

Detailed In Vitro Pharmacological Characterization of the Clinically Viable Nociceptin/Orphanin FQ Peptide Receptor Antagonist BTRX-246040

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ABSTRACT

The peptide nociceptin/orphanin FQ (N/OFQ) is the natural ligand of the N/OFQ receptor (NOP), which is widely expressed in the central and peripheral nervous system. Selective NOP antagonists are worthy of testing as innovative drugs to treat depression, Parkinson disease, and drug abuse. The aim of this study was to perform a detailed in vitro characterization of BTRX-246040 (also known as LY2940094, [2-[4-[(2-chloro-4,4-difluoro-spiro[5H-thieno[2,3-c]pyran-7,4'-piperidine]-1'-yl)methyl]-3-methyl-pyrazol-1-yl]-3-pyridyl]methanol), a novel NOP antagonist that has been already studied in humans. BTRX-246040 has been tested in vitro in the following assays: calcium mobilization in cells expressing NOP and classic opioid receptors and chimeric G proteins, bioluminescence resonance energy transfer assay measuring NOP interaction with G proteins and β -arrestins, the label-free dynamic mass redistribution assay, and the electrically stimulated

mouse vas deferens. BTRX-246040 was systematically compared with the standard NOP antagonist SB-612111. In all assays, BTRX-246040 behaves as a pure and selective antagonist at human recombinant and murine native NOP receptors displaying 3–10-fold higher potency than the standard antagonist SB-612111. BTRX-246040 is an essential pharmacological tool to further investigate the therapeutic potential of NOP antagonists in preclinical and clinical studies.

SIGNIFICANCE STATEMENT

NOP antagonists may be innovative antidepressant drugs. In this research, the novel clinically viable NOP antagonist BTRX-246040 has been deeply characterized in vitro in a panel of assays. BTRX-246040 resulted a pure, potent, and selective NOP antagonist.

Introduction

The peptide nociceptin/orphanin FQ (N/OFQ) is the natural ligand of the N/OFQ receptor (NOP), which is widely expressed in the central and peripheral nervous system (Lambert, 2008). Through the activation of the NOP receptor, N/OFQ regulates different biologic functions and pathologic states (Lambert, 2008). NOP receptor agonists are now under clinical development for the treatment of hypertension (Kantola et al., 2017), urinary incontinence (Angelico et al., 2019), and pain (Calo and Lambert, 2018; Tzschentke et al., 2019). Several chemically diverse NOP antagonists have been described in the literature, including the peptides [Nphe¹]N/OFQ(1-13)-NH₂ (Calo et al., 2000) and [Nphe¹,Arg¹⁴,Lys¹⁵]N/OFQ-NH₂ (UFP-101) (Calo et al., 2002) and the small molecules J-113397 (Ozaki et al., 2000), SB-612111 (Zaratin et al., 2004), and Compound 24 (C-24) (Goto et al., 2006) [for a review, see Toll et al. (2016) and Zaveri

and Meyer (2019)]. The crystal structure of the NOP receptor in complex with SB-612111 and C-24 has been described (Thompson et al., 2012; Miller et al., 2015). The availability of selective antagonists together with mice (Nishi et al., 1997) and rats (Hombert et al., 2009; Rizzi et al., 2011) knockout for the NOP gene allowed researchers to study the consequences of blocking NOP signaling in animal models of pathology. A large series of studies suggest that selective NOP antagonists may represent innovative drugs to treat depression (Gavioli and Calo, 2013) and Parkinson disease (Mercatelli et al., 2019). Moreover, recent findings suggest that treatment of drug abuse might be an additional indication for NOP antagonists (Rorick-Kehn et al., 2016; Kallupi et al., 2017) [see (Ciccocioppo et al., 2019) for a detailed discussion of this topic].

Recently, a novel NOP receptor antagonist, BTRX-246040 (also known as LY2940094, [2-[4-[(2-chloro-4,4-difluoro-spiro[5H-thieno[2,3-c]pyran-7,4'-piperidine]-1'-yl)methyl]-3-methyl-pyrazol-1-yl]-3-pyridyl]methanol) has been discovered (Toledo et al., 2014). BTRX-246040 displays high affinity, functional potency, and selectivity for the NOP receptor versus classic opioid proteins (Toledo et al., 2014; Statnick et al., 2016). In rodents,

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ABBREVIATIONS: BRET, bioluminescence resonance energy transfer; BTRX-246040, [2-[4-[(2-chloro-4,4-difluoro-spiro[5H-thieno[2,3-c]pyran-7,4'-piperidine]-1'-yl)methyl]-3-methyl-pyrazol-1-yl]-3-pyridyl]methanol; CL, confidence limit; C-24, compound 24; DMR, dynamic mass redistribution; DPDPE, [D-Pen²,D-Pen⁵]enkephalin; HBSS, Hanks' balanced salt solution; HEK, human embryonic kidney; mVD, mouse vas deferens; N/OFQ, nociceptin/orphanin FQ; NOP, N/OFQ peptide receptor; RGFP, recombinant green fluorescent protein; RLuc, Renilla-luciferin 2-monoxygenase; UFP-101, [Nphe¹,Arg¹⁴,Lys¹⁵]N/OFQ-NH₂.

BTRX-246040 produced robust antidepressant effects (Post et al., 2016b; Witkin et al., 2016) and displayed potential therapeutic utility in treating alcohol addiction (Rorick-Kehn et al., 2016) and disorders of appetitive behavior (Statnick et al., 2016). Moreover, in small proof-of-concept clinical studies, BTRX-246040 was safe and well tolerated and showed efficacy in depressed (Post et al., 2016b) and alcohol-dependent (Post et al., 2016a) patients. For an updated review on this NOP ligand, see Witkin et al. (2019).

Despite increasing information regarding the *in vivo* actions of BTRX-246040, our knowledge of the *in vitro* pharmacological profile of this clinically viable NOP antagonist is very limited. Because a well-grounded knowledge of the pharmacological profile of a receptor ligand is based on the analysis of its actions in different assays and preparations, the aim of this study was to carry out a detailed *in vitro* pharmacological characterization of BTRX-246040 using multiple assays, including calcium mobilization in cells coexpressing NOP or classic opioid receptors and chimeric G proteins (Camarda et al., 2009; Camarda and Calò, 2013), bioluminescence resonance energy transfer (BRET) assay measuring NOP interaction with G proteins and β -arrestins (Malfacini et al., 2015), and the label-free dynamic mass redistribution (DMR) assay (Malfacini et al., 2018). Moreover, to obtain information on the action of BTRX-246040 at native NOP receptors, the last series of experiments were done in the electrically stimulated mouse *vas deferens* (mVD) (Calò et al., 1996). In all the above mentioned assays, the effects of BTRX-246040 were compared with those of the standard NOP antagonist SB-612111 (Zaratin et al., 2004; Spagnolo et al., 2007).

Material and Methods

Drugs. N/OFQ, dermorphin, and dynorphin A were synthesized in house. [D-Pen²,D-Pen⁵]enkephalin (DPDPE) and naltrexone were from Tocris Bioscience (Bristol, UK). SB-612111 and BTRX-246040 were provided by BlackThorn Therapeutics. A total of 1 mM stock solution of N/OFQ, dermorphin, DPDPE, and dynorphin A were made in bidistilled water, whereas BTRX-246040, SB-612111, and naltrexone were solubilized in DMSO (10 mM). Stock solutions were kept at -20°C until use.

Calcium Mobilization Assay. CHO cells expressing the human NOP, kappa, or mu receptor and the C-terminally modified G α_{q15} as well as CHO cells expressing the delta receptor and the G α_{q66D15} protein were generated and cultured as previously described (Camarda et al., 2009; Camarda and Calò, 2013). Cells were seeded at a density of 50,000 cells/well into 96-well black, clear-bottom plates 20 hours before the experiment. The day of the experiment, cells were loaded with 2.5 mM probenecid, 3 μ M of the calcium-sensitive fluorescent dye Fluo-4 AM, and 0.01% pluronic acid in Hanks' balanced salt solution (HBSS). After 30 minutes, the loading solution was removed, and 100 μ l/well of HBSS containing 20 mM HEPES, 2.5 mM probenecid, and 500 μ M Brilliant Black (Sigma Aldrich) were added. Serial dilutions were carried out in HBSS/HEPES (20 mM) buffer (containing 0.02% bovine serum albumin fraction V). The plate reader FlexStation II (Molecular Devices, Sunnyvale, CA) was used to measure fluorescence changes. Online additions were carried out in a volume of 50 μ l/well. All the experiments were performed at 37°C. To facilitate drug diffusion into the wells, in antagonism experiments, three cycles of mixing (25 μ l from each well moved up and down three times) were performed immediately after antagonist injection. Antagonists were injected 24 minutes before agonists. Agonist effects were expressed as maximum change in percent over the baseline fluorescence. Baseline fluorescence was measured in wells treated with vehicle.

BRET Assay. HEK293 cells coexpressing the different pairs of fusion proteins (NOP-Renilla-luciferin 2-monooxygenase (RLuc)/G β 1-recombinant green fluorescent protein (RGFP) and NOP-RLuc/ β -arrestin 2-RGFP) were generated and cultured as previously described (Malfacini et al., 2015). Enriched plasma membrane aliquots, used for G-protein experiments, were prepared from transfected cells as described by Vachon et al. (1987). The protein concentration in membrane preparations was determined with the QPRO-BCA kit (Cyanagen Srl, Bologna, IT) and Beckman DU 520 spectrophotometer (Brea, CA). Membrane experiments were performed in 96-well untreated white opaque microplates, whereas whole-cell experiments were performed in 96-well sterile poly-D-lysine-coated white opaque microplates (PerkinElmer, Waltham, MA). The luminometer Victor 2030 (PerkinElmer) was used. For NOP/G-protein interaction evaluation, cell membranes (3 μ g proteins/well) were suspended in Dulbecco's phosphate-buffered saline. For the determination of NOP/ β -arrestin 2 interaction, whole cells (100,000 cells/well) were seeded 24 hours before the experiment. The day of the experiment, cell medium was removed, and PBS containing MgCl₂ (0.5 mM) and CaCl₂ (0.9 mM) was added. Coelenterazine (5 μ M) was injected 15 minutes prior to reading the cell plate. Ligands were added 5 minutes before luminescence reading. In antagonism experiments, BTRX-246040 and SB-612111 were injected into the wells 15 minutes before N/OFQ, and luminescence reading was started 60 minutes after N/OFQ injection. Count per second measured for the RGFP and RLuc light emitted using 510(10) and 460(25) filters (Perkin Elmer), respectively, were used to calculate BRET ratio. All data are expressed as stimulated BRET ratio, i.e., the ratio between count per second from RGFP and RLuc in the presence of ligands minus the BRET ratio in vehicle-treated wells. Maximal agonist effects were expressed as fraction of the N/OFQ maximal effect, which was determined in each plate. All the experiments were performed at room temperature.

DMR Assay. We would like to thank D.G. Lambert (University of Leicester, UK) for kindly providing CHO cells stably expressing the human NOP. Cells were cultured as described by Malfacini et al. (2018). The EnSight Multimode Plate Reader (Perkin Elmer) was used for measuring DMR. Twenty hours before the experiment, cells were seeded (15,000 cells/well/30 μ l) into fibronectin-coated 384-well DMR microplates. Cells were starved in assay buffer (HBSS with 20 mM HEPES, 0.01% bovine serum albumin fraction V) for 90 minutes before the test. Serial dilutions of ligands were made in the assay buffer. After reading baseline, compounds were added in a volume of 10 μ l. Antagonists were incubated 30 minutes before N/OFQ injection, and then DMR changes were recorded for 60 minutes. Responses were described as picometer shifts over time (seconds) following subtraction of values from vehicle-treated wells. Maximum picometer modification (Peak) was used to generate concentration-response curves. All the experiments were carried out at 37°C.

Electrically Stimulated mVD Assay. All animal care and experimental procedures conformed to the European Communities Council directives (2010/63/EU) and national regulations (D.L. 26/2014). Studies involving animals are reported in accordance with the Animal Research: Reporting of In Vivo Experiments (ARRIVE) guidelines (Kilkenny et al., 2010). This study was approved by the Italian Ministry of Health (authorization number 302/2017-PR). The experiments were performed on the mVD. The tissues were taken from male, specific-pathogen-free, CD-1 mice (40 g, Laboratory for Preclinical Research of the University of Ferrara, Italy). Mice were housed under standard conditions (22°C, 55% humidity, 12-hour light/dark cycle, light on at 7:00 AM) with free access to food and water. Appropriate environmental enrichment was present in each cage. Mice were killed with CO₂ overdose. Bioassay experiments were performed as previously described (Calò et al., 1996). The electrically evoked contractions were measured by means of Basile strain gauge isotonic transducers (Basile 7006; Ugo Basile srl, Varese, Italy) and recorder with the Power Laboratory 8 system (AD Instruments, Colorado Springs, CO). After 60 minutes equilibration, the contractions induced by electrical field stimulation were stable. At this time, cumulative concentration-response curves to agonists were performed

(0.5 log unit steps). Antagonists were added 15 minutes before agonists. Data are expressed as percent of the control twitch. The injection of vehicle did not modify the electrically induced twitch. A total number of 10 mice has been used for this study.

Data Analysis and Terminology. The pharmacological terminology adopted in this paper is consistent with the International Union of Basic and Clinical Pharmacology recommendations (Neubig et al., 2003). All data are expressed as the mean \pm S.E.M. of at least three experiments. For potency values, 95% confidence limits ($CL_{95\%}$) were indicated. Agonist potencies are given as pEC_{50} , which is the negative logarithm to base 10 of the molar concentration of an agonist that produces 50% of the maximal effect of that agonist. Concentration-response curves to agonists were fitted to the classic four-parameter logistic nonlinear regression model as follows: $Effect = Baseline + (E_{max} - Baseline)/(1 + 10^{(LogEC_{50} - Log(compound)) * Hillslope})$. Curves fitting were performed using PRISM 6.0 (GraphPad Software Inc., San Diego, CA). SB-612111 was assayed at single concentrations against the concentration-response curve to N/OFQ, and its potency was expressed as pA_2 derived from the following equation: $pA_2 = -\log[(CR - 1)/[A]]$, assuming a slope value equal to unity, where CR indicates the ratio between agonist potency in the presence and absence of antagonist, and [A] is the molar concentration of the antagonist (Kenakin, 2004). BTRX-246040 was tested using the classic Schild protocol. Antagonist potencies were expressed either as pA_2 or pK_B , depending on the type of antagonism. For competitive type of antagonism, pA_2 values were derived from the classic Schild protocol. For noncompetitive types of antagonism, pK_B values were obtained by the Gaddum method (Gaddum et al., 1955). In practice,

equiactive concentrations of the agonist in the absence ([A]) and presence ([A']) of a noncompetitive antagonist ([B]) are compared in a double reciprocal plot describing a straight line, and pK_B was derived from the following equation: $pK_B = \log[(slope - 1)/[B]]$. Data have been statistically analyzed using one-way ANOVA followed by the Dunnett's post hoc test. $P < 0.05$ was considered statistically significant.

Results

In $CHO_{NOP} + G_{\alpha q15}$ cells N/OFQ increased calcium levels in a concentration-dependent manner, showing high potency [pEC_{50} value of 9.65 (9.03–9.98)] and maximal effects (E_{max} 353% \pm 15%), whereas SB-612111 and BTRX-246040 were inactive up to 10 μ M (Fig. 1A). SB-612111, 100 nM, shifted to right the concentration-response curve to N/OFQ without changing the agonist maximal effect. A pA_2 value of 8.86 ($CL_{95\%}$ 8.31–9.41) was calculated from these experiments (Fig. 1B). BTRX-246040, in the range of concentrations 1–1000 nM, elicited a concentration-dependent dextral displacement of the concentration-response curve to N/OFQ, with a slight depression of maximal effects at the higher concentrations tested (Fig. 1C). A pA_2 value of 9.27 ($CL_{95\%}$ 8.85–9.69) was extrapolated from the Schild plot (Fig. 1D). In CHO cells expressing chimeric G proteins and classic opioid receptors, standard agonists (dermorphin for mu, DPDPE for delta, and

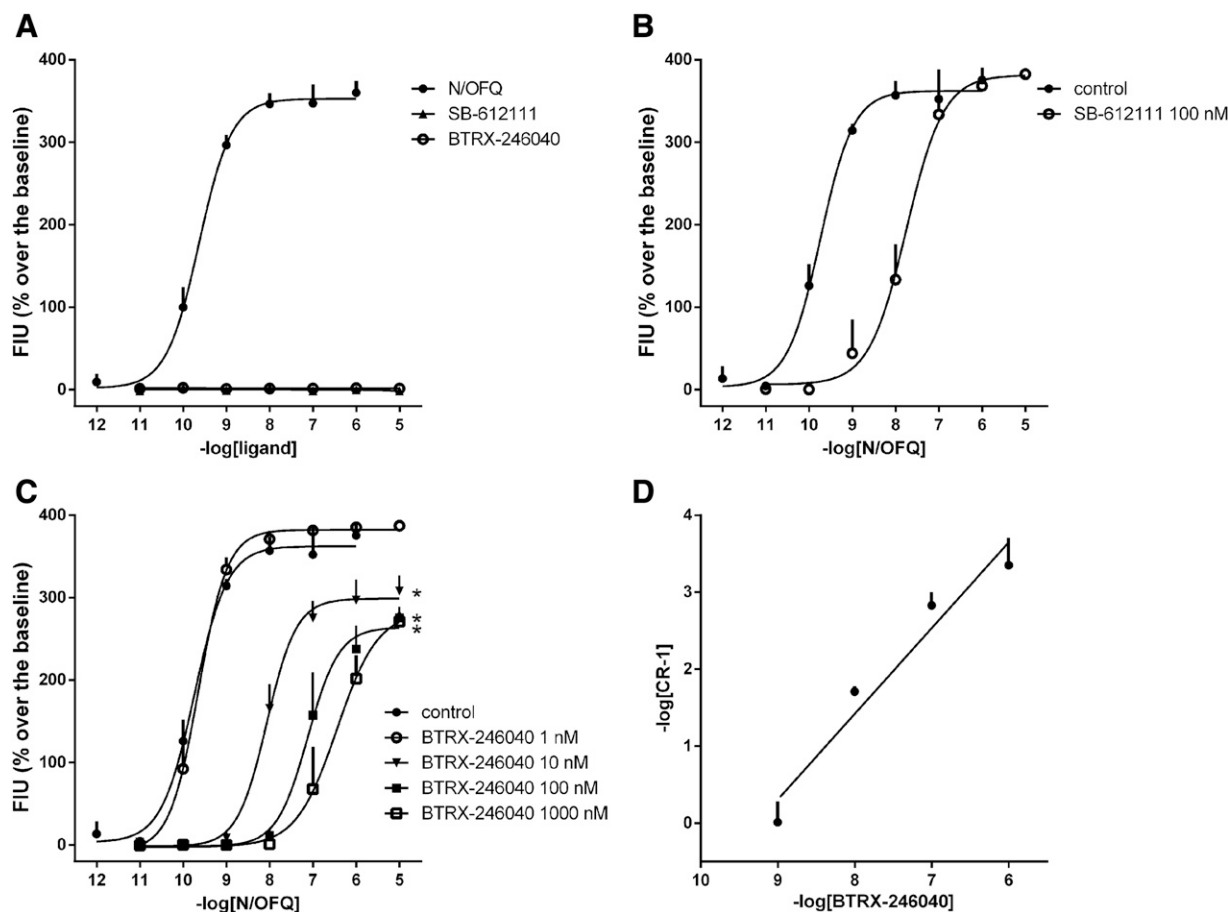


Fig. 1. Calcium mobilization assay. Concentration-response curves to N/OFQ, SB-612111, and BTRX-246040 are displayed in (A). Concentration-response curves to N/OFQ in the absence and presence of SB-612111 100 nM and BTRX-246040 1–1000 nM are shown in (B and C), respectively. The corresponding Schild plot is shown in (D). Data are mean \pm S.E.M. of three (A) or four (B–D) separate experiments performed in duplicate. * $P < 0.05$ vs. control, according to one-way ANOVA followed by the Dunnett post hoc test.

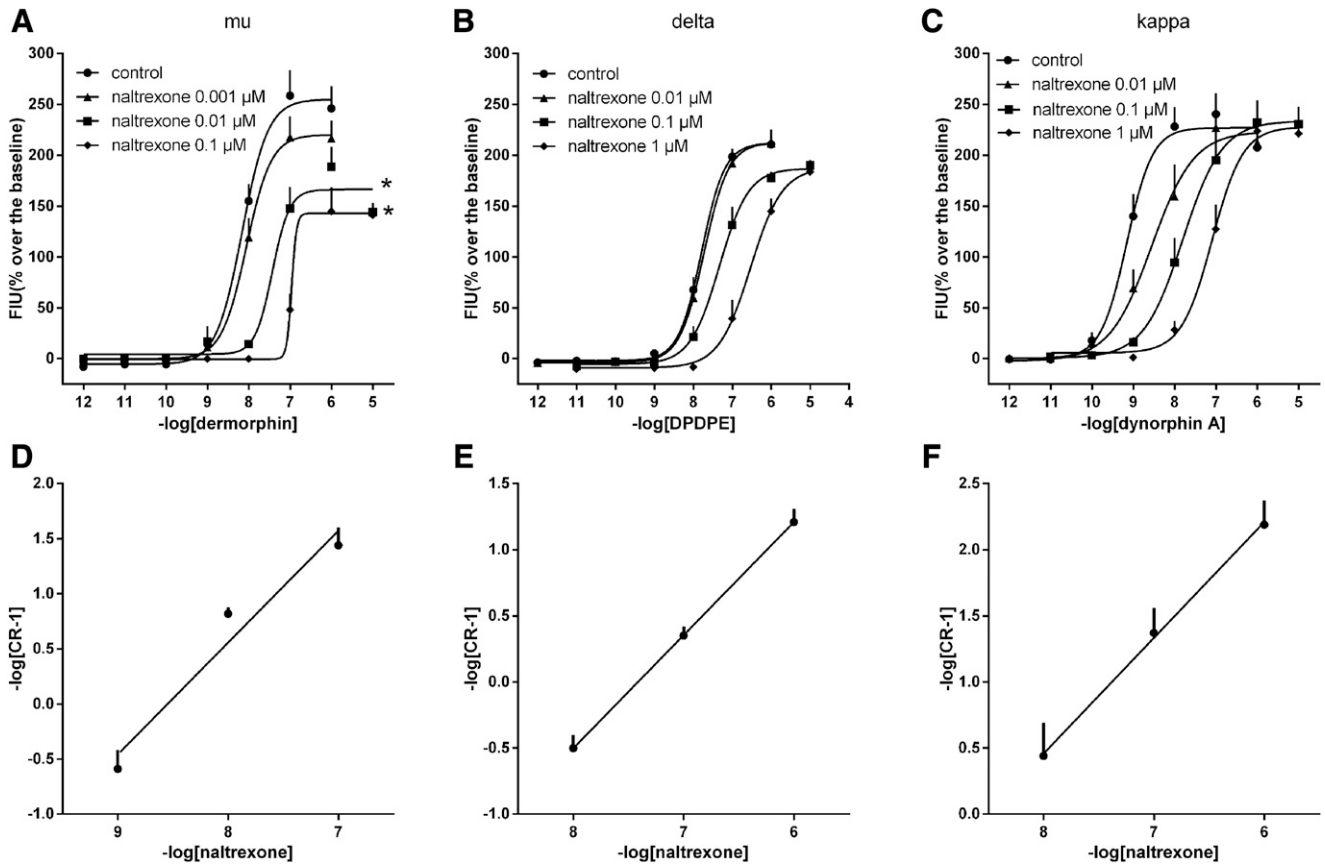


Fig. 2. Calcium mobilization assay in CHO cells coexpressing the mu (left panels), delta (middle panels), and kappa (right panels) receptors and chimeric G proteins. Concentration-response curves to dermorphin (A), DPDPE (B), and dynorphin A (C) in absence and presence of increasing concentrations (0.01–1 μ M) of naltrexone. The corresponding Schild plots are shown in (D–F). Data are mean \pm S.E.M. of five separate experiments performed in duplicate. * $P < 0.05$ vs. control, according to one-way ANOVA followed by the Dunnett post hoc test.

dynorphin A for kappa receptors) elicited concentration-dependent stimulatory effects that were competitively antagonized by naltrexone with pA_2 values of 8.55 (CL_{95%} 8.07–9.03), 7.42 (CL_{95%} 7.17–7.67), and 8.53 (CL_{95%} 7.78–9.28), respectively (Fig. 2). At 1 μ M, SB-612111 was inactive at mu and delta receptors but behaved as a low potency antagonist at the kappa receptor (pA_2 6.14, 5.69–6.59). One micromolar BTRX-246040 was inactive at delta and kappa receptors while producing a small but consistent shift of the concentration-response curve to dermorphin (pA_2 6.60, 6.34–6.85) (Fig. 3).

In membranes prepared from HEK293 cells stably coexpressing the NOP/RLuc and G β 1/RGFP fusoproteins, N/OFQ promoted receptor/G protein interaction in a concentration-dependent manner, showing high potency [pEC₅₀ 8.94 (8.73–9.15)] and maximal effects. In parallel experiments, SB-612111 was inactive, whereas BTRX-246040 elicited a very small reduction of the BRET ratio in a concentration-dependent manner (Fig. 4A). SB-612111, 10 nM, produced a rightward shift of the concentration-response curve to N/OFQ with no changes of the E_{max} . A pA_2 value of 8.96 (CL_{95%} 8.05–9.87) was calculated from these experiments (Fig. 4C). BTRX-246040, in the range of concentrations 1–1000 nM, elicited a concentration-dependent dextral displacement of the concentration-response curve to N/OFQ with a significant reduction of N/OFQ E_{max} (Fig. 4E). A pK_B value of 9.53 (8.65–10.41) was calculated for BTRX-246040 from these experiments. In HEK293 cells stably coexpressing the NOP/RLuc and the β -arrestin 2/RGFP fusoproteins, N/OFQ

promoted receptor/ β -arrestin 2 interaction in a concentration-dependent manner with high potency [pEC₅₀ value of 8.23 (8.16–8.30)] and maximal effects, whereas SB-612111 and BTRX-246040 were inactive up to micromolar concentrations (Fig. 4B). SB-612111, 10 nM, produced a dextral displacement of the concentration-response curve to N/OFQ without modifying agonist maximal effect, with a pA_2 of 8.70 (CL_{95%} 7.91–9.49) (Fig. 4D). BTRX-246040, 1–1000 nM, elicited a concentration-dependent rightward shift of the concentration-response curve to N/OFQ, with a large depression of maximal effects (Fig. 4F). A pK_B value of 9.16 (8.00–10.32) was calculated from these experiments.

In CHO_{NOP} cells, N/OFQ elicited a concentration-dependent positive DMR response, whereas SB-612111 was inactive up to micromolar concentrations. BTRX-246040 elicited a very small negative DMR response in a concentration dependent manner (Fig. 5A). In the presence of 100 nM SB-612111, the curve to N/OFQ was rightward shifted with no changes of the agonist maximal effects. A pA_2 value of 8.21 (CL_{95%} 7.32–8.75) was derived for SB-612111 from these experiments (Fig. 5B). BTRX-246040, in the 10–1000 nM range, promoted a concentration-dependent dextral displacement of the concentration-response curve to N/OFQ, associated with a small reduction of maximal effects (Fig. 5C). The Schild analysis of these data yielded a pA_2 of 8.88 (CL_{95%} 8.63–9.13) (Fig. 5D).

In the isolated mVD, N/OFQ inhibited the electrically induced twitch in a concentration-dependent manner with

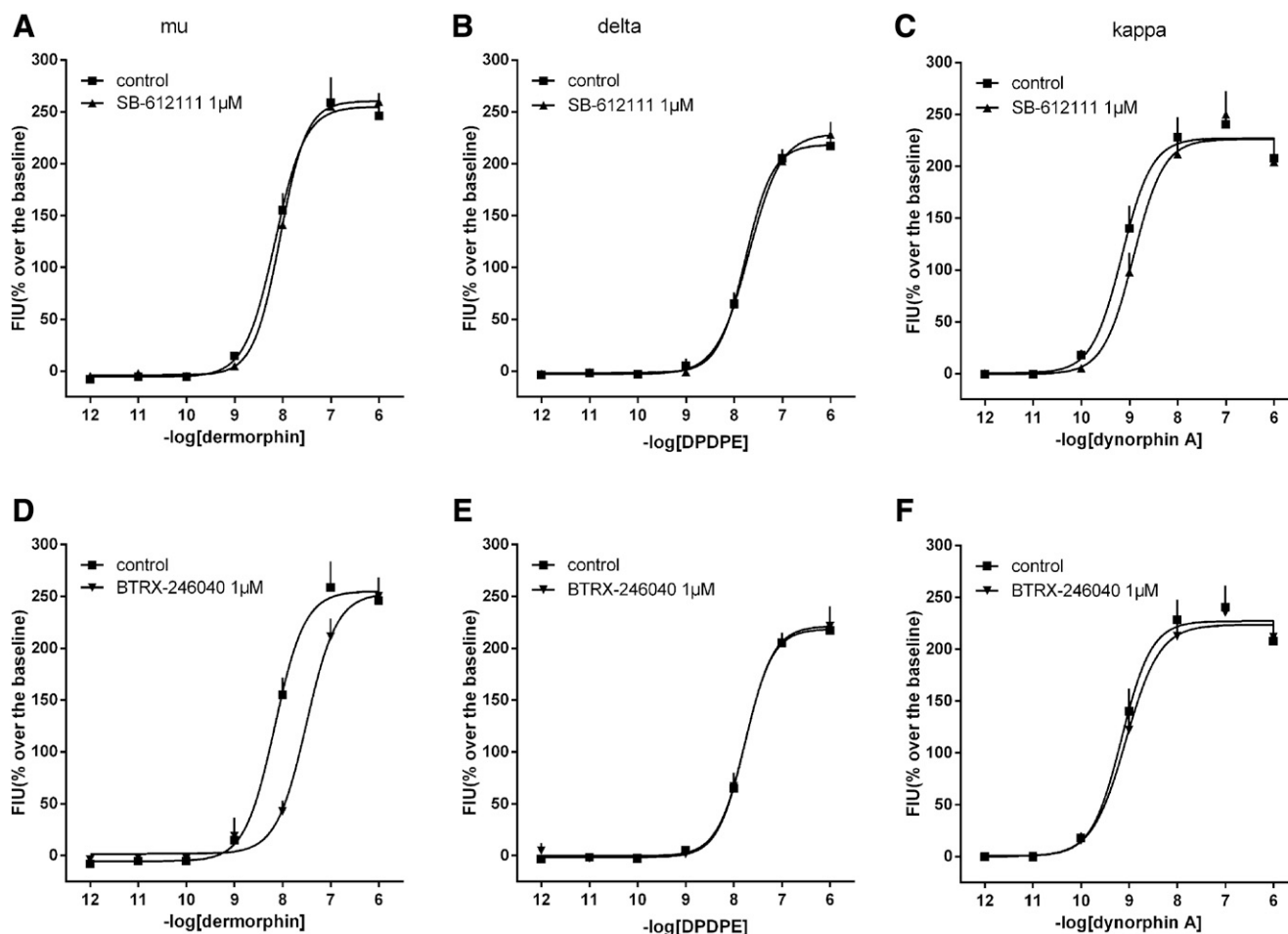


Fig. 3. Calcium mobilization assay in CHO cells coexpressing the mu (left panels), delta (middle panels), and kappa (right panels) receptors and chimeric G proteins. Concentration-response curves to dermorphin, DPDPE, and dynorphin A in absence and presence of SB-612111 1 μM (A–C, respectively) and BTRX-246040 1 μM (D–F, respectively). Data are mean ± S.E.M. of five separate experiments performed in duplicate.

a pEC_{50} of 7.45 (7.28–7.62), whereas SB-612111 and BTRX-246040 were inactive up to 1 μM. At 100 nM, SB-612111 elicited a rightward shift of the curve to N/OFQ, displaying a pA_2 value of 7.89 ($CL_{95\%}$ 7.59–8.19) (Fig. 6A). BTRX-246040, in the 10–1000 nM range, elicited a concentration-dependent dextral displacement of the concentration-response curve to N/OFQ without changing its E_{max} (Fig. 6B). The Schild analysis of this set of data were compatible with a competitive type of interaction and yielded a pA_2 value of 8.45 ($CL_{95\%}$ 7.91–8.99) (Fig. 6C). Finally, under the same experimental conditions, 1 μM BTRX-246040 did not modify the inhibitory effects elicited by the delta agonist DPDPE (Fig. 6D).

The results obtained in this study, together with data from literature, have been summarized in Table 1.

Discussion

The present study extends previous findings (Toledo et al., 2014; Statnick et al., 2016) demonstrating that BTRX-246040 behaves as a pure and selective antagonist at human recombinant and murine native NOP receptors, displaying 3–10-fold higher potency than the standard antagonist SB-612111.

BTRX-246040 behaved as a pure NOP antagonist; this is demonstrated by the lack of effect per se in the calcium mobilization and bioassay studies as well as in previous

GTPγS-binding experiments (Statnick et al., 2016). In NOP/G protein BRET and DMR experiments, BTRX-246040 elicited very small effects opposite to those of N/OFQ, thus suggesting an inverse agonist behavior of this NOP ligand. However, the putative effects of BTRX-246040 were too small to be further investigated. It should be mentioned in this regard that the NOP receptor has very low liability to display constitutive activity. This has been clearly demonstrated in previous receptor/G protein BRET studies in which, under the same experimental conditions, GDP inhibited in a concentration-dependent manner mu and particularly delta opioid receptor interaction with G protein (Vezzi et al., 2013) while producing negligible effects on NOP/G protein interaction (Malfacini et al., 2015). Thus, to investigate the possible inverse agonist activity of BTRX-246040, further studies employing constitutively active mutants of the NOP receptor, e.g., NOP_{N133W} (Kam et al., 2002), or overexpression of NOP receptors via microinjection of cDNA plasmids (Mahmoud et al., 2010) are needed.

As far as type of antagonism is concerned, contrasting results were obtained in the various assays. In fact, although a typical competitive interaction for BTRX-246040 and N/OFQ has been measured in the mVD bioassay, a small but statistically significant depression of N/OFQ maximal effects has been induced by high concentrations of BTRX-246040 in calcium

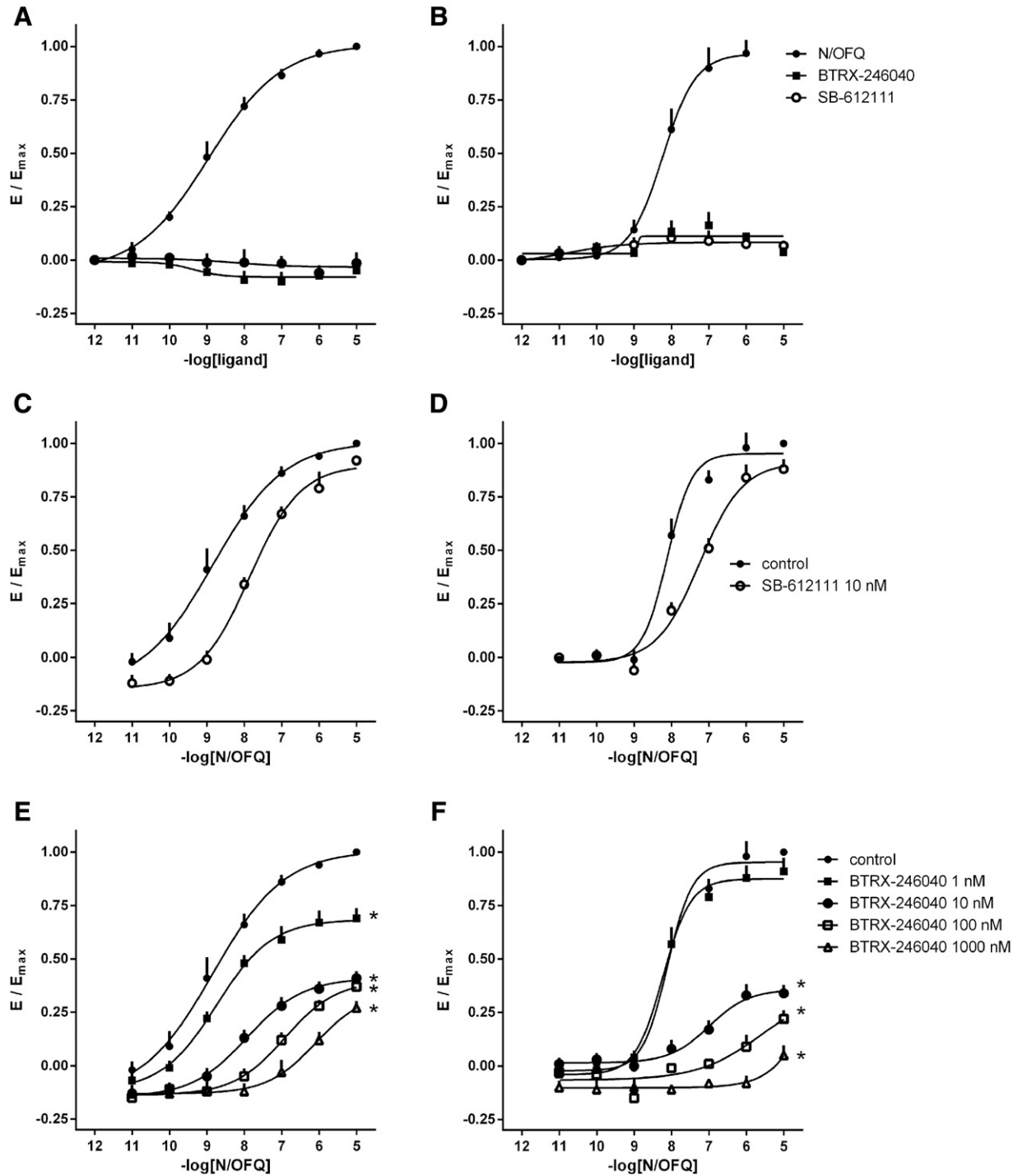


Fig. 4. BRET assay. NOP/G-protein (left panels) and NOP/ β -arrestin 2 interaction (right panels) experiments. Concentration-response curves to N/OFQ, SB-612111, and BTRX-246040 are displayed in (A and B). Concentration-response curves to N/OFQ in the absence and presence of SB-612111 10 nM (C and D) and BTRX-246040 1–1000 nM (E and F). Data are mean \pm S.E.M. of five separate experiments performed in duplicate. * $P < 0.05$ vs. control, according to one-way ANOVA followed by the Dunnett post hoc test.

mobilization and DMR experiments, whereas a more profound reduction of agonist maximal effects was measured in BRET studies. These variable results deserve attention. For calcium mobilization experiments, it is well known that the rapid and transient nature of calcium peaks causes hemi-equilibrium conditions, and this may lead to an apparent insurmountable behavior of competitive antagonists (Charlton and Vauquelin, 2010). In calcium mobilization studies, the NOP competitive

antagonist C-24 generated results very similar to those reported here with BTRX-246040 (Fischetti et al., 2009). In previous NOP/G protein BRET studies, it has been reported that after 5 minutes of incubation with N/OFQ, the antagonists UFP-101 and J-113397 displayed a surmountable type of antagonism, whereas C-24 and SB-612111 caused a significant depression of N/OFQ maximal effects; this depression was no longer evident by prolonging the incubation time with N/OFQ

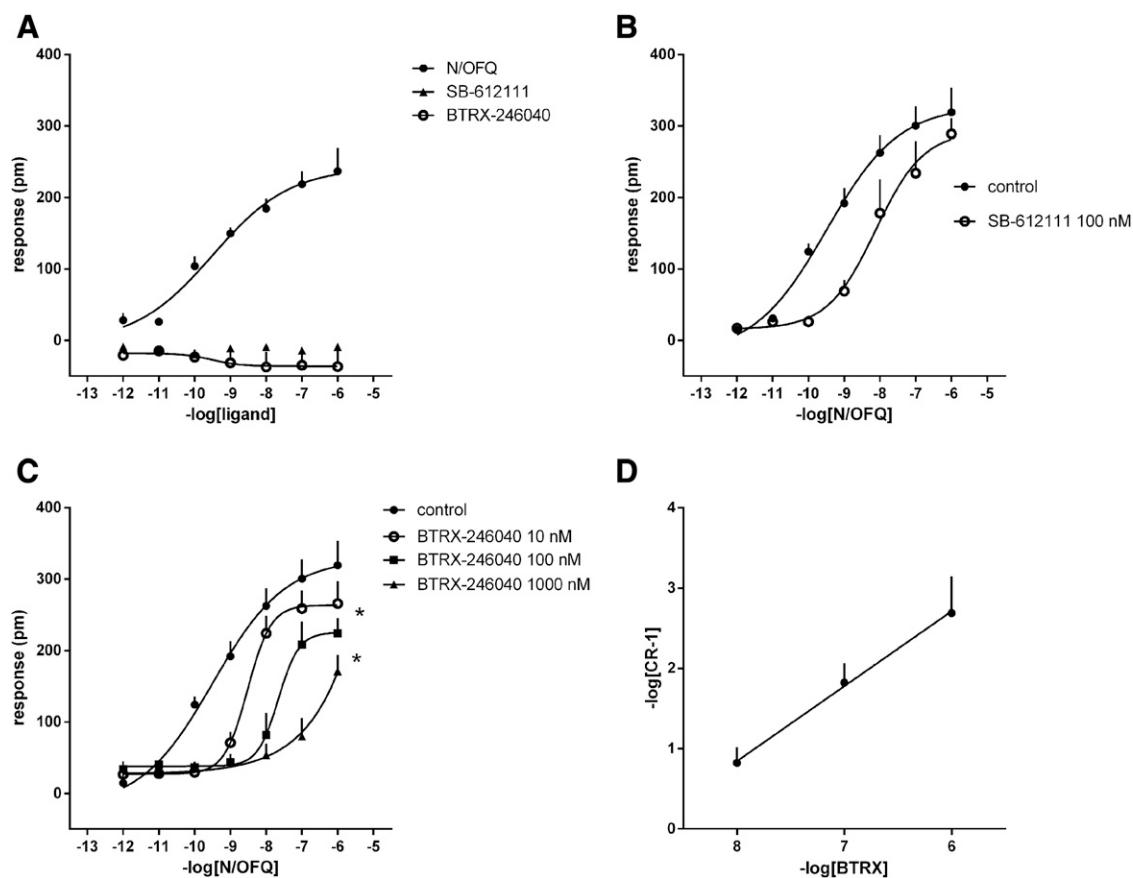


Fig. 5. DMR assay. Concentration-response curves to N/OFQ, SB-612111, and BTRX-246040 are displayed in (A). Concentration-response curves to N/OFQ in the absence and presence of SB-612111 100 nM and BTRX-246040 10–1000 nM are shown in (B and C), respectively. The corresponding Schild plot is shown in (D). Data are mean \pm S.E.M. of four separate experiments performed in duplicate. * $P < 0.05$ vs. control, according to one-way ANOVA followed by the Dunnett post hoc test.

to 15 minutes (Malfacini et al., 2015). Under these experimental conditions (15 minutes incubation time with the agonist), BTRX-246040 displayed an insurmountable type of antagonism (data not shown). Based on the previous findings obtained with SB-612111, we further prolonged incubation time to 30 and 60 minutes. However, even though the depression of N/OFQ E_{max} was smaller with longer incubation times, a depression of N/OFQ maximal effects was still clearly evident after 1 hour of incubation. Because multiflexible docking studies (Della Longa and Arcovito, 2018) clearly demonstrated that BTRX-246040 binds the NOP receptor in the same binding pocket recognizing competitive antagonists such as C-24 (Thompson et al., 2012) and SB-612111 (Miller et al., 2015), it is reasonable to suggest that the BTRX-246040 unsurmountable behavior derives from a very slow rate of offset that does not allow equilibrium to be reached even with prolonged incubation times (Kenakin, 2004). The involvement of kinetic factors in the insurmountable behavior of BTRX-246040 is also suggested by the fact that the maximal effects of N/OFQ were more depressed measuring receptor/ β -arrestin 2 than receptor/G protein interaction. This may be due to the fact that the kinetics of receptor/G protein interaction is much faster (half time in the range 6–12 seconds) than that of receptor/ β -arrestin 2 interaction (half time in the range 60–130 seconds) (Molinari et al., 2010). The above considerations may also explain the insurmountable behavior displayed by BTRX-246040 in DMR experiments; however,

they go against the typical competitive interaction displayed by the antagonist in the electrically stimulated mVD assay. Although a large series of studies demonstrated that the murine NOP receptor displays a pharmacological profile very similar if not identical to that of the human NOP receptor [see table 1 in Malfacini and Calo (2019)], it is still possible that subtle species-specific receptor differences may explain the different behavior of BTRX-246040.

As far as antagonist potency is concerned, BTRX-246040 displayed very high and consistent potency (range 8.45–9.58) in the various assays; these results are in line with the potency (9.77) previously reported in stimulated GTP γ S-binding studies as well as with the affinity (9.98) obtained in receptor-binding experiments (Statnick et al., 2016). Importantly, compared with the standard NOP antagonist SB-612111 in parallel experiments, BTRX-246040 was always found to be 3–10-fold more potent. Of note, the values of SB-612111 potency obtained in the present study in the different assays were very close to those previously reported in the literature (Spagnolo et al., 2007; Malfacini et al., 2015, 2018; Ferrari et al., 2017). Therefore, based on present and previous findings [reviewed in Toll et al. (2016), Malfacini and Calo (2019)], we may propose a rank order of potency of antagonists to be BTRX-246040 > SB-612111 > J-113397 > UFP-101 as a NOP pharmacological fingerprint. Moreover, a strong correlation between NOP antagonist potency and receptor thermostability has been previously demonstrated that is consistent with a reduction

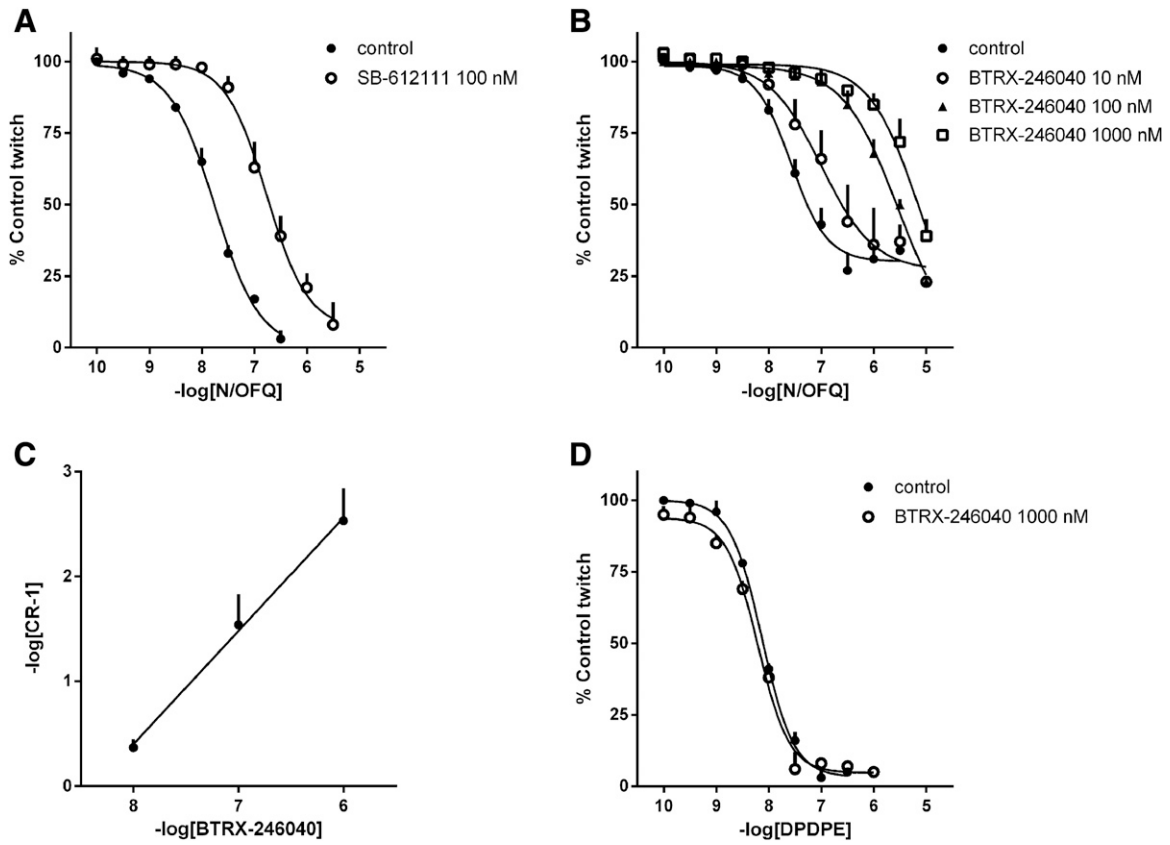


Fig. 6. mVD assay. Concentration-response curves to N/OFQ in the absence and presence of SB-612111 100 nM (A) and of BTRX-246040 10–1000 nM (B). The corresponding Schild plot is shown in (C). (D) The concentration-response curves to DPDPE in absence and presence of BTRX-246040 1000 nM. Data are mean \pm S.E.M. of five separate experiments performed in duplicate.

in receptor flexibility by shifting the conformational equilibrium exclusively to the receptor inactive state (Miller et al., 2015). On this basis, it is reasonable to suggest that BTRX-246040 is an ideal candidate for further NOP crystalization studies.

Together with potency, selectivity of action is another key feature of a receptor antagonist. In calcium mobilization studies, BTRX-246040 was approximately 1000-fold selective for NOP over classic opioid receptors, and in bioassay studies, the estimated NOP selectivity of BTRX-246040 over delta receptor was >300 . These results are perfectly in line with published receptor-binding data (Statnick et al., 2016) that demonstrated that BTRX-246040 affinity is >3000 -fold higher for NOP than opioid receptors. In parallel experiments,

SB-612111 displayed high NOP selectivity, confirming previous findings (Zaratin et al., 2004; Spagnolo et al., 2007). Thus, collectively, these findings suggest that BTRX-246040 is more potent and as NOP selective as SB-612111. The high NOP selectivity of BTRX-246040 demonstrated in vitro is corroborated by in vivo findings; in fact, the antidepressant (Witkin et al., 2016) and anorectic (Statnick et al., 2016) actions of BTRX-246040 were no longer evident in mice knockout for the NOP receptor gene.

In conclusion, the results of the present study demonstrated that BTRX-246040 is a pure, potent, and selective antagonist of the NOP receptor. Compared with the standard NOP antagonist SB-612111, BTRX-246040 displays similar high NOP selectivity but is 3–10-fold more potent. These pharmacodynamic features are associated with great pharmacokinetic properties demonstrated in rodents and humans (Raddad et al., 2016). In addition, the tolerability and safety of BTRX-246040 once-daily dosing of 40 mg up to 8 weeks have been already demonstrated in clinical studies (Witkin et al., 2019). Collectively, these features candidate BTRX-246040 as an essential pharmacological tool to further investigate the therapeutic potential of NOP antagonists as innovative drugs to treat depression (Gavioli and Calo', 2013), Parkinson disease (Mercatelli et al., 2019), and possibly drug abuse (Ciccocioppo et al., 2019) as well as to identify novel conditions/diseases for which the blockade of the NOP receptor is associated with beneficial effects, including traumatic injuries of the central nervous system (Awwad et al., 2018; Sekine et al., 2018), post-traumatic stress disorders (Zhang et al., 2015;

TABLE 1

Comparison of the NOP affinity and antagonistic potency of BTRX-246040 and SB-612111

Receptor-binding and stimulated GTP γ S-binding data are taken from Statnick et al. (2016) for BTRX-246040 and from Spagnolo et al. (2007) for SB-612111. Receptor-binding data are expressed as pK_B , GTP γ S-binding and BRET data are expressed as pK_B , and other data are expressed as pA_2 .

	BTRX-246040	SB-612111
Receptor binding	9.98	9.18
Stimulated GTP γ S binding	9.77	9.70
Calcium mobilization	9.27	8.86
BRET G protein	9.53	8.96
BRET/ β -arrestin 2	9.16	8.70
DMR	8.88	8.21
Mouse vas deferens	8.45	7.89

Genovese and Dobre, 2017), and sepsis (Carvalho et al., 2008; Williams et al., 2008).

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Authorship Contributions

Participated in research design: Ferrari, Ruzza, Calo.

Conducted experiments: Ferrari, Rizzo.

Performed data analysis: Ferrari, Rizzo, Ruzza.

Wrote or contributed to the writing of the manuscript: Ruzza, Calo.

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