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Inhibition of morphine-induced cAMP overshoot: a cell-based assay model in a high-throughput format

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Abstract

Opiates are potent analgesics but also drugs of abuse mainly because they produce euphoria. Chronic use of opiates results in the development of tolerance and dependence. Dr Marshall Nirenberg's group at the National Institutes of Health (NIH) was the first to use a cellular model system of Neuroblastoma×Glioma hybrid cells (NG108-15) to study morphine addiction. They showed that opiates affect adenylyl cyclase (AC) by two opposing mechanisms mediated by the opiate receptor. Although the cellular mechanisms that cause addiction are not yet completely understood, the most observed correlative biochemical adaptation is the upregulation of adenylyl cyclase. This model also provides the opportunity to look for compounds which could dissociate the acute effect of opiates from the delayed response, upregulation of AC, and thus lead to the discovery of non-addictive drugs. To identify small molecule compounds that can inhibit morphine-induced cAMP overshoot, we have validated and optimized a cell-based assay in a high throughput format that measures cellular cAMP production after morphine withdrawal. The assay performed well in the 1536-well plate format. The LOPAC library of 1280 compounds was screened in this assay on a quantitative high throughput screening (qHTS) platform. A group of compounds that can inhibit morphine induced cAMP overshoot were identified. The most potent compounds are eight naloxone related compounds, including levallorphan tartrate, naloxonazine dihydrochloride, naloxone hydrochloride, naltrexone hydrochloride, and naltriben methanesulfonate. The qHTS approach we used in this study will be useful in identifying novel inhibitors of morphine induced addiction from a larger scale screening.

Keywords

adenylyl cyclase (AC); Adenosine- 3',5'-monophosphate (cAMP); quantitative high- throughput screening (qHTS); μ opioid receptor (morphine receptor); Human embryonic kidney293- μ opioid receptor cell line (HEK-MOR); Homogeneous Time-Resolved Fluorescence (HTRF)

Introduction

Opiates derived from opium poppy have been used for centuries (Kritikos P G, 1967; Way, 1967) to treat severe pain. However their use in patients is limited due to the side effects such as nausea, vomiting, constipation, and respiratory depression. In addition, continued

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use of opiates results in the development of tolerance and physical dependence (O'Brien, 2005). A major goal in opiate research is to continue to develop drugs that may have analgesic effects but do not produce tolerance and dependence. Although a large number of studies have been done in vivo, it is difficult to interpret the data due to the many effects these drugs exert on a complex organ such as the brain.

In the late 1970s Dr. Nirenberg's laboratory at the National Institutes of Health (NIH) studied the effect of opiates on cultured neuroblastoma cells. Among several cell lines screened (Klee and Nirenberg, 1974), a Neuroblastoma×Glioma hybrid cell line (NG108-15) was found to have abundance of opiate receptors. Nirenberg and his colleagues (Sharma *et al.*, 1975a, b) discovered that opiate receptors in NG108-15 cells were coupled to adenylyl cyclase (AC) and the acute effect of morphine resulted in a decrease in AC activity and reduced cAMP levels in the cells. Similar observations were made with brain homogenates but the heterogeneity of the cell types and the apparent lability of brain enzymes made it difficult to draw solid conclusions (Collier and Roy 1974). Sharma et al further showed that chronic exposure of NG108-15 cells to morphine resulted in an increase in AC activity (Sharma *et al.*, 1975a,b; Sharma *et al.*, 1977). Sharma et al in 1975 first observed the compensatory increase of AC on morphine withdrawal either by adding the antagonist, naloxone, or washing the cells. This phenomenon is now known as hypertrophy of the cAMP system, cAMP overshoot, superactivation, supersensitization or heterologous sensitization and is postulated to be responsible for the development of tolerance and dependence (Koob and Bloom, 1988; Nestler and Aghajanian, 1997; Watts, 2002; Charles and Hales, 2004). Pharmacological studies have defined three types of opioid receptors, μ, δ and k (Loh and Smith, 1990). Activation of all three types of receptors leads to inhibition of AC and this effect is mediated through G proteins (Law and Loh, 1999; Law *et al.*, 2000)

There is substantial evidence that opiate dependence is a result of adaptive changes in signal transduction networks in several brain regions. Upregulation of AC is one of the most robust signaling adaptations to repeated morphine exposure in the locus coeruleus (Zachariou *et al.*, 2008). Activation of the μ opioid receptors in the locus coeruleus by acute morphine addition decreases cAMP and AC activity, protein kinase A levels and ultimatelyphosphorylation of cAMP response element binding protein (Nestler and Aghajanian, 1997). In the nucleus accumbens/striatal neurons, morphine withdrawal induces cAMP superactivation, which requires upregulation of an activator of G protein signaling 3 (AGS3), and cAMP increases as a function of morphine withdrawal time (Fan *et al.*, 2009). Opiate dependence has also been suggested to be linked to molecular chaperones such as heat shock proteins (Hsp90). Inhibition of Hsp90 attenuates AC sensitization after chronic morphine treatment (Koshimizu *et al.* 2010).

In order to identify potential inhibitors of morphine induced cAMP overshoot, we have validated our cellular model of opiate addiction. This cell-based assay was optimized and miniaturized first in 384 and later in 1536-well plate format. Using this optimized assay, we have screened the LOPAC (Library of Pharmacologically Active Compounds) collection of 1280 compounds in a quantitative high-throughput screening (qHTS) format, which provides concentration dependent pharmacological information on all compounds directly from the screen (Xia *et al.*, 2009a; Xia *et al.*, 2009b). The assay was highly robust in a 1536-well format. From the primary screen, we have identified 24 inhibitors with IC_{50}s ranging from 0.12 to 18 μM. Therefore, this cell-based assay model can be useful in identifying the inhibitors of morphine induced addiction.

Materials and methods

Cell line and materials

HEK 293-MOR cell line (HEK-MOR), kindly provided by Dr. Horace Loh (Department of Pharmacology, Medical School at University of Minnesota), stably express mu opioid receptor-1. These cells were maintained in advanced DMEM (Invitrogen, Carlsbad, CA, USA) containing 5% fetal bovine serum (HyClone, Logan, UT, USA), 2 mM glutaMAX (Invitrogen), 100 units/ml penicillin/100 μg/ml streptomycin (Invitrogen) and 0.2 mg/ml G418 (Invitrogen) - complete culture medium. Ro20-1724, 3-isobutyl methyl xanthine (IBMX), naloxone hydrochloride dihydrate and morphine sulfate pentahydrate were from Sigma-Aldrich St-Louis, MO, USA. NKH 477 was from Tocris Bioscience (Ellisville, MO, USA).

Morphine tolerance cell model and cAMP assay

For a 384-well plate format, HEK-MOR cells were suspended in the complete culture medium and were dispensed in 10 μl aliquots in white/solid bottom 384 well plates (Greiner Bio-One North America, NC, USA). These were incubated for 18 hr at 37°C in 5% CO_2 and 95 % air atmosphere. cAMP levels were measured in presence of NKH477 and phosphodiesterase inhibitors. Final concentrations of Ro20-1724 and IBMX were 0.5mM. Ro20-1724 and IBMX stock solutions were prepared in DMSO and diluted with complete culture media to a final concentration of 0.3 % DMSO in the assay. NKH477, morphine and naloxone solutions were prepared in complete culture media.

The cAMP production from cells was measured using cAMP dynamic HTRF kits (Cisbio Bioassays, Bedford, MA, USA) according to manufacturer instructions. Using the HTRF technology, the assay is based on competition between native cAMP produced by cells and cAMP labelled with the dye d2. The tracer binding is visualized by an anti-cAMP antibody labeled with cryptate. Briefly, after the compound treatment, 12.5 μl each of d2 and cryptate were added into the assay plates. The plates were sealed and incubated at room temperature (RT) in the dark for 1 hr. The fluorescence intensity of the assay plates was measured at 314 nm excitation, and 668 and 620 nm emission in a FlexStation 3 Microplate Reader (Molecular Devices, Sunnyvale, CA, USA). Data was expressed as the ratio of 668nm/620nm emissions, and then was converted into cAMP levels according to cAMP standard curve. The concentration response curves were fitted using the non-linear regression analysis program, GraphPad Prism (GraphPad Software, La Jolla, CA, USA).

For a 1536-well plate format, HEK-MOR cells were suspended in the complete culture medium and dispensed at 1000 cells per 4 μl volume per well in 1536-well tissue culture treated white/solid bottom plates (Aurora Biotechnologies, Carlsbad, CA) using a Flying Reagent Dispenser (FRD, Aurora Discovery, Carlsbad, CA). After the cells were incubated at 37°C with 5% CO_2 for 6 hrs, 1 μl of 1 μM morphine (final concentration) was added, followed by addition of 23 nL of compound or DMSO into the assay plates using a pin tool (Kalypsys, San Diege, CA). The plates were incubated at 37°C with 5% CO_2 overnight (18 hrs). The next day 1 μl of stimulation mixture containing 0.5 mM Ro 20-1724, 0.5 mM IBMX, 0.1 mM naloxone and 25 nM NKH 477 (final concentration) was added into the plates. After the assay plates were incubated at 37°C for 10 min, 2 μl of 5.2X d2 and cryptate was added. The plates were incubated at RT in the dark for 1 hr. The fluorescence intensity of the assay plates was measured at 340 nm excitation and 665 and 620 nm emission by an Envision plate reader (Perkin Elmer, Boston, MA). Data was expressed as the ratio of 665nm/620nm emissions in the primary screen.

qHTS and Data Analysis

The Library of Pharmacologically Active Compounds (LOPAC, Sigma-Aldrich) which contains 1280 compounds was screened in this study. Compound plates were prepared as inter-plate titrations of seven concentrations with the first four columns in each plate reserved for controls. Pin tool transfer of compounds to assay plates resulted in a 217-fold dilution. The final compound concentration in the 5 μl assay volume ranged from 3 nM to 46 μM. Analysis of the primary screening data was performed as previously described (Xia *et al.*, 2009a). Briefly, raw plate reads for each titration point were first normalized relative to the morphine control (1 μM, 100%) in the presence of 25 nM NKH 477 and DMSO without morphine in the presence of 25 nM NKH 477 wells (basal, 0%), and then corrected by applying a pattern correction algorithm using compound-free control plates (DMSO plates). Concentration-response titration points for each compound were fitted to the Hill equation yielding concentrations of half maximal inhibition (IC_{50}) and maximal response (efficacy) values.

Cell viability assay

In order to exclude the compounds which attenuate morphine tolerance due to the cytotoxic effect of the compound, the LOPAC library was also tested in a cell viability assay that measures intracellular ATP content using a luciferase-coupled ATP quantitation assay (CellTiter-Glo®, Promega, Madison, WI). HEK-MOR cells were dispensed at 1000 cells per well in 1536-well white/solid bottom assay plates (Greiner Bio-One North America, Monroe, NC) using a FRD. The assay plates were incubated for 5 hr and followed by addition of compounds via a pin tool. The assay plates were incubated for 18 hr at 37°C. At the end of the incubation period, 5 μl of CellTiter-Glo® reagent was added, plates were incubated at RT for 30 minutes, and luminescence intensity determined using a ViewLux plate reader (PerkinElmer).

Results and Discussion

Assay validation and optimization in 384 well plates

Acute effect of morphine on cAMP levels—HEK-MOR cells were used in the studies due to the homogeneous expression of mu opioid receptors and the high magnitude of overshoot in cAMP on withdrawal as shown in the previous studies (El Kouhen *et al.*, 1999). NKH477 stimulated cAMP production in concentration dependent manner with an EC_{50} of 0.13 μM. In the presence of 1 μM morphine, the concentration response curve of NKH 477 was shifted to the right with an EC_{50} of 0.83 μM (Fig 1). The acute inhibition of morphine on cAMP production confirmed the previous studies (Sharma et al 1975 a,b).

Cell density optimization—At the cellular level, morphine dependence is characterized by a significant elevation of cAMP levels after morphine withdrawal. To find optimal concentration of cells three different cell numbers were treated with or without morphine (1μM) for 18 hrs. Morphine withdrawal was mimicked by treating the cells with naloxone (1 mM) for 10 min (without washing away morphine in the plates) in presence of NKH 477 (0.1μM) and Ro20-1724+ IBMX (0.5 mM each). As shown in Fig 2, the cAMP levels increased significantly after prolonged morphine exposure compared to the control at all three cell densities. Withdrawal of the opiate agonist resulting in cAMP overshoot was also reported in human neuroblastoma cell line, SHSY5Y, which expresses both the μ and δ receptors (Yu *et al.*, 1990; Ammer and Schulz, 1993; Wang and Gintzler, 1994) and the μ opioid transfected CHO cells (Avidor-Reiss *et al.*, 1995). In the neuroblastoma N2A cells, short and long term regulation of AC by δ opioid receptors are mediated by Gαi2 (Zhang *et al.*, 2006).

Time course of morphine withdrawal—After the cells were treated with or without morphine for 18 hrs, morphine withdrawal was tested at 10, 30 and 60 min after naloxone (1 mM) treatment. cAMP overshoot was observed in the morphine treated group as compared to the control group at all three time points (Fig 3). In separate experiments, cAMP levels (at zero time) were similar (2–4 nM) in both control (without morphine) and morphine treated groups (data not shown in the figure). The assay window as defined by the cAMP level difference between the untreated control and the morphine treated cells is much larger in the 10 min treatment group than the 30 and 60 min treatment groups (Fig 3). The decrease of cAMP in 30 min treatment group might be due to the degradation of phosphodiesterase inhibitors in the buffer. Therefore, 10 min was the optimal time point for morphine withdrawal in this cell model. It is pertinent to mention that in the homogeneous assay where cells are neither washed nor morphine is removed the withdrawal at time points longer than 10 min is not meaningful

Optimization of Naloxone concentration during withdrawal—Naloxone concentrations were further optimized. While an increase in cAMP is observed at all three concentrations of naloxone, at the 5 mM concentration cAMP levels are significantly lower in both cells treated with or without morphine (Fig 4). This could be due to non-specific effects of naloxone which may not be related to opioid receptors. In the absence of naloxone, there was no difference of cAMP levels (20 – 22 nM) in both untreated control and morphine treated cells (data not shown in the figure). Therefore, 0.1 mM of naloxone along with 10 min incubation time to induce cAMP overshoot was chosen to further optimize the assay in the 1536-well plate format.

Assay optimization and miniaturization in the 1536-well plate format—The assay was miniaturized into the 1536-well plate format with a final assay volume of 5μl. To find the optimal assay window in a 1536-well plate, several concentrations of NKH 477 were tested either in the absence or presence of morphine. During data analysis, the ratio of 665nm/620nm was used instead of converting the ratio to cAMP concentrations. As shown in Table 1, the highest signal-to-background ratio of 2.5-fold and the best Z factor of 0.65 were observed in the 25 nM NKH 477 treatment group. These results indicate that this assay in a 1536-well plate format was robust and ready for high throughput screening. During the screen, 25 nM NKH 477 was used to stimulate cAMP production either with or without morphine treatment.

Identification of compounds that can attenuate morphine induced cAMP overshoot from the LOPAC library screen—The LOPAC library, containing 1280 compounds with known pharmacological activities, was screened in the cAMP assay in the presence of 25 nM of NKH477. The average signal-to-background ratio from 9 plates was 2.0 ± 0.1 and the average CV (%) from the DMSO controls and low concentration (up to 9.2 μM) plates was 6.9 ± 0.5. The average Z factor was 0.58 ± 0.03 from the primary screen.

From the LOPAC library screening, 24 of the 1280 LOPAC compounds were identified with < 20 μM potency and >40% efficacy. The IC_{50} values of these 24 compounds are listed in Table 2. Among these 24 compounds, eight were structural analogs of naloxone, including levallorphan tartrate, naloxonazine dihydrochloride, naloxone benzoylhydrazone, naloxone hydrochloride, naltrexone hydrochloride, naltriben methanesulfonate, naltrindole hydrochloride. The most potent compound identified from the screening was naltrexone hydrochloride with an IC_{50} of 0.11 μM, followed by naloxone hydrochloride (IC_{50}=0.20μM), levallorphan tartrate (IC_{50}=0.32μM), naloxone benzoylhydrazone (IC_{50}=0.41μM), naloxonazine dihydrochloride (IC_{50}=0.46μM), and naltrindole hydrochloride (IC_{50}=0.81μM). Naloxone and its analogs, such as naltrexone (Advokat, 1981) and naltrindole (Hepburn *et al.*, 1997), have been shown effective in treating

morphine tolerance in rats. All other compounds identified as active from the screening showed potencies (IC_{50} > 5 μM) markedly lower than the naloxone analogs and are of less interest. The data from the present study suggests that this assay can be used to screen large compound collections to identify small molecule compounds that can attenuate morphine tolerance.

Conclusions

We have developed and validated a cell-based assay model of-inhibition of morphine-induced cAMP overshoot in a high throughput format. This cell-based assay has been optimized into a 384-well plate format and further miniaturized into a 1536-well plate format. To validate this assay, we have screened the LOPAC library. From the primary screen, we have identified 24 inhibitors. Among these compounds, the most potent compounds are naloxone dervatives/analogs with IC_{50}s ranging from 0.12 to 4.61 μM. These data suggest that this assay can be used for large scale library screening, and a larger compound screen may identify more potent compounds that inhibit morphine-induced cAMP overshoot. The identification of compounds that inhibit the overshoot of cAMP without affecting the acute effect of morphine on cAMP levels will open a plethora of research activities to understand the mechanism of their action at behavioral, cellular and molecular levels. Such future studies may help in better understanding of the precise role played by different pathways that contribute to opiate tolerance and dependence and ultimately to addiction.

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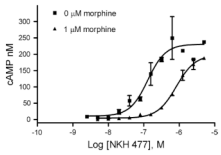

Fig 1. Acute effect of morphine on cAMP levels

HEK-MOR cells (at 2000) per well suspended in 10μl complete culture media were plated into each well in 384 well plates. After incubation for 18 hrs, 15μl of NKH477 at various concentrations with Ro20-1724 (0.5 mM) and IBMX (0.5mM) in the absence or presence of 1 μM morphine were added and the plates were incubated for 10 mins. cAMP levels in the cells were measured. Values are mean ± SD of the results from 12 wells or more (in triplicate lanes) in a single experiment.

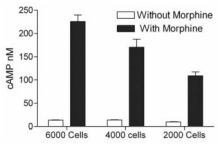

Fig 2. Cell density optimization

HEK-MOR cells at different densities were dispensed into 384 well plates. The assay plates were treated with or without morphine (1μM) for 18 hr. Withdrawal was mimicked by addition of mix with NKH477 (0.1μM), naloxone (1mM) and phosphodiesterase inhibitors. The plates were incubated for 10 min and cAMP levels in the cells were measured. Values are mean ± SD of the results from 12 wells or more (in triplicate lanes) in a single experiment.

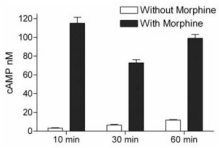

Fig 3. Time course of cAMP overshoot

Conditions were the same as in Fig 2 except that 2000 cells were plated per well and withdrawal was done at different time intervals. Values are mean ± SD of the results from 12 wells or more (in triplicate lanes) in a single experiment.

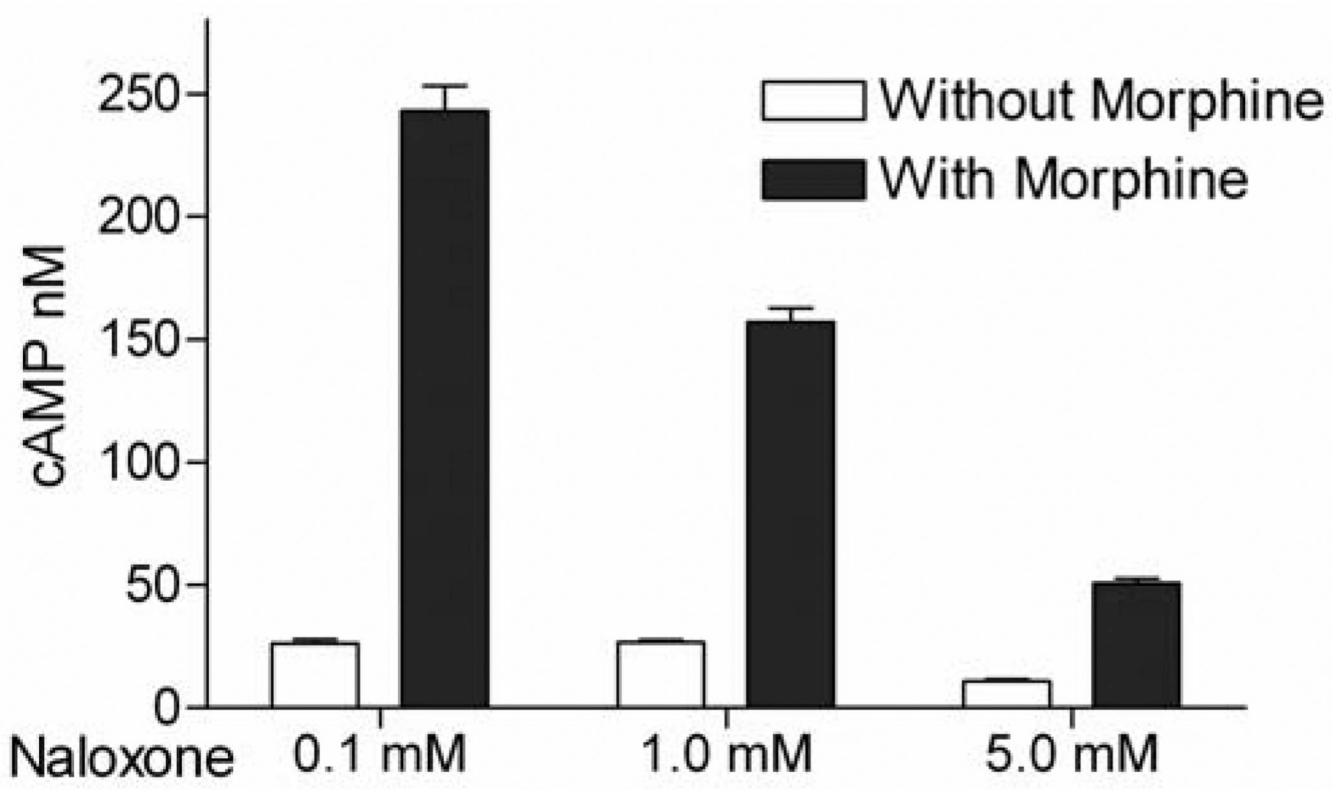

Fig 4. Effect of different concentrations of naloxone on cAMP

Conditions were the same as in Fig 2 except that 2000 cells were plated per well and withdrawal was mimicked in the presence of different concentrations of Naloxone. Values are mean ± SD of the results from 12 wells (in triplicate lanes) in a single experiment.

Table 1

Assay performance in a 1536-well plate format

NKH 477, nM	Control	Morphine	S/B, fold	Z factor
10	3.34 ± 0.17	1.63 ± 0.13	2.05	0.45
25	2.74 ± 0.13	1.08 ± 0.05	2.52	0.65
50	2.06 ± 0.12	0.88 ± 0.04	2.33	0.56
100	1.16 ± 0.07	0.75 ± 0.03	1.55	0.21

Each value from control (without morphine treatment) and morphine treatment is the mean of ratio (665nm/620nm) ± SD from 288 wells in 1536-well plates.

Table 2

Summary of compounds with potency and efficacy identified from LOPAC library screen

Compound name	Potency (μM)	Efficacy (%)
Cantharidic Acid	14.58	86
Cantharidin	12.99	63
ET-18-OCH3	18.35	144
Ifenprodil tartrate	8.20	87
I-OMe-Tyrphostin AG 538	16.35	67
L-687,384 hydrochloride	14.58	138
Levallorphan tartrate	0.33	70
ML-7	6.35	61
Naloxonazine dihydrochloride	0.46	95
Naloxone benzoylhydrazone	0.41	48
Naloxone hydrochloride	0.21	91
Naltrexone hydrochloride	0.12	102
Naltriben methanesulfonate	2.31	128
Naltrindole hydrochloride	0.82	72
nor-Binaltorphimine dihydrochloride	4.61	119
N-p-Tosyl-L-phenylalanine chloromethyl ketone	18.35	49
Parthenolide	16.35	191
Propionylpromazine hydrochloride	16.35	41
R(−)-N-Allylnorapomorphine hydrobromide	16.35	65
rac-2-Ethoxy-3-octadecanamido-1-propylphosphocholine	18.35	101
Retinoic acid p-hydroxyanilide	18.35	46
Ropinirole hydrochloride	18.35	92
SCH-202676 hydrobromide	16.35	85
TBB	14.58	54

Efficacy is defined as % to the morphine control (1 μM) in the presence of 25 nM NKH 477.