix (F12) (1:1) medium supplemented with 10 % FBS, 2 mM l-glutamine, 200 mg/ml G418, 100 mg/ml Hygromycin B, 100 IU/ml penicillin, and 100 IU/ml streptomycin. Cells were maintained at 37 °C in a humidified atmosphere with 5 % CO_2 and were seeded at 50,000 cells/well into 96-well black, clear-bottom plates 24 h prior to test. Loading for 45 min with a solution consisting of Hank's Balanced Salt Solution (HBSS) supplemented with 2.5 mM probenecid, 3 μM Fluo-4 AM, and 0.01 % pluronic acid ensure the calcium-sensitive dye (Fluo-4) to reach the needed concentration inside the cell, while the exchange of the solution with 100 μL/well of buffer consisting of HBSS with 20 mM N-2-Hydroxyethylpiperazine-N'-2-ethanesulfonic acid (HEPES), 2.5 mM probenecid, and 500 μM Brilliant Black allows for fluorescence background to decrease. Serial dilutions of ligands were prepared in HBSS buffer with 20 mM HEPES and 0.02 % bovine serum albumin (BSA) to reduce the affinity of ligands for plasticware. The automated microplate reader FlexStation II (Molecular Device, CA, US) was set to 37 °C and changes in fluorescence intensity detected. Maximum change in percentage over the baseline fluorescence of the vehicle were computed to express the effects of all compounds.

2.3. Inhibition of Forskolin-induced cAMP levels

The bioluminescence-based real-time cAMP accumulation assay (GloSensor-22F, Promega, WI, US) was used to detect cAMP levels in Human Embryonic Kidney (HEK293) cells stably overexpressing the human NOP receptor. Cells were grown in DMEM medium supplemented with 10 % FBS, 2 mM L-Glutamine, 100 mg/ml Hygromycin B, 100 IU/ml penicillin, and 100 IU/ml streptomycin in a humidified atmosphere with 5 % CO_2 at 37 °C. The GloSensor-22F plasmid was transiently transfected in cells by a mixture of DNA (8 μg) and polyethyleneimine (1:3 μg). These experiments were carried out in the presence of 1-hour preincubation in 100 μM 3-isobutyl-1-methylxanthine (IBMX) and 450 μg/mL luciferine (NanoLight Technology, CA, US) in a solution of HBSS. 1 μM forskolin (FSK) was added to the cells to increase cAMP levels that were depressed by increasing concentrations of NOP agonists. FSK was used also as positive control for successful transfection. The effects of all compounds were expressed as change in percentage of the effect of FSK (set to 100 %).

2.4. NOP receptor − G protein and − β-Arrestin 2 interaction

A bioluminescence resonance energy transfer (BRET) interaction assay was used, as previously detailed [32], to study the propensity of ligands to evoke interaction between the NOP receptor to G protein and to β-Arrestin 2. In brief, HEK293 cells stably co-expressing two pairs of fusion proteins were employed i) Renilla Luciferase (RLuc)-tethered human NOP receptor, ii) the bovine $G\beta_1$ tagged with Renilla Green Fluorescent Protein (RGFP), and iii) the human β-arrestin 2 tagged with RGFP. To measure NOP receptor-G protein interaction, membranes taken from HEK293 cells stably co-expressing the fusoproteins NOP-RLuc and Gβ1-RGFP were used, while NOP receptor-β-arrestin 2 coupling was assayed on living cells. Such cells were grown in DMEM medium supplemented with 10 % FBS, 2 mM L-Glutamine, 200 mg/ml G418, 100 mg/ml Hygromycin B, 100 IU/ml penicillin, and 100 IU/ml streptomycin in a humidified atmosphere with 5 % CO_2 at 37 °C. NOP-β-arrestin 2 coupling experiments were performed on living cells that were seeded at a cell density of 100,000 cells per well approx. 24 h prior

to assaying BRET in poly-D-lysine (1 μg/mL) treated white opaque 96 wells microplates (PerkinElmer, CT, US). The day of the experiment, medium was discarded, and cells were incubated in PBS (plus Mg^{2+} 0.5 mM, Ca^{2+} 0.9 mM) and 2 μM Prolume Purple Coelenterazine (NanoLight Technology. CA, US) before agonist injections and bioluminescence reading. Thawed cell membranes were resuspended in PBS (supplemented with 0.01 % BSA) and an amount of 3 μg of total protein was dispensed in each of the 96 well in the presence of 2 μM Prolume Purple Coelenterazine (at room temperature). In agonism experiments, ligands were added and averaged BRET ratios from 10 min measurements computed. BRET ratios were computed as counts per second (CPS) obtained on a Victor Nivo (PerkinElmer, CT, US) with 405 (10) nm and 510 (30) nm bandpass filters. BRET ratios were derived from light passed through 510 divided by that passed through 405 filters. Vehicles BRET values were subtracted from all computations and datasets normalized as a fraction of N/OFQ maximal effects. In antagonism experiments, a 10 min preincubation with vehicle or antagonist preceded the injection of the agonist.

2.5. Bioimpedance studies by XCelligence SP

Complete medium consisting of DMEM supplemented with 10 % FBS, 2 mM L-Glutamine, 100 mg/ml Hygromycin B, 100 IU/ml penicillin, and 100 IU/ml streptomycin was added in a volume of 50 μL to each of 96 wells in a poly-D-lysine coated RTCA E-Plate (Agilent, CA, US) and cell-free baseline recorded by XCelligence SP (Agilent, CA, US). Afterwards, HEK293 cells stably expressing the human NOP receptor were seeded at a density of 75,000 cells / well in the same plate in a volume of 50 μL of complete medium (tot volume 100 μL). Cells were kept in a humid atmosphere with 5 % CO_2 at 37 °C. After approx. 20 h medium was exchanged to HBSS/HEPES 20 mM, variation of the dimensionless Cell Index (CI) was recorded in each well, different concentrations of ligands injected, and values normalized to that in the absence of treatment. CI is function of the relative change in impedance as the experiment progresses and is described as $CI = (Z_t - Z_0) / Z_0$, where Z_t is the impedance at time t and Z_0 is the baseline impedance.

2.6. Mouse Vas Deferens

All animal care and experimental procedures conformed to the standards of the European Communities Council directives (2010/63/EU) and national regulations (D.L. 26/2014). This study was approved by the Animal Welfare Body of the University of Ferrara and by the Italian Ministry of Health (authorization number CBCC2.N.BXI). Experiments were performed on isolated mouse vas deferens. All experiments were conducted with mice bred and housed in the University of Ferrara's animal facility under specific pathogen-free conditions (SPF). All mice were housed in cages with individual ventilation, with a constant temperature of 21°C, 60 % humidity, and with a 12-hour light/dark cycle. Food and water were provided ad libitum. CD-1 male mice, aged 9 to 12 months were used. Animals were sacrificed on the day of the experiment with CO_2 overdose. Bioassay experiments were performed as previously described [35]. The tissues were suspended in 5 mL organ bath containing Krebs solution (NaCl 118.5 nM, KCl 4.7 nM, KH_2PO_4 1.2 nM, $NaHCO_3$ 25 nM, $CaCl_2$ 2.5 nM, glucose 10 nM). The Krebs solution was oxygenated with 95 % O_2 and 5 % CO_2 and temperature set at 33 °C with a resting tension 0.3 g applied to the tissues. Tissues were stimulated through two platinum electrodes with supramaximal rectangular pulse of 1 msec duration, 0.05 Hz frequency, and 80 V of amplitude. The electrically evoked contractions were measured isotonically by means of Basile strain gauge transducers (Basile, IT) and recorder with Power Lab 8 (ADInstruments, CO, US). Following an equilibration period of approx. 60 min, the contractions induced by electrical field stimulation were stable. At this time, cumulative concentration response curves were carried out.

2.7. Data Analysis, Statistics, and terminology

Concentration-response curves to agonists were analyzed by a four-parameter logistic nonlinear regression model: Effect = baseline + (Emax – baseline) / (1 + 10^((log$_{EC50}$ – log[ligand]) slope)). The slope factor, also known as the Hill slope, describes the steepness of the concentration–response curve. In our analysis, the slope was not significantly different from 1. In BRET and bioimpedance experiments the effects were normalized to that of N/OFQ (Emax = 1). Experimental data were expressed as mean ± sem of at least 5 experiments. Potency values were expressed as mean and $CL_{95\%}$. To facilitate maximal effects confrontation, data are represented as α ± sem in Table 1 (α is defined as the intrinsic activity of a given molecule where the standard, N/OFQ, is 1.00). GraphPad Prism 10.0 (GraphPad Software, CA, US) software was used for all the analyses. The terminology employed is consistent with the International Union of Basic and Clinical Pharmacology (IUPHAR) recommendations [40].

3. Results

3.1. NOP receptor activation and selectivity by calcium mobilization assaying

First, we adopted the calcium mobilization assay, which employs NOP receptor stably overexpressed in cells together with a chimeric G protein that force the coupling to Ca^{2+} release (Fig. 1A). N/OFQ produced a robust, concentration-dependent, increase of Ca^{2+} with high potency and maximal effect (pEC_{50} 9.03, Emax 265 %, Fig. 1B). Sunobinop and UFP-113 were tested in parallel experiments and displayed both approx. 10-fold lower potency and slightly reduced maximal effects as compared to N/OFQ (Fig. 1B). Selectivity to the NOP receptor was carried out in cells overexpressing in turn the mu (Fig. 1C), the delta (Fig. 1D), and the kappa (Fig. 1E) receptors. Dermorphin, DPDPE, and Dynorphin A produced a robust concentration-dependent stimulation of calcium release in cells expressing mu, delta and kappa receptors, respectively with high maximal effects and potency values (Fig. 1C–E). Sunobinop and UFP-113 were inactive as agonist at the mu, delta, and kappa receptor (Fig. 1C–E).

3.2. Fsk-stimulated cAMP inhibition assay

Secondly, we employed the cAMP inhibition assay which uses NOP receptor stably overexpressed in cells together with a transiently expressed cAMP sensor (Fig. 2A). N/OFQ could reduce the effect of 1 μM FSK (set to 100 %) to 40 % (Emax −60 %) and displayed very high potency (pEC_{50} 10.39, Fig. 2B). Sunobinop and UFP-113 exhibited reduced maximal effects (Emax ~ -40 %). Potency value for UFP-113 (pEC_{50} 8.80) was approx. 10-fold higher than that of sunobinop (Fig. 2B).

3.3. NOP receptor – Transducer interaction

G protein and β-Arrestin 2 – We then took advantage of a well-established BRET-based approach to capture NOP receptor interaction with G protein or β-Arrestin 2 (Fig. 3A). NOFQ potency was high in mediating NOP receptor to G protein interaction (pEC_{50} 8.93, Fig. 3B). Sunobinop and UFP-113 maximal effects were clearly reduced (0.29 and 0.53, respectively, with N/OFQ set to 1, Fig. 3B). As far as potency is concerned, sunobinop was 125-fold less potent than N/OFQ, while UFP-113 was about 3-fold more potent (Fig. 3B). In a separate approach, N/OFQ was able to foster β-Arrestin 2 recruitment to the NOP receptor with high potency (pEC_{50} 8.40, Fig. 3C), under the present conditions, no agonist activity was detected for both sunobinop and UFP-113 (Fig. 3B). Importantly, we also tested sunobinop against increasing concentrations of N/OFQ in the NOP − β-Arrestin 2 interaction assay. Sunobinop shifted to right the concentration–response curve to N/OFQ without altering its

Table 1
Agonist potency (pEC_{50}) and efficacy (α), and antagonist potency (pA_2) obtained in the indicated assays.

	N/OFQ		sunobinop			UFP-113		
	pEC_{50} ($CL_{95\%}$)	α	pEC_{50} ($CL_{95\%}$)	α ± sem	pA_2 ($CL_{95\%}$)	pEC_{50} ($CL_{95\%}$)	α ± sem	pA_2
$G\alpha_{qi5}$ Ca^{2+}	9.03 (8.37–9.68)	1.00	7.99 (7.43–8.56)	0.71 ± 0.08	–	8.04 (7.32–8.76)	0.83 ± 0.09	–
cAMP inh.	10.39 (10.15–10.62)	1.00	7.62 (7.19–8.04)	0.72 ± 0.09	–	8.80 (8.40–9.20)	0.75 ± 0.08	–
$G\beta_1$	8.93 (7.59–10.30)	1.00	7.22 (6.08–8.36)	0.29 ± 0.10	–	9.51 (8.53–10.50)	0.53 ± 0.10	–
β-Arr. 2	8.40 (8.18–8.57)	1.00	inactive		7.28 (6.97–7.59)	inactive		9.42[a]
Ω	8.00 (6.52–9.47)	1.00	8.07 (6.90–9.24)	0.19 ± 0.05	8.12 (6.82–9.42)	8.84 (8.24–9.46)	0.85 ± 0.08	–
mVD	7.66 (7.42–7.90)	1.00	inactive		7.66 (7.15 – 8.16)	inactive		9.10[b]

α represents the fraction of Emax compared to that of N/OFQ. Inactive: compound was found inactive as agonist. Antagonist potency of UFP-113 is taken from the literature [a] [32], [b] [36].

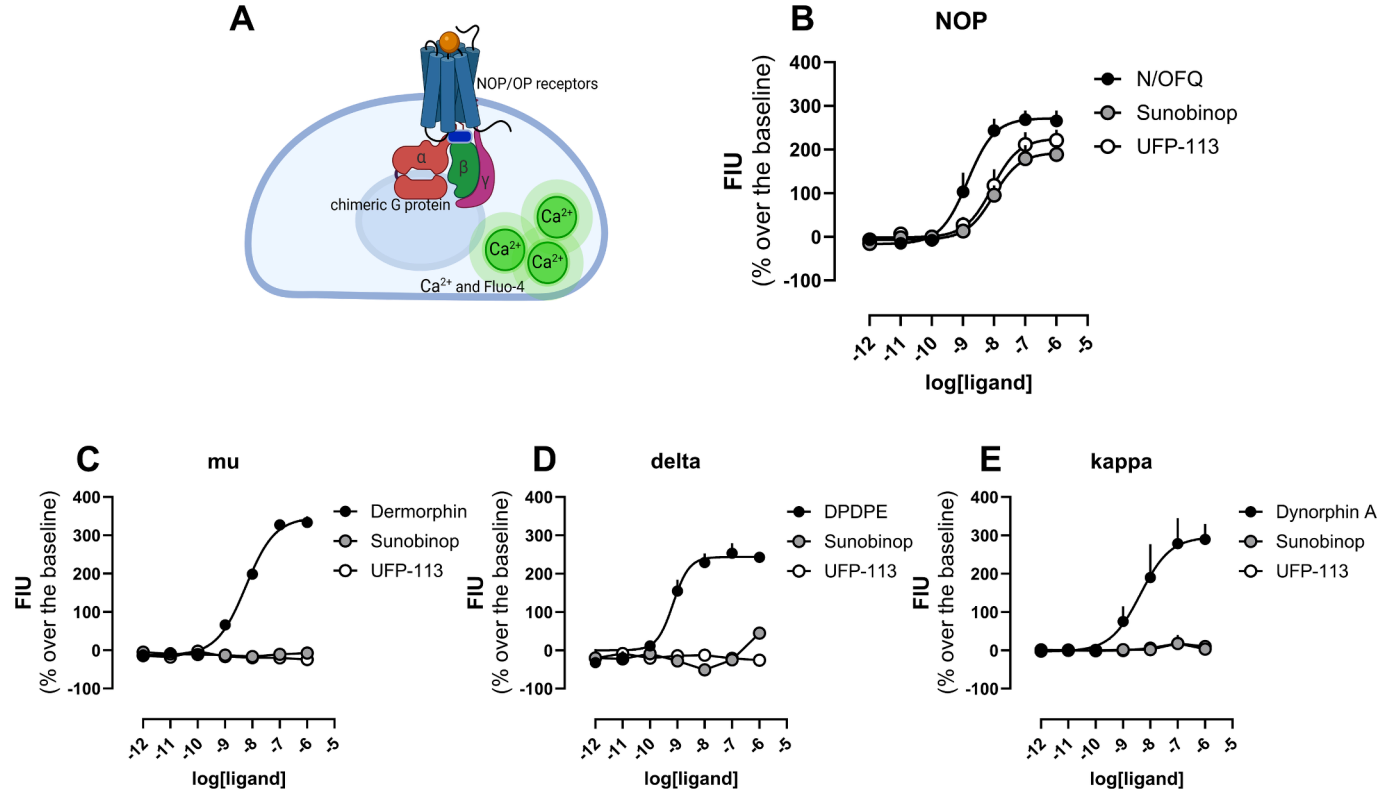

Fig. 1. Sunobinop selectivity of action at the NOP receptor by calcium mobilization – scheme of calcium mobilization assay (A). Concentration-response curves to N/OFQ, dermorphin, DPDPE, and dynorphin A (i.e. standards) on CHO cells stably expressing the NOP (B), mu (C), delta (D), and kappa (E) receptors, respectively. Sunobinop and UFP-113 were tested together with standards from 1 pM to 1 μM. Calcium mobilization is measured in cells stably expressing the receptors and a chimeric G protein. Data are the mean sem of 5 experiments conducted in duplicate. Image A was created with Biorender.

maximal effects (Fig. 3C), and, in agreement with a surmountable type of antagonism, the Schild plot line depicted a slope near to one (Fig. 3D). Sunobinop antagonist potency (pA_2 7.28, Fig. 3D)) in NOP – β-Arrestin 2 interaction experiments was similar to that obtained for NOP – G protein interaction as agonist (Fig. 3B).

3.4. Bioimpedance studies

We could also employ a newly established bioimpedance-based assay, which take advantage of capacity of the XCelligence system to sense variations in bioimpedance (computed as the dimensionless CI) that are function of different types of cellular alterations (Fig. 4A). Here, we adopted HEK293 cells stably expressing the human NOP receptor, and evaluated the effects of N/OFQ, UFP-113, and sunobinop on CI (Fig. 4B–D). All molecules generated a concentration-dependent increase in CI, with potency rank order being UFP-113 > N/OFQ = sunobinop (Fig. 4E). In terms of efficacy, the rank order of the compounds was N/OFQ > UFP-113 ≫ sunobinop (Fig. 4E). In addition, we also carried out antagonism experiments where sunobinop at 0.1 μM displaced to the right the concentration–response curve to N/OFQ with derived potency (pA_2) of 8.12.

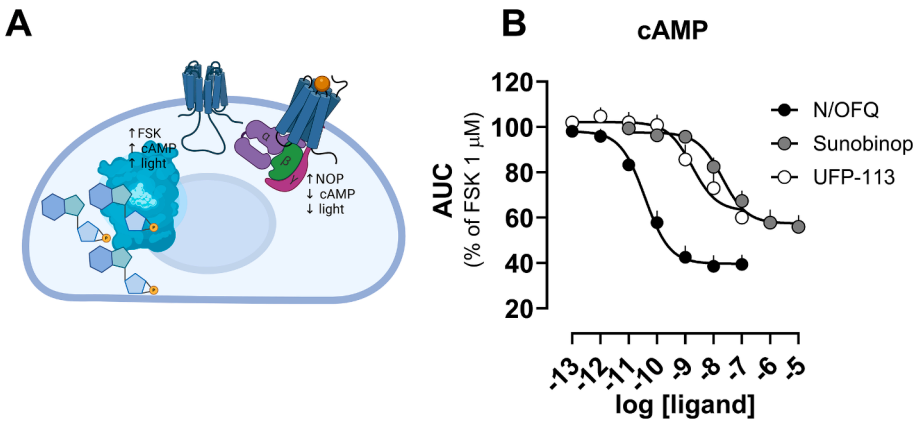

Fig. 2. Effects of sunobinop and standards in inhibiting FSK stimulated cAMP levels – scheme of the cAMP-inhibition assay (A). Increasing concentrations of N/OFQ (0.1 pM – 0.1 μM), UFP-113 (0.1 pM – 0.1 μM), and sunobinop (10 pM – 10 μM) where co-applied with FSK (1 μM) to HEK293 cells stably expressing the NOP receptor and transiently transfected with the cAMP-sensitive GloSensor (B). 30 min effects were summed up by computing each concentration AUC and data represented are the mean ± sem of 5 experiments conducted in duplicate. Image A was created with Biorender.

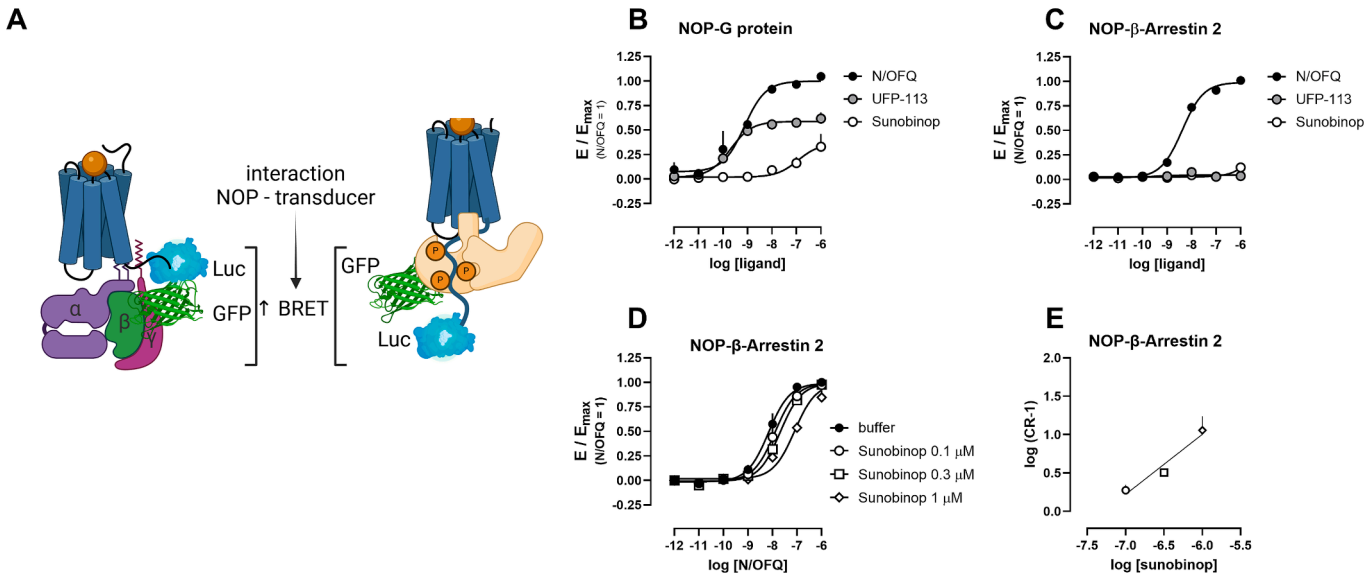

Fig. 3. Effects of sunobinop and standards at NOP receptor – G protein and β-Arrestin 2 interaction – Scheme of the BRET-based NOP-transducer interaction approach (A) Effects of increasing concentrations of N/OFQ (1 pM – 1 μM), UFP-113 (1 pM – 1 μM), and sunobinop (1 pM – 1 μM) on fostering the NOP- G protein (B) and – β-Arrestin 2 (C) interaction. Selected concentrations of sunobinop (0.1, 0.3, 1 μM) were challenged in the presence of increasing concentrations of N/OFQ (1 pM – 1 nM) (D) and data analyzed following the classical Schild's analysis (E). Experiments were carried out in preparations (i.e. membranes for G protein and cells for arrestin) obtained by HEK cells stably expressing the NOP-RLuc fusoproteins and the transducer-RGFP tethered proteins. Data are normalized to N/OFQ Emax and represented as the mean ± sem of 5 experiments conducted in duplicate. Image A was created with Biorender.

3.5. *Electrically stimulated mouse Vas Deferens*

Finally, we adopted a classical bioassay to study sunobinop pharmacological effect in native tissue ex vivo, the electrically stimulated mVD, where the activation of presynaptic NOP receptors leads to inhibition of neurotransmitter release and consequent reduction of the twitch response (Fig. 5A). In brief, both the delta opioid selective agonists DPDPE and N/OFQ concentration dependently inhibited the electrically induced contractions with high maximal effects, and with DPDPE approx. 10-fold more potent than N/OFQ (Fig. 5B). Sunobinop and UFP-113 were inactive as agonists (Fig. 5B). Increasing concentrations of sunobinop shifted to the right the concentration–response curve to N/OFQ without altering its maximal effect (Fig. 5C), while 1 μM sunobinop did not modify the concentration–response curve to DPDPE (Fig. 5D). When the rightward shift of N/OFQ concentration–response curve by sunobinop is computed as Schild analysis (Fig. 5E), results (slope 1.02, pA_2 7.66) suggest that sunobinop competitively antagonizes N/OFQ action in this preparation. All pharmacological parameters obtained in the different assays are summed up in Table 1.

4. Discussion

Sleep-related diseases affect a broad subset of the global population with chronic insomnia alone accounting for something 10–15 % of individuals. Subsequently to classical and recently approved therapeutics to treat sleep disorders such as benzodiazepines and Z-drugs (e.g. zolpidem), histamines and orexin receptors antagonists, NOP receptor agonism is now a subject of current research. NOP receptor selective agonist Ro 64–6198 demonstrated a profound effect on sleep/wake circles in mice, rats, and non-human primates [7], with similar results found in rats with sunobinop, together with the first evidence of safety and hypnotic efficacy in humans [8]. Here, we focused on a thorough pharmacological characterization of sunobinop, in parallel with the endogenous peptide N/OFQ, and the synthetic partial agonist UFP-113,

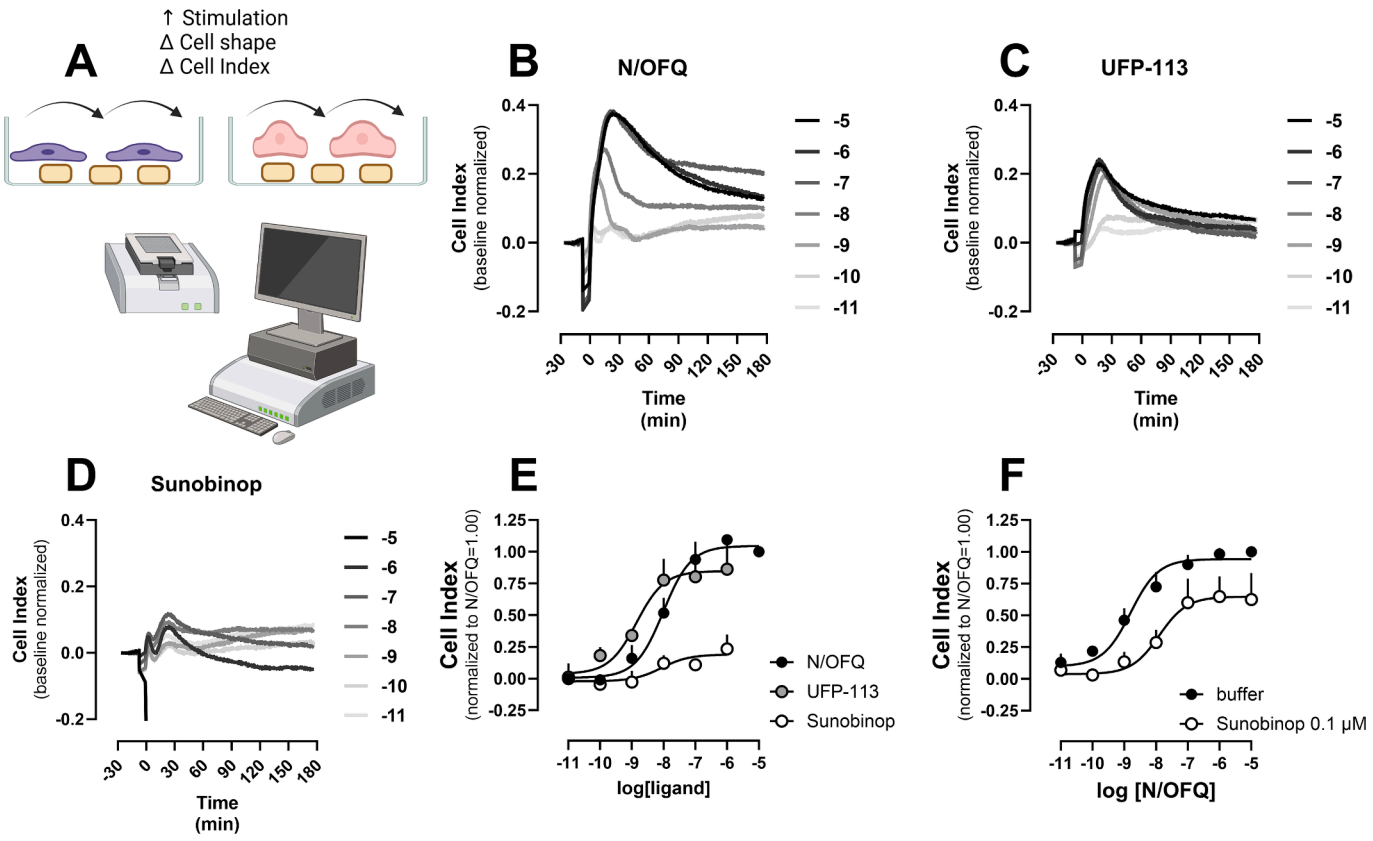

Fig. 4. Effects of sunobinop and standards at NOP receptor by bioimpedance assaying – Scheme of the bioimpedance-based assay (A), Effects of increasing concentrations (represented as Log of the molar concentration) of N/OFQ (10 pM – 10 μM) (B), UFP-113 (10 pM – 10 μM) (C), and sunobinop (10 pM – 10 μM) (D) on cell index in NOP expressing HEK cells. Corresponding concentration–response curves are represented (E). Effects of sunobinop (0.1 μM) on the concentration–response curve to N/OFQ (data are shown as normalized to N/OFQ Emax, F). Curves in panels B-D are single representative experiments conducted in duplicate, while data in E and F are mean ± sem of 5 experiments performed in duplicate. Image A was created with Biorender.

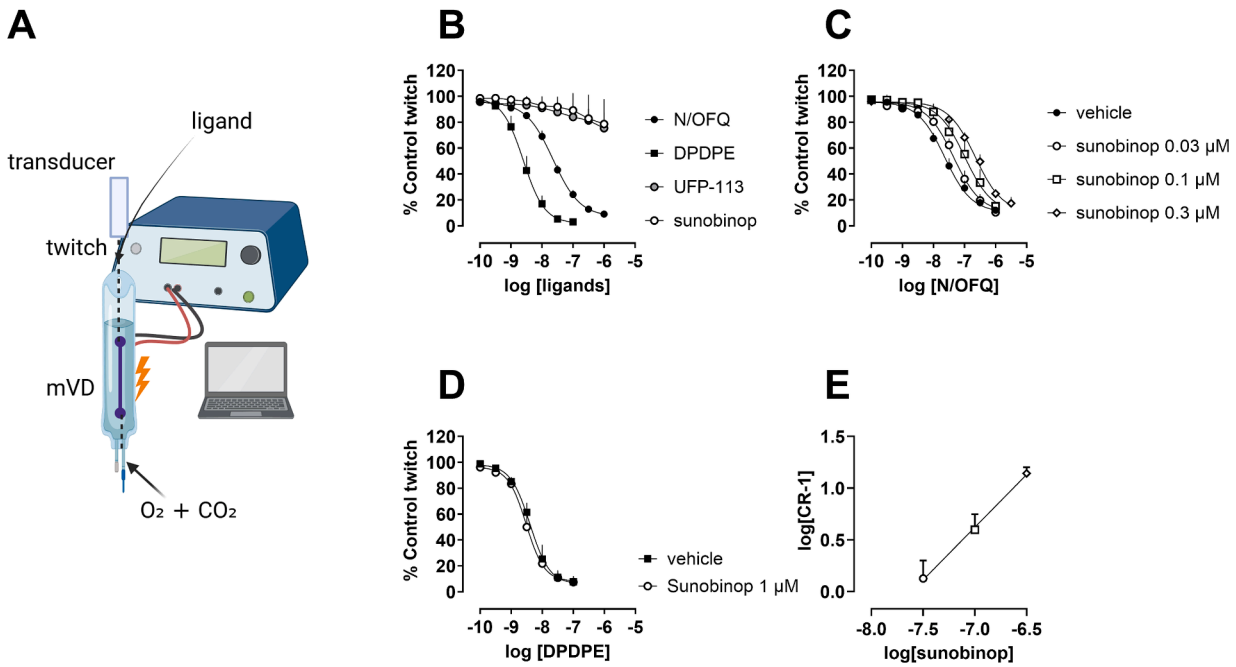

Fig. 5. Effects of sunobinop and NOP standards on the mVD – Scheme of the electrical stimulation of the mVD (A). N/OFQ (0.1 nM – 1 μM), sunobinop (0.1 nM – 1 μM), UFP-113 (0.1 nM – 1 μM), and DPDPE (0.1 nM – 100 nM) concentration–response curves on the mVD (B). Concentration-dependent rightward shift of the concentration–response curve to N/OFQ (0.1 nM – 3 μM) by increasing concentrations of sunobinop (0.03 μM, 0.1 μM, 0.3 μM) (C). Lack of effect for sunobinop (0.1 μM) against the concentration–response curve to DPDPE (0.1 nM – 100 nM) (D). Schild analysis of the data presented in C (E). Data are mean and sem of 5 separate experiments. Image A was created with Biorender.

by employing a wide array of in vitro and ex vivo approaches. In brief, sunobinop behaved as selective partial agonist at the NOP receptor in the calcium-mobilization assays. Sunobinop behavior as partial agonist was then confirmed in NOP – G protein interaction, label-free bioimpedance, and cAMP levels inhibition assays. In addition, antagonism experiments were carried out when the compound displayed no or very low efficacy, as in NOP – β-Arrestin 2 interaction, label-free bioimpedance, and mVD assays. Overall, our results corroborate previous findings [8] and robustly demonstrate that sunobinop, compared to N/OFQ, is a moderate potency, highly selective NOP receptor partial agonist.

Calcium mobilization is a classical assay in industrial drug discovery studies targeting GPCRs coupling to Ca^{2+} and also otherwise cAMP modulating GPCRs [41], thanks to the seminal work of Conklin and colleagues [42] in understanding Gq – GPCRs coupling determinants. Despite the limitations due to the transient nature of the calcium signaling [43] and the aberrant nature of the "unnatural" signaling we extensively applied this approach in several reports with overall preserved pharmacological fingerprints [44–48]. In this study, with calcium mobilization assays, we obtained values of potency and efficacy for standards N/OFQ and UFP-113 at the NOP receptor in line with what was previously reported in the literature [30]. Sunobinop behavior was very similar to that of UFP-113, moderate potency and reduced maximal effects. In addition, Dermorphin, DPDPE, and Dynorphin A displayed high maximal effects and potency at mu, delta, and kappa opioid receptors, respectively, in line with previous datasets [38] while UFP-113 and sunobinop were inactive at classical opioid receptors. The lack of affinity of sunobinop for the delta opioid receptor was also confirmed in mVD bioassay studies where the compound did not modify DPDPE effects. Thus, these results demonstrate high selectivity of action of sunobinop for the NOP receptor in line with previous findings generated by receptor binding and stimulation of GTPγS binding [8]. Importantly, sunobinop selectivity for the NOP receptor was confirmed in vivo in rats where sunobinop hypnotic effects were no longer evident in NOP receptor knock out animals [8].

Then, we employed a bioluminescence-based sensor (GloSensor) to measure the effect of N/OFQ, UFP-113 and sunobinop in inhibiting cAMP levels. As widely described in the literature, to study Gi – coupled GPCRs, cAMP levels must be artificially augmented with FSK and preserved from degradation with IBMX, under these conditions N/OFQ was very potent and both UFP-113 and sunobinop displayed partial agonist effects (α 0.7) with relatively high potency. Values of potency for N/OFQ was in line with what reported with radioactive-based approaches [49,50], the potency of UFP-113 was approx. 50-fold lower than that of N/OFQ, thus aligning with results obtained in calcium mobilization (10-fold loss). As far as sunobinop potency is concerned, it displayed almost 500-fold lower potency than N/OFQ in these experiments. We also took advantage of a BRET-based assay to measure NOP – G protein interaction and NOP – β-Arrestin recruitment, which was based on the seminal work carried out by Molinari and co-workers [51,52] and then largely validated in several previous studies [32,46,53,54]. Here, N/OFQ displayed high potency in fostering G protein and β-Arrestin 2 interactions with the NOP receptor, while UFP-113 behaved as potent partial agonist in NOP – G protein experiments being unable to elicit β-Arrestin 2 recruitment. These results are in full agreement with previously described datasets, where UFP-113 also behaved as antagonist in NOP – β-Arrestin 2 interaction [32]. Sunobinop behaved as moderate potency weak efficacy agonist in mediating G protein interaction, the compound was totally inactive in inducing β-Arrestin 2 recruitment; however, it behaved as competitive antagonist when challenged versus N/OFQ. These results are relevant because, on one hand, they demonstrate that sunobinop exerts its effect in an orthosteric fashion, and on the other, they suggest that sunobinop should be classified as NOP receptor agonist with a bias toward G protein signaling. In this regard it is worth noting that the available information regarding NOP receptor functional selectivity is very limited [55–57] thus the biological implications of NOP biased agonism are at present unknown however, this attribute could impact the clinical profile.

Bioimpedance assaying of sunobinop, in parallel with N/OFQ and UFP-113 was carried out via the XCelligence SP device. We already reported the use of optic-based label-free approaches to study different GPCRs [47,58–60] including the NOP receptor [61]. Notably in this study it has been demonstrated that the dynamic mass redistribution responses to N/OFQ and several different NOP receptor agonists were sensitive to pertussis toxin thus highlighting the involvement of Gi in label free measurements. The present investigation is, however, the first report of assaying the NOP receptor pharmacology by bioimpedance. Nonetheless, this type of label-free system was applied to other GPCRs elsewhere (e.g. [33,62–65]); therefore, we took advantage of the same approach and obtained the following rank order of potency UFP-113 > N/OFQ = sunobinop. Sunobinop efficacy was very low; therefore, the compound could be also tested against N/OFQ where it behaved as competitive antagonist displaying moderate potency. We also adopted a classical ex-vivo approach, the electrically stimulated mVD bioassay which is sensitive to N/OFQ [34,35] and has been consistently demonstrated to be a very useful preparation for investigating the pharmacological properties of novel NOP receptor ligands [66–70]. In this preparation N/OFQ behaved as a moderately potent agonist, while UFP-113 was inactive as an agonist, in line with previous findings showing antagonist behavior [36]. Like UFP-113, sunobinop was inactive as an agonist and behaved as a selective and competitive NOP antagonist in this preparation.

Collectively our findings obtained in different assays allow us to definitively characterize the pharmacological properties of sunobinop. In terms of potency, sunobinop displayed moderate values of potency at the human and mouse receptors both as agonist and antagonist (ranging 8–60 nM). As summarized in Fig. 6A the potency of sunobinop was consistently lower than that of N/OFQ with the only exception of the mVD and bioimpedance studies. The mVD findings could be explained considering that the potency of N/OFQ is increased using peptidase inhibitors [71]; therefore, the preparation contains peptidases that recognize N/OFQ (but not sunobinop) as substrate thus reducing peptide potency. On the contrary, for the relatively low potency of N/OFQ in the bioimpedance studies we do not have a meaningful explanation. Importantly, the moderate potency displayed by sunobinop in the various in vitro assays could impact effective doses in vivo [8].

In terms of agonist efficacy of sunobinop, different results were obtained in the different preparations and assays from pure antagonism to low efficacy partial agonist to almost full agonism. These apparent discrepant effects must be interpreted considering that the maximal effect measured in a given preparation depends both on ligand efficacy and on the efficiency of the stimulus/response coupling of that given preparation (see scheme in Fig. 6B, [72]). This is true in general, and for the NOP receptor has been also experimentally validated in the study by McDonald and co-workers, who used a NOP inducible expression system to measure [F/G]N/OFQ(1–13)–NH_2 (a NOP partial agonist [73]) maximal effect variations [50]. Thus based on these considerations we can classify sunobinop as a NOP partial agonist which displays very low if any efficacy in preparations characterized by low efficiency of the stimulus/response coupling such as the mVD and the β-Arrestin 2 assay, intermediate efficacy in the G protein interaction assay, and almost full efficacy in the calcium and cAMP assays characterized by rather large amplification phenomena that make the efficiency of the stimulus/response coupling very high. Moreover, the maximal effects elicited by sunobinop in the different preparations were similar or slightly lower than those of UFP-113 suggesting no major differences in agonist efficacy between the two ligands. However, in the label free experiments UFP-113 displayed much higher efficacy than sunobinop. Thus, the label free assay seems to be a test very sensitive to difference in ligand efficacy.

Taken together, sunobinop partial agonist activity was thefore confirmed; in addition, we could clearly identify the orthosteric nature

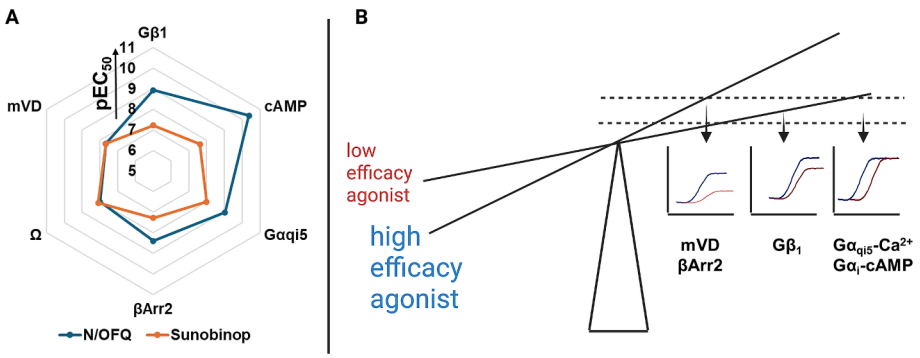

Fig. 6. Comparison of N/OFQ and sunobinop effects across different assays – Potency (A) and efficacy (B) comparison, with emphasis at stimulus-coupling efficiency and signal amplification. This image was created with Biorender. Panel B is inspired by Fig. 2.15 in [72].

of sunobinop interaction with the NOP receptor, thanks to antagonism experiments carried out under specific conditions.

Very intriguingly, the hypnotic effect of sunobinop in rats [8] appears comparable to that evoked by the NOP full agonist Ro 64–6198 [7], while opposite to that of the NOP antagonist LY2940094 [22], though directly comparing results from separate studies remains inherently challenging. We propose that the similar in vivo hypnotic efficacy of sunobinop and Ro 64–6198, one a partial agonist and the other a full agonist, may be attributed to highly efficient stimulus–response coupling in NOP-expressing neurons that regulate sleep-wake functions. Interestingly, the partial agonist properties of sunobinop could also explain the lack of gastrointestinal effects [8] in contrast to the robust constipation measured in response to the full agonists N/OFQ and UFP-112 [74]. It is possible that the inhibitory effect on gastrointestinal functions associated with NOP receptor stimulation is characterized by a low efficiency of stimulus–response coupling. Alternatively, we demonstrated that sunobinop act as a NOP agonist biased toward G protein; therefore, it can be proposed that arrestin signaling can be important for the N/OFQ control on gastrointestinal functions but not sleep. However, these interpretations remain speculative and require experimental validation in future studies.

In conclusion, this study provides a detailed investigation of the pharmacodynamic properties of sunobinop, a novel partial agonist highly selective for the NOP receptor. Sunobinop is currently under clinical evaluation, and it has shown promising potential in the treatment of sleep-related disorders, including insomnia, with significant improvements in sleep efficiency, latency, and overall sleep quality reported in early studies. Additionally, clinical trials are assessing sunobinop effectiveness in addressing moderate to severe alcohol use disorders and incontinence due to overactive bladder. These advancements highlight the therapeutic potential of sunobinop as a novel drug and extend the potential of NOP receptor ligands to treat multiple disorders and pathological conditions [75].

Funding sources

This work was supported by funds from the Italian Ministry of University (PRIN Bando 2022 PNRR (P20225HP4C) to Girolamo Calo'), the University of Padova (DOR grants to Girolamo Calo' and Davide Malfacini), and the University of Ferrara (FAR grant to Chiara Ruzza).

CRediT authorship contribution statement

Riccardo Camilotto: Writing – review & editing, Methodology, Investigation, Formal analysis, Data curation. **Davide Malfacini:** Writing – review & editing, Writing – original draft, Resources, Project administration, Funding acquisition, Conceptualization. **Pietro Pola:** Writing – review & editing, Methodology, Investigation, Formal analysis, Data curation. **Erika Morrone:** Writing – review & editing, Methodology, Investigation, Formal analysis, Data curation. **Alessia Frezza:** Writing – review & editing, Methodology, Investigation, Formal analysis, Data curation. **Rahul Ramalingam:** Writing – review & editing, Methodology, Investigation, Formal analysis, Data curation. **Chiara Sturaro:** Writing – review & editing, Methodology, Investigation, Formal analysis, Data curation. **Salvatore Pacifico:** Writing – review & editing, Resources, Methodology, Investigation. **Chiara Ruzza:** Writing – review & editing, Project administration, Funding acquisition, Conceptualization. **Remo Guerrini:** Writing – review & editing, Resources. **Garth Whiteside:** Writing – review & editing, Writing – original draft, Resources, Conceptualization. **Girolamo Calo':** Writing – review & editing, Writing – original draft, Resources, Project administration, Funding acquisition, Conceptualization.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Acknowledgements

We would like to acknowledge the Department of Pharmaceutical and Pharmacological Sciences, University of Padova for granting access to cell culture and screening facilities. Imbrium Pharmaceuticals L.P. provided the sunobinop tosylate for these experiments.

Data availability

Data will be made available on request.

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