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Discovery, structure–activity relationship studies, and anti-nociceptive effects of 1-phenyl-3,6,6-trimethyl-1,5,6,7-tetrahydro-4*H*-indazol-4-one as novel opioid receptor agonists

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ABSTRACT

The μ-opioid receptor (MOR) is the major opioid receptor targeted by most analgesics in clinical use. However, the use of all known MOR agonists is associated with severe adverse effects. We reported that the 1-phenyl-3,6,6-trimethyl-1,5,6,7-tetrahydro-4*H*-indazol-4-ones are novel opioid receptor agonists. Subsequent structural modification resulted in the potent MOR/KOR (κ-opioid receptor) agonists **19**, **20**, and **21**. Testing the analgesic effect of these in WT B6 mice (tail-flick test) gave ED_{50} values of 8.4, 10.9, and 26.6 mg/kg, respectively. The 1-phenyl-3,6,6-trimethyl-1,5,6,7-tetrahydro-4*H*-indazol-4-one core could be addressed in 1 or 2 synthetic steps with moderate to high percent of yield. In the adenylyl cyclase assay, compound **19** displayed a MOR/KOR agonist profile, with IC_{50} values of 0.73 and 0.41 μM, respectively. Current results suggest that compound **19** is a promising lead to go further development and in vitro/in vivo adverse effects studies.

1. Introduction

Opioids, including morphine, primarily act on opioid receptors, of which there are three well-defined subtypes: MOR, DOR (δ-opioid receptor) and KOR. These opioid receptors are G_i-coupled receptors, possessing the same general structure of an extracellular N-terminal region, seven transmembrane domains, and an intracellular C-terminal tail structure. Neuronal cells in the central nervous system (CNS) broadly express these receptors, which regulate numerous complex, partially overlapping physiological and neurobiological functions.[1–5] Currently, most opioids in clinical use for analgesia are either non-selective or selective MOR agonists,[6] and it has been proven that the analgesic effect of opioids is mediated principally by activation of MOR. However, use of MOR also results in tolerance, dependence, and addiction.[7–10] DOR are implicated in morphine tolerance, dependence and rewarding effects, and DOR agonists have been regarded as very weak analgesics.[11–13] Activation of KOR in non-human primates causes severe adverse effects such as dysphoria, water diuresis, salivation and emesis, which may limit the utility of KOR agonists for clinical pain management.[14] However, recent studies of mixed MOR/DOR agonists for analgesia with reduced tolerance was reported.[15] In addition, the relevance of that mixed MOR/KOR activity is useful in reducing cocaine self-administration, and displays fewer side effects has been established recently.[16–20]

Most of the non-peptide MOR/DOR and MOR/KOR agonists are morphine-like compounds or morphine analogs. High-throughput screening (HTS) has the potential to discover new scaffolds of non-peptide opioid receptor agonists, which may result in novel analgesics that work without severe adverse effects. 1-(2-Chlorophenyl)-3,6,6-trimethyl-1,5,6,7-tetrahydro-4*H*-indazol-4-one (**1**) was identified to activate μ-opioid receptor with activity in the micromolar range (Fig. 1) by HTS. The EC_{50} of compound **1** in the FLIPR® calcium assay at MOR is 2.28 μM. Compound **1** is based on a tetrahydro-4*H*-indazol-4-one core structure, and can be synthesized by the condensation of hydrazines and 2-acylcyclohexane-1,3-diones under acidic conditions. The 1-phenyl-3,6,6-trimethyl-1,5,6,7-tetrahydro-4*H*-indazol-4-one core is not a morphine-like structure, may has the potential to distinguish from the morphine-like MOR/DOR or MOR/KOR agonists, and the synthesis of it is easy to address. Although some of the 1-phenyl-3,6,6-trimethyl-1,5,6,7-tetrahydro-4*H*-indazol-4-one analogs in this study are

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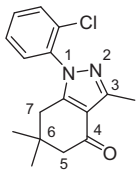


FLIPR calcium assay EC_{50} = 2.28 μM

1

Figure 1. Initial hit from HTS.

commercially available, the application of these analogs is unapparent.[21–23] In this study, 1-phenyl-3,6,6-trimethyl-1,5,6,7-tetrahydro-4*H*-indazol-4-ones are reported as novel opioid receptor agonists, and their structure–activity relationships and pharmacological studies discussed.

2. Chemistry

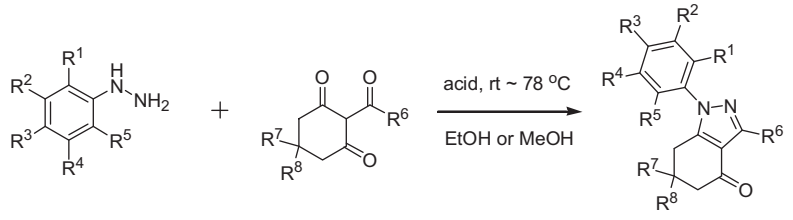
The regioselective condensation of phenylhydrazines with 2-acylcyclohexane-1,3-diones in the presence of a catalytic amount of acid at room temperature or under reflux temperature of methanol or ethanol gave tetrahydroindazol-4-ones **2**–**22**, Scheme 1. A variety of commercially available phenylhydrazines were utilized to this reaction, and a series of tetrahydroindazol-4-one derivatives **2**–**21** bearing phenyl substituents at N_1-, and methyl groups at C_3- and C_6-positions were prepared (Scheme 1). Phenylhydrazines for preparing compound **10** and compound **11** were synthesized from the corresponding hydrazinobenzoic acids through esterification. According to literature procedures, several specific phenylhydrazines (2,4-dibromo **23a**, 2-bromo-4-chloro **23b**, 2-bromo-4-methyl **23c**) were obtained from diazotization of the corresponding anilines with sodium nitrite, followed by reduction of the resulting diazonium salts with tin(II) chloride.[24] Phenylhydrazines **23a**–**23c** were utilized to prepare compound **19**–**21**, respectively.

Scheme 2 depicts the synthesis of 3-trifluoromethyl- and 3-cyclopropyl-tetrahydroindazol-4-ones **26** and **27** from the corresponding 2-acylcyclohexane-1,3-diones (**25a** and **25b**) under acidic and basic conditions, respectively. Compounds **25a** and **25b** were prepared by C-acylation of 5,5-dimethyl-1,3-cyclohexanedione with trifluoroacetic acid and cyclopropanecarbonyl chloride, respectively.[25,26] In addition, compound **28** was synthesized from hydrazine and 2-acetyl-5,5-dimethyl-1,3-cyclohexanedione under neutral conditions.[27] Tetrahydroindazol-4-ol **29** was synthesized by reduction of **19** (Scheme 3). The purities of all final compounds were measured by reverse phase HPLC, and determined to be more than 95%.

3. Biological evaluation

3.1. Structure–activity relationship (SAR) studies of tetrahydro-4*H*-indazol-4-ones

A variety of tetrahydro-4*H*-indazol-4-one derivatives were prepared and their structure–activity relationships explored using a

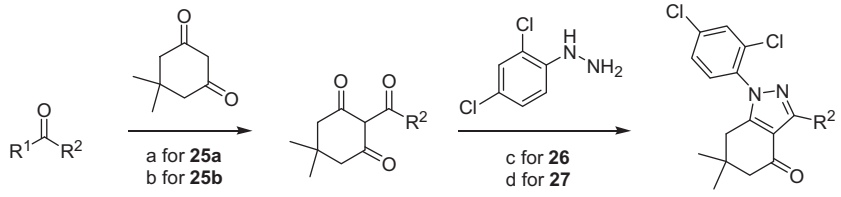


1–**22**

R^6, R^7, R^8 = Me (**1**–**21**)

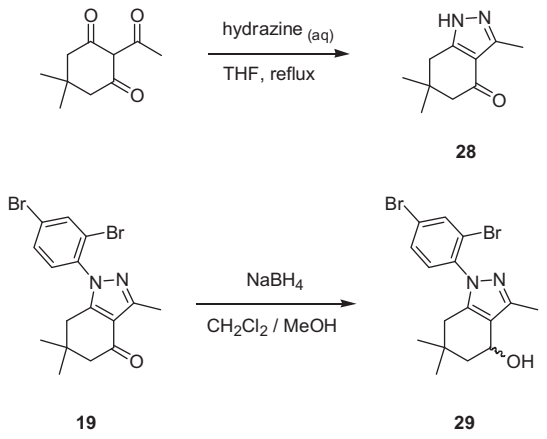
1 R^1 = Cl, R^2 ~ R^5 = H
2 R^1 ~ R^5 = H
3 R^1 = H, R^2 = Cl, R^3 ~ R^5 = H
4 R^1, R^2 = H, R^3 = Cl, R^4, R^5 = H
5 R^1 = Br, R^2 ~ R^5 = H
6 R^1, R^2 = H, R^3 = Br, R^4, R^5 = H
7 R^1 = Me, R^2 ~ R^5 = H
8 R^1 = Et, R^2 ~ R^5 = H
9 R^1, R^2 = H, R^3 = OMe, R^4, R^5 = H
10 R^1 = COOEt, R^2 ~ R^5 = H
11 R^1, R^2 = H, R^3 = COOMe, R^4, R^5 = H
12 R^1 = Me, R^2 = H, R^3 = Me, R^4, R^5 = H
13 R^1, R^2 = Me, R^3 ~ R^5 = H
14 R^1 = Me, R^2, R^3 = H, R^4 = Me, R^5 = H
15 R^1 = Me, R^2 = H, R^3 = Cl, R^4, R^5 = H
16 R^1 = F, R^2 = H, R^3 = F, R^4, R^5 = H
17 R^1 = Cl, R^2, R^3 = H, R^4 = Cl, R^5 = H
18 R^1 = Cl, R^2 = H, R^3 = Cl, R^4, R^5 = H
19 R^1 = Br, R^2 = H, R^3 = Br, R^4, R^5 = H
20 R^1 = Br, R^2 = H, R^3 = Cl, R^4, R^5 = H
21 R^1 = Br, R^2 = H, R^3 = Me, R^4, R^5 = H
22 R^1 = Cl, R^2 = H, R^3 = Cl, R^4, R^5 = H, R^6 = Me, R^7, R^8 = H

Scheme 1. Synthesis of tetrahydroindazol-4-one derivatives.



24a R^1 = OH, R^2 = CF_3
24b R^1 = Cl, R^2 = *cyclo*-C_3H_5
25a R^2 = CF_3
25b R^2 = *cyclo*-C_3H_5
26 R^2 = CF_3
27 R^2 = *cyclo*-C_3H_5

Scheme 2. Modification of 2-acylcyclohexane-1,3-diones and synthesis of tetrahydroindazol-4-ones. Reagents and conditions: (a) CDI, imidazole, $CHCl_3$, rt; (b) DMAP, DIPEA, CH_2Cl_2, reflux; (c) H_2SO_4, EtOH, reflux; (d) NaOH, EtOH, rt.

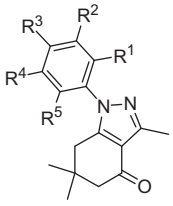


Scheme 3. Synthesis of **28** and **29**.

FLIPR® calcium assay in CHO-K1 cells, stably expressing hMOR and Gα15. Activation of MOR elicits an intracellular calcium release leading to an increase in the relative fluorescence units (RFU).[28] As shown in Table 1, the substituted groups at C_3- and C_6-positions of tetrahydro-4*H*-indazol-4-one were methyl groups and various substituents of phenyl group at N_1-position were introduced to investigate the relationships between substituents R^1–R^5 and biological activity. Compound **2**, without substituents on the benzene ring, eliminated agonistic activity. This result reveals the importance of substituents on the benzene ring. Then, several analogs bearing mono-substituents at either R^1, R^2 or R^3, were synthesized (**3**–**11**). Changing the position of the chlorine atom to the *meta*- (**3**: EC_{50} >100 μM) or *para*- (**4**: EC_{50} = 13.6 μM) position of the benzene ring resulted in a significant drop in potency. Replacing the *ortho*-chlorine atom of **1** with bromine to gave analog **5**, the potency of which was 2-fold higher than **1** (**5**: EC_{50} = 1.12 μM vs **1**: EC_{50} = 2.28 μM). In comparison, substituting the chlorine for methyl (to give **7**) and ethyl (to give **8**) devastated the potency EC_{50} = 4.72 and 19 μM, respectively. Introduction of methoxy and ester groups was found to have no benefit; the EC_{50} values for compounds **9**–**11** was in double digit μM range (**9**: EC_{50} = 18.4 μM, **10**: EC_{50} = 30.2 μM, **11**: EC_{50} = 10 μM). To expand the scope of SAR study, *ortho*- and *para*-di-substituted phenyl groups were introduced in tetrahydro-4*H*-indazol-4-one and a dramatic enhancement of activation of the μ-opioid receptor was observed. For example, compound **12** (EC_{50} = 0.37 μM) exhibited higher potency than compounds **13** (R^1, R^2 = Me, EC_{50} = 8.9 μM) and **14** (R^1, R^4 = Me, EC_{50} = 10 μM). Replacing the methyl groups with halogens to give compounds **15** and **18** resulted in an improvement in EC_{50} values (0.37 and 0.15 μM, respectively). Introduction of a bromine atom at the *ortho*-position of an *ortho*-, *para*-di-substituted benzene ring gave compounds **19**, **20**, and **21** (**19**, **20**, **21**: EC_{50} = 0.041 - μM), which were 27-fold more potent than compound **5**, bearing only one bromo substituent at the *ortho*-position of benzene ring. These results suggest that the μ-opioid receptor favors *ortho*-, *para*-di-substituted benzene on the N_1-position of tetrahydro-4*H*-indazol-4-one. The activation ability of *ortho*-, *para*-di-halogen benzene on the N_1-position of tetrahydro-4*H*-indazol-4-one was increased in the order; F, Cl, Br (**16**: EC_{50} = 10.3 μM, **18**: EC_{50} = 0.15 μM, **19**: EC_{50} = 0.041 μM).

To investigate additional SARs, several more derivatives were synthesized, Table 2. Analog **22** (EC_{50} = 13.6 μM), in which the methyl groups at the R^2 and R^3 positions were replaced with hydrogen atoms, displayed 91-fold less potency than compound **18**, revealing the di-methyl group at the C_6-position of tetrahydro-4*H*-indazol-4-one to be necessary for agonistic activity. Introduction of trifluoromethyl and cyclopropyl groups at the C_3-position of tetrahydro-4*H*-indazol-4-one gave compounds **26** (EC_{50} = 1.54 μM) and **27** (EC_{50} = 0.37 μM), both of which were less active than compound **18**. Removing the benzene ring to give compound **28** (EC_{50} >100 μM) eliminated all agonistic activity, emphasizing the importance of the benzene ring at the N_1-position. When the carbonyl group at the C_4 position was reduced to a hydroxyl group to give compound **29**, the agonistic activity was 2-fold less than compound **19** (EC_{50} = 0.1 μM). In summary, the *ortho*, *para*-di-halogen/dimethyl-substituted (except fluorine atom) benzene ring at the N_1-position of the tetrahydro-4*H*-indazol-4-one, and

Table 1
Structure–activity relationships of tetrahydroindazol-4-ones **1**–**21**

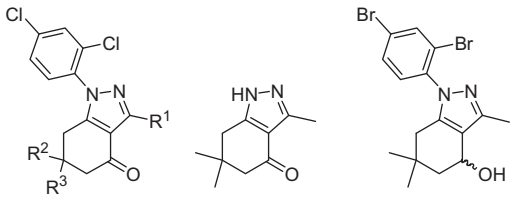


1–**21**

Compound	R^1	R^2	R^3	R^4	R^5	EC_{50}[a] (μM)
1	Cl	H	H	H	H	2.28
2	H	H	H	H	H	>100
3	H	Cl	H	H	H	>100
4	H	H	Cl	H	H	13.6
5	Br	H	H	H	H	1.12
6	H	H	Br	H	H	9.91
7	Me	H	H	H	H	4.72
8	Et	H	H	H	H	19
9	H	H	OMe	H	H	18.4
10	CO_2Et	H	H	H	H	30.2
11	H	H	CO_2Me	H	H	10
12	Me	H	Me	H	H	0.37
13	Me	Me	H	H	H	8.9
14	Me	H	H	Me	H	10
15	Me	H	Cl	H	H	0.37
16	F	H	F	H	H	10.3
17	Cl	H	H	Cl	H	2.89
18	Cl	H	Cl	H	H	0.15
19	Br	H	Br	H	H	0.041
20	Br	H	Cl	H	H	0.041
21	Br	H	Me	H	H	0.041
Morphine	—	—	—	—	—	0.01

[a] EC_{50} values are the means of at least two independent experiments.

Table 2
Structure–activity relationships of tetrahydroindazol-4-ones **22**, **26**–**29**



22, **26**, **27** **28** **29**

Compound	R^1	R^2	R^3	EC_{50}[a] (μM)
22	Me	H	H	13.6
26	CF_3	Me	Me	1.54
27	Cyclo-C_3H_5	Me	Me	0.37
28	—	—	—	>100
29	—	—	—	0.1
Morphine	—	—	—	0.01

[a] EC_{50} values are the mean of at least two independent experiments.

Table 3
[^{3}H]naloxone displacement assay and cAMP inhibition assay for functional selectivity

Compound	K_i (μM) [a]	hMOR IC_{50} [b] (μM)	hDOR IC_{50} [b] (μM)	hKOR IC_{50} [b] (μM)
12	3.93 ± 0.14	ND[c]	ND[c]	ND[c]
15	>100	ND[c]	ND[c]	ND[c]
18	ND[c]	7.89	10	2.65
19	0.47 ± 0.028	0.73	1.69	0.41
20	0.61 ± 0.074	3.3	3.38	0.57
21	0.74 ± 0.036	6	>100	0.37
27	1.3 ± 0.88	ND[c]	ND[c]	ND[c]
29	>100	3.38	>100	1.1
Naloxone	0.002 ± 0.0005	ND[c]	ND[c]	ND[c]
DAMGO	ND[c]	0.005	ND[c]	ND[c]
DPDPE	ND[c]	ND[c]	0.0009	ND[c]
U50488	ND[c]	ND[c]	ND[c]	0.0003

[a] K_i values are the average ± SEM from three independent experiments.
[b] IC_{50} values are the mean of at least two independent experiments.
[c] ND = not determined.

the dimethyl groups at the C_6-position, contribute greatly to the human μ opioid receptor agonism.

3.2. Binding affinities determination and measurement of adenylyl cyclase-mediated effects

Binding affinities of the new compounds for MOR were determined by displacement of [^{3}H]naloxone binding in HEK cells expressing recombinant rat MOR. Compounds **19**, **20**, **21** had significant binding for the MOR with K_i value in submicromolar range (**19**, K_i = 0.47 μM; **20**, K_i = 0.61 μM; **21**, K_i = 0.74 μM) (Table 3) in radioligand displacement studies. The poor correlation between K_i of [^{3}H]naloxone displacement assay and EC_{50} of FLIPR® calcium assay revealed that the cellular response to a pharmacological agonist was related to the efficacy of agonist, which is the intrinsic character for individual agonist, and the sensitivity of the assay.[29,30] The typical signaling pathway of opioid receptors is through G_i/G_o proteins for inhibiting adenylyl cyclase.[31] To investigate the selectivity of new compounds in activating opioid receptors, the ability of new compounds in affecting forskolin-stimulated adenylyl cyclase activity in HEK293 cells stably expressing the μ, δ, and κ opioid receptors was measured. As shown in Table 3, compound **19** was both MOR and KOR agonist (MOR IC_{50} = 0.73 μM; KOR IC_{50} = 0.41 μM), whereas compound **20** and **21** were selective KOR agonist (**20**, MOR IC_{50} = 3.3 μM, KOR IC_{50} = 0.57 μM; **21**, MOR IC_{50} = 6 μM, KOR IC_{50} = 0.37 μM). Thus compounds **19**, **20** and **21** were novel agonists of both MOR and KOR.

3.3. Compounds 19, 20 and 21 activate G protein-coupled inwardly-rectifying potassium channel in the AtT-20 pituitary cells

Upon activation of the opioid receptor and coupling to trimeric G proteins, the βγ subunit of the G protein is released from Gαβγ complex to activate the G protein-coupled inwardly-rectifying potassium (GIRK) channels.[32] Spinal GIRK1/GIRK2 heterotetrameric channels modulate nociception and contribute to morphine analgesia.[33] In this study, AtT-20 cells endogenously expressing GIRK1/GIRK2,[34] were transiently transfected with myc-MOR expression plasmid, to detect GIRK-mediated membrane potential hyperpolarization after morphine treatment. By using the FLIPR® membrane potential assay, **19**, **20** and **21** treatment was found to alter the membrane potential in myc-tagged MOR expressing AtT-20 cells (Fig. 2). The EC_{50} value of **19** was 5.49 μM, and that of **20** and **21** was 0.1 μM and 0.2 μM, respectively. Finally, naloxone was found to reverse compound-stimulated membrane poten-

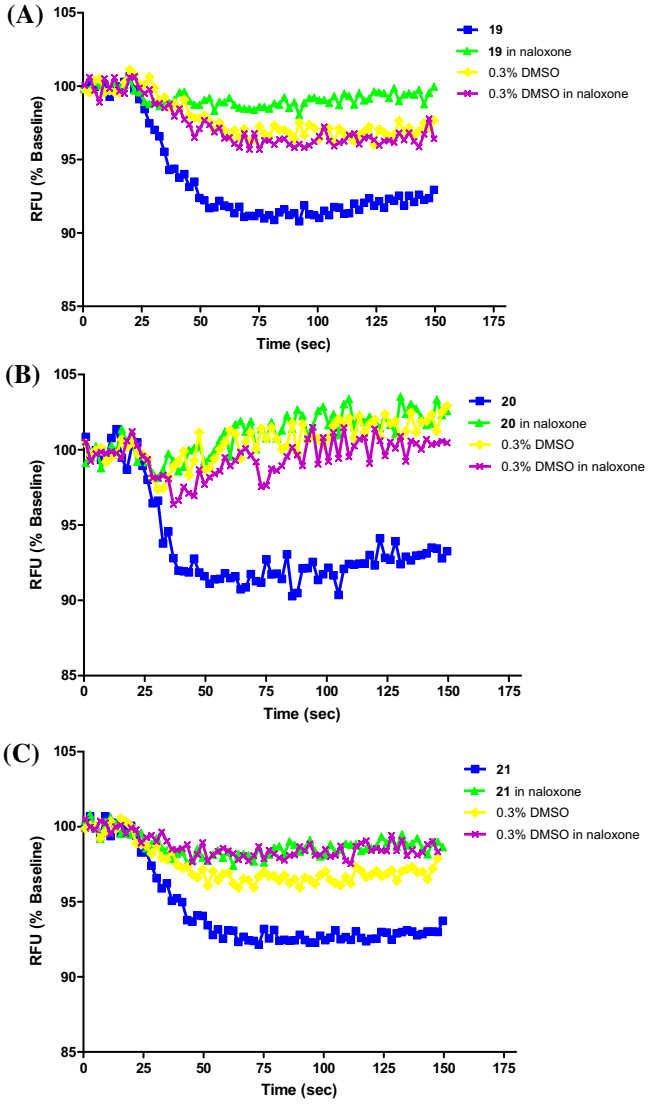


Figure 2. Effects of compound-mediated membrane potential hyperpolarization in pituitary AtT-20 cells. Compounds **19**, **20** and **21** induced a membrane potential hyperpolarization in MOR expressing AtT-20 cells. The pituitary AtT-20 cells were transiently transfected with vehicle or myc-MOR, the effects of compounds on membrane potential were detected in the presence or absence of naloxone (1 μM) treatment.

tial hyperpolarization in myc-tagged MOR expressing AtT-20 cells, indicating that the effects of compounds were dependent on opioid receptor activation.

Table 4
ED_{50} of **19**, **20**, and **21**

	19	20	21
ED_{50} (mg/kg)	8.4	10.9	26.6

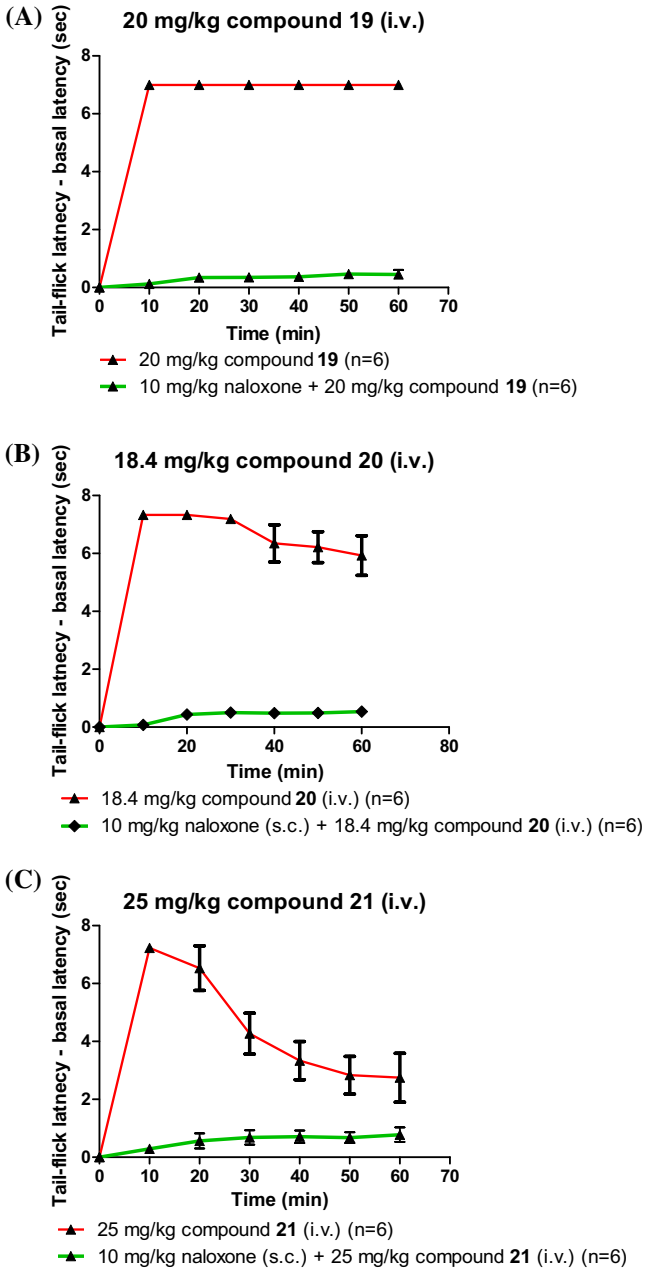


Figure 3. Compounds **19**, **20** and **21** show analgesic effects through opioid receptor activation in B6 mice. Analgesic effect of **19** (A), **20** (B) and **21** (C) in the WT B6 mice. After detection of basal latencies, each group of mice were injected with **19** (20 mg/kg, iv), **20** (18.4 mg/kg, iv) and **21** (25 mg/kg, iv), respectively, to detect tail-flick latencies at the indicated time points (10 min, 20 min, 30 min, 40 min, 50 min, and 60 min). Mice were injected with naloxone (10 mg/kg, sc) 5 min before compound injection. Quantitative results were calculated from tail-flick test (tail-flick latency-basal latency). Neither the vehicle nor naloxone alone was found to alter tail-flick latency (Supplementary Fig. S1). $^{***}p$ <0.001 versus sham-control group. (Statistical analysis was carried out using one-way ANOVA with appropriate post hoc tests.)

3.4. Compounds 19, 20, 21 elicited anti-nociceptive effects in B6 mice

To further evaluate the activity of compounds **19**–**21**, mice were injected with **19**, **20**, and **21** and their tail-flick latencies at the indicated time points (0, 0.5 and 1 h) measured. The extent of thermal withdrawal was evaluated by measuring the AUC of the tail-flick test to study the effect of the compounds on pain-related behavior in B6 mice. All three compounds, especially **19**, were found to exert strong analgesia compared to vehicle control (Fig. 3). The ED_{50} of three compounds were listed in Table 4. It was also found that this effect could be attenuated using the opioid antagonist naloxone (Fig. 3).

4. Conclusions

The tetrahydro-4*H*-indazol-4-one containing opioid receptor agonist **1** was identified by high-throughput screening. Structural modification of this scaffold resulted in the novel opioid receptor agonist **19**, a >50 fold level potency improvement compared with compound **1**, in the FLIPR® assay at MOR. Radioligand displacement study of **19** exhibited moderate binding affinity. The effect of **19** on cAMP accumulation assay in HEK293 cells stably expressing the μ, δ, and κ opioid receptors revealed that **19** possesses a mixed MOR/KOR agonistic activity. Recent studies emerged the relevance of MOR/KOR agonists or partial agonists in treating cocaine abuse or in pain relief with fewer adverse effects.[16–20] The membrane potential hyperpolarization assay in pituitary AtT-20 cell indicated that **19** altered the cell membrane potential, suggesting that **19** effectively activated MOR. Compound **19** elicited strong anti-nociceptive effect in WT B6 mice (ED_{50} = 8.4 mg/kg). Synthesis of **19** could be easily addressed in 2 synthetic steps with 61% of yield. These results demonstrate that compound **19** is a promising lead for further development and in vitro/in vivo adverse effects studies.

5. Experimental

5.1. Chemistry methods

All the reagents and solvents were reagent grade and were used without further purification unless otherwise specified. All materials used were commercially available and used as supplied. Merck silica gel 60 F_{254} sheets were used for analytical thin-layer chromatography (TLC). Column chromatography was performed on silica gel (230–400 mesh) or used an Isco CombiFlash Companion system (Teledyne Isco) with prepacked silica gel cartridges. ^{1}H NMR spectra were recorded on a Varian Mercury-300 or Varian Mercury-400 spectrometers. Chemical shifts (δ) are given in parts per million (ppm) relative to the solvent peak. High-resolution electrospray ionization mass spectra were measured with a VARIAN 901-MS (FT-ICR Mass) mass spectrometer. Purity of all tested compounds was over 95% and determined on a Hitachi 2000 series HPLC system equipped with a C-18 column (Agilent ZORBAX Eclipse XDB-C18 5 μm, 4.6 mm × 150 mm) at 254 nm, eluting with mobile phase A (acetonitrile) and mobile phase B (10 mM NH_4OAc aqueous solution containing 0.1% formic acid) from 0 min (A/B, 10%) to 45 min (A/B, 90%) at 25 °C.

5.2. Representative procedure for the synthesis of tetrahydroindazol-4-ones

To a mixture of 1-(2-bromo-4-chlorophenyl)hydrazine hydrochloride **23b** (103.2 mg, 0.40 mmol) and 2-acetyl-5,5-dimethyl-1,3-cyclohexanedione (72.9 mg, 0.40 mmol) in ethanol (1.2 mL) was treated with a few drops of concd H_2SO_4. The mixture was stirred at room temperature for 24 h. After the reaction was

finished, the mixture was diluted with EtOAc (8.0 mL) and washed with water (4.0 mL), satd $NaHCO_{3(aq)}$ (4.0 mL) and brine (4.0 mL). The organic layer was dried over $MgSO_4$ and concentrated under reduced pressure. The residue was purified by Isco CombiFlash Companion column chromatography (silica gel, 0–70% EtOAc/hexane) to give 1-(2-bromo-4-chlorophenyl)-3,6,6-trimethyl-1,5,6,7-tetrahydro-4*H*-indazol-4-one **20** (65.0 mg, 44%) as a powder. ^{1}H NMR (CD_3OD, 400 MHz) δ 7.93 (d, J = 2.0 Hz, 1H), 7.61 (dd, J = 2.0, 8.4 Hz, 1H), 7.51 (d, J = 8.4 Hz, 1H), 2.54 (s, 2H), 2.46 (s, 3H), 2.40 (s, 2H), 1.10 (s, 6H); HRMS calcd for $C_{16}H_{17}BrClN_2O$ (M+H) 367.0213, found 367.0211.

5.2.1. 1-(2-Chlorophenyl)-3,6,6-trimethyl-1,5,6,7-tetrahydro-4*H*-indazol-4-one (1)

The reaction of 1-(2-chlorophenyl)hydrazine hydrochloride (71.6 mg, 0.40 mmol) with 2-acetyl-5,5-dimethyl-1,3-cyclohexanedione (72.9 mg, 0.40 mmol) in ethanol (1.2 mL) at room temperature overnight, by a procedure similar to that for **20** using concd HCl (0.40 mL) instead of H_2SO_4 and the crude product was purified by flash chromatography on silica gel eluting with EtOAc/hexane (4:1), gave 1-(2-chlorophenyl)-3,6,6-trimethyl-1,5,6,7-tetrahydro-4*H*-indazol-4-one **1** (45.2 mg, 39%) as a powder. ^{1}H NMR (CD_3OD, 400 MHz) δ 7.68 (d, J = 7.6 Hz, 1H), 7.60–7.52 (m, 3H), 2.54 (s, 2H), 2.47 (s, 3H), 2.40 (s, 2H), 1.09 (s, 6H); HRMS calcd for $C_{16}H_{18}ClN_2O$ (M+H) 289.1108, found 289.1117.

5.2.2. 3,6,6-Trimethyl-1-phenyl-1,5,6,7-tetrahydro-4*H*-indazol-4-one (2)

The reaction of 1-phenylhydrazine (43.3 mg, 0.40 mmol) with 2-acetyl-5,5-dimethyl-1,3-cyclohexanedione (72.9 mg, 0.40 mmol) in ethanol (1.2 mL) at room temperature overnight, by a procedure similar to that for **20** using concd HCl (0.40 mL) instead of H_2SO_4, gave 3,6,6-trimethyl-1-phenyl-1,5,6,7-tetrahydro-4*H*-indazol-4-one **2** (39.2 mg, 39%) as a powder. ^{1}H NMR (CD_3OD, 300 MHz) δ 7.59–7.45 (m, 5H), 2.84 (s, 2H), 2.47 (s, 3H), 2.41 (s, 2H), 1.08 (s, 6H); HRMS calcd for $C_{16}H_{19}N_2O$ (M+H) 255.1497, found 255.1501.

5.2.3. 1-(3-Chlorophenyl)-3,6,6-trimethyl-1,5,6,7-tetrahydro-4*H*-indazol-4-one (3)

The reaction of 1-(3-chlorophenyl)hydrazine hydrochloride (71.6 mg, 0.40 mmol) with 2-acetyl-5,5-dimethyl-1,3-cyclohexanedione (72.9 mg, 0.40 mmol) in ethanol (1.2 mL) under reflux for 5 h, by a procedure similar to that for **20** using concd HCl (0.40 mL) instead of H_2SO_4, gave 1-(3-chlorophenyl)-3,6,6-trimethyl-1,5,6,7-tetrahydro-4*H*-indazol-4-one **3** (45.5 mg, 39%) as a powder. ^{1}H NMR (CD_3OD, 400 MHz) δ 7.63 (dd, J = 1.2, 3.6 Hz, 1H), 7.60–7.47 (m, 3H), 2.88 (s, 2H), 2.47 (s, 3H), 2.41 (s, 2H), 1.09 (s, 6H); HRMS calcd for $C_{16}H_{18}ClN_2O$ (M+H) 289.1108, found 289.1108.

5.2.4. 1-(4-Chlorophenyl)-3,6,6-trimethyl-1,5,6,7-tetrahydro-4*H*-indazol-4-one (4)

The reaction of 1-(4-chlorophenyl)hydrazine hydrochloride (71.6 mg, 0.40 mmol) with 2-acetyl-5,5-dimethyl-1,3-cyclohexanedione (72.9 mg, 0.40 mmol) in ethanol (1.2 mL) at room temperature for 2.5 days, by a procedure similar to that for **20** using concd HCl (0.40 mL) instead of H_2SO_4, gave 1-(4-chlorophenyl)-3,6,6-trimethyl-1,5,6,7-tetrahydro-4*H*-indazol-4-one **4** (45.5 mg, 39%) as a powder. ^{1}H NMR (CD_3OD, 300 MHz) δ 7.56 (m, 4H), 2.86 (s, 2H), 2.47 (s, 3H), 2.41 (s, 2H), 1.09 (s, 6H); HRMS calcd for $C_{16}H_{18}ClN_2O$ (M+H) 289.1108, found 289.1109.

5.2.5. 1-(2-Bromophenyl)-3,6,6-trimethyl-1,5,6,7-tetrahydro-4*H*-indazol-4-one (5)

The reaction of 1-(2-bromophenyl)hydrazine hydrochloride (89.4 mg, 0.40 mmol) with 2-acetyl-5,5-dimethyl-1,3-cyclohexanedione (72.9 mg, 0.40 mmol), by a procedure similar to that for **20**, gave 1-(2-bromophenyl)-3,6,6-trimethyl-1,5,6,7-tetrahydro-4*H*-indazol-4-one **5** (92.4 mg, 69%) as a powder. ^{1}H NMR (CD_3OD, 400 MHz) δ 7.84 (dd, J = 2.4, 7.6 Hz, 1H), 7.60–7.50 (m, 3H), 2.53 (s, 2H), 2.47 (s, 3H), 2.40 (s, 2H), 1.10 (s, 6H); HRMS calcd for $C_{16}H_{18}BrN_2O$ (M+H) 333.0602, found 333.0606.

5.2.6. 1-(4-Bromophenyl)-3,6,6-trimethyl-1,5,6,7-tetrahydro-4*H*-indazol-4-one (6)

The reaction of 1-(4-bromophenyl)hydrazine hydrochloride (89.4 mg, 0.40 mmol) with 2-acetyl-5,5-dimethyl-1,3-cyclohexanedione (72.9 mg, 0.40 mmol), by a procedure similar to that for **20**, gave 1-(4-bromophenyl)-3,6,6-trimethyl-1,5,6,7-tetrahydro-4*H*-indazol-4-one **6** (34.6 mg, 26%) as a powder. ^{1}H NMR (CD_3OD, 300 MHz) δ 7.71 (d, J = 8.4 Hz, 2H), 7.48 (d, J = 8.4 Hz, 2H), 2.86 (s, 2H), 2.47 (s, 3H), 2.41 (s, 2H), 1.08 (s, 6H); HRMS calcd for $C_{16}H_{18}BrN_2O$ (M+H) 333.0602, found 333.0606.

5.2.7. 3,6,6-Trimethyl-1-(2-methylphenyl)-1,5,6,7-tetrahydro-4*H*-indazol-4-one (7)

The reaction of 1-(2-methylphenyl)hydrazine hydrochloride (63.5 mg, 0.40 mmol) with 2-acetyl-5,5-dimethyl-1,3-cyclohexanedione (72.9 mg, 0.40 mmol), by a procedure similar to that for **20**, gave 3,6,6-trimethyl-1-(2-methylphenyl)-1,5,6,7-tetrahydro-4*H*-indazol-4-one **7** (55.0 mg, 51%) as a powder. ^{1}H NMR (CD_3OD, 400 MHz) δ 7.49–7.36 (m, 3H), 7.28 (d, J = 7.6 Hz, 1H), 2.50 (s, 2H), 2.47 (s, 3H), 2.40 (s, 2H), 2.09 (s, 3H), 1.08 (s, 6H); HRMS calcd for $C_{17}H_{21}N_2O$ (M+H) 269.1654, found 269.1656.

5.2.8. 1-(2-Ethylphenyl)-3,6,6-trimethyl-1,5,6,7-tetrahydro-4*H*-indazol-4-one (8)

The reaction of 1-(2-ethylphenyl)hydrazine hydrochloride (69.1 mg, 0.40 mmol) with 2-acetyl-5,5-dimethyl-1,3-cyclohexanedione (72.9 mg, 0.40 mmol), by a procedure similar to that for **20**, gave 1-(2-ethylphenyl)-3,6,6-trimethyl-1,5,6,7-tetrahydro-4*H*-indazol-4-one **8** (52.1 mg, 46%) as a powder. ^{1}H NMR (CD_3OD, 400 MHz) δ 7.52–7.47 (m, 2H), 7.40–7.36 (m, 1H), 7.26 (d, J = 7.6 Hz, 1H), 2.51 (s, 2H), 2.48–2.42 (m, 5H), 2.40 (s, 2H), 1.09–1.06 (m, 9H); HRMS calcd for $C_{18}H_{23}N_2O$ (M+H) 283.1810, found 283.1810.

5.2.9. 1-(4-Methoxyphenyl)-3,6,6-trimethyl-1,5,6,7-tetrahydro-4*H*-indazol-4-one (9)

The reaction of 1-(4-methoxyphenyl)hydrazine hydrochloride (69.9 mg, 0.40 mmol) with 2-acetyl-5,5-dimethyl-1,3-cyclohexanedione (72.9 mg, 0.40 mmol) in ethanol (1.2 mL) under reflux for 5 h, by a procedure similar to that for **20** using concd HCl (0.40 mL) instead of H_2SO_4, gave 1-(4-methoxyphenyl)-3,6,6-trimethyl-1,5,6,7-tetrahydro-4*H*-indazol-4-one **9** (45.8 mg, 40%) as an oil. ^{1}H NMR (CD_3OD, 300 MHz) δ 7.42 (d, J = 9.0 Hz, 2H), 7.08 (d, J = 9.0 Hz, 2H), 3.87 (s, 3H), 2.77 (s, 2H), 2.46 (s, 3H), 2.40 (s, 2H), 1.08 (s, 6H); HRMS calcd for $C_{17}H_{21}N_2O_2$ (M+H) 285.1603, found 285.1604.

5.2.10. Ethyl 2-(3,6,6-trimethyl-4-oxo-4,5,6,7-tetrahydro-1*H*-indazol-1-yl)benzoate (10)

The reaction of 2-hydrazinobenzoic acid hydrochloride (56.6 mg, 0.30 mmol) with 2-acetyl-5,5-dimethyl-1,3-cyclohexanedione (54.7 mg, 0.30 mmol) in ethanol (0.9 mL) under reflux for 6 h, by a procedure similar to that for **20** using concd HCl (0.30 mL) instead of H_2SO_4, gave ethyl 2-(3,6,6-trimethyl-4-oxo-4,5,6,7-tetrahydro-1*H*-indazol-1-yl)benzoate **10** (27.7 mg, 28%) as a powder. ^{1}H NMR ($CDCl_3$, 300 MHz) δ 8.01 (dd, J = 1.5, 7.5 Hz, 1H), 7.67–7.54 (m, 2H), 7.36 (dd, J = 1.5, 7.5 Hz, 1H), 4.15 (q, J = 7.2 Hz, 2H), 2.52 (s, 3H), 2.50 (s, 2H), 2.38 (s, 2H), 1.13 (t,

J = 7.2 Hz, 3H), 1.09 (s, 6H); HRMS calcd for $C_{19}H_{23}N_2O_3$ (M+H) 327.1709, found 327.1703.

5.2.11. Methyl 4-(3,6,6-trimethyl-4-oxo-4,5,6,7-tetrahydro-1*H*-indazol-1-yl)benzoate (11)

The reaction of 4-hydrazinobenzoic acid hydrochloride (60.9 mg, 0.40 mmol) with 2-acetyl-5,5-dimethyl-1,3-cyclohexanedione (72.9 mg, 0.40 mmol) in MeOH (1.2 mL) under reflux for 3 days, by a procedure similar to that for **20**, gave methyl 4-(3,6,6-trimethyl-4-oxo-4,5,6,7-tetrahydro-1*H*-indazol-1-yl)benzoate **11** (27.7 mg, 28%) as a powder. ^{1}H NMR (CD_3OD, 400 MHz) δ 8.19 (d, *J* = 8.0 Hz, 2H), 7.70 (d, *J* = 8.0 Hz, 2H), 3.94 (s, 3H), 2.94 (s, 2H), 2.48 (s, 3H), 2.43 (s, 2H), 1.09 (s, 6H); HRMS calcd for $C_{18}H_{21}N_2O_3$ (M+H) 313.1552, found 313.1558.

5.2.12. 1-(2,4-Dimethylphenyl)-3,6,6-trimethyl-1,5,6,7-tetrahydro-4*H*-indazol-4-one (12)

The reaction of 1-(2,4-dimethylphenyl)hydrazine hydrochloride (69.1 mg, 0.40 mmol) with 2-acetyl-5,5-dimethyl-1,3-cyclohexanedione (72.9 mg, 0.40 mmol) in ethanol (1.2 mL) at room temperature overnight, by a procedure similar to that for **20** using concd HCl (0.40 mL) instead of H_2SO_4 and the crude product was purified by flash chromatography on silica gel eluting with EtOAc/hexane (4:1), gave 1-(2,4-dimethylphenyl)-3,6,6-trimethyl-1,5,6,7-tetrahydro-4*H*-indazol-4-one **12** (48.5 mg, 42%) as a powder. ^{1}H NMR (CD_3OD, 400 MHz) δ 7.25 (s, 1H), 7.18 (d, *J* = 7.6 Hz, 1H), 7.13 (d, *J* = 7.6 Hz, 1H), 2.48 (s, 2H), 2.46 (s, 3H), 2.39 (s, 5H), 2.03 (s, 3H), 1.07 (s, 6H); HRMS calcd for $C_{18}H_{23}N_2O$ (M+H) 283.1810, found 283.1817.

5.2.13. 1-(2,3-Dimethylphenyl)-3,6,6-trimethyl-1,5,6,7-tetrahydro-4*H*-indazol-4-one (13)

The reaction of 1-(2,3-dimethylphenyl)hydrazine hydrochloride (69.1 mg, 0.40 mmol) with 2-acetyl-5,5-dimethyl-1,3-cyclohexanedione (72.9 mg, 0.40 mmol) in ethanol (1.2 mL) at room temperature overnight, by a procedure similar to that for **20** using concd HCl (0.40 mL) instead of H_2SO_4 and the crude product was purified by flash chromatography on silica gel eluting with EtOAc/hexane (4:1), gave 1-(2,3-dimethylphenyl)-3,6,6-trimethyl-1,5,6,7-tetrahydro-4*H*-indazol-4-one **13** (50.8 mg, 45%) as a powder. ^{1}H NMR (CD_3OD, 400 MHz) δ 7.37 (d, *J* = 7.6 Hz, 1H), 7.26 (dd, *J* = 7.6, 7.6 Hz, 1H), 7.11 (d, *J* = 7.6 Hz, 1H), 2.46 (s, 5H), 2.39 (s, 2H), 2.37 (s, 3H), 1.94 (s, 3H), 1.07 (s, 6H); HRMS calcd for $C_{18}H_{23}N_2O$ (M+H) 283.1810, found 283.1814.

5.2.14. 1-(2,5-Dimethylphenyl)-3,6,6-trimethyl-1,5,6,7-tetrahydro-4*H*-indazol-4-one (14)

The reaction of 1-(2,5-dimethylphenyl)hydrazine hydrochloride (69.1 mg, 0.40 mmol) with 2-acetyl-5,5-dimethyl-1,3-cyclohexanedione (72.9 mg, 0.40 mmol), by a procedure similar to that for **20**, gave 1-(2,5-dimethylphenyl)-3,6,6-trimethyl-1,5,6,7-tetrahydro-4*H*-indazol-4-one **14** (31.1 mg, 27%) as a powder. ^{1}H NMR (CD_3OD, 300 MHz) δ 7.30–7.29 (m, 2H), 7.10 (s, 1H), 2.50 (s, 2H), 2.46 (s, 3H), 2.40 (s, 2H), 2.37 (s, 3H), 2.02 (s, 3H), 1.08 (s, 6H); HRMS calcd for $C_{18}H_{23}N_2O$ (M+H) 283.1810, found 283.1811.

5.2.15. 1-(4-Chloro-2-methylphenyl)-3,6,6-trimethyl-1,5,6,7-tetrahydro-4*H*-indazol-4-one (15)

The reaction of 1-(4-chloro-2-methylphenyl)hydrazine hydrochloride (77.2 mg, 0.40 mmol) with 2-acetyl-5,5-dimethyl-1,3-cyclohexanedione (72.9 mg, 0.40 mmol) in ethanol (1.2 mL) at room temperature overnight, by a procedure similar to that for **20** using concd HCl (0.40 mL) instead of H_2SO_4 and the crude product was purified by flash chromatography on silica gel eluting with EtOAc/hexane (4:1), gave 1-(4-chloro-2-methylphenyl)-3,6,6-trimethyl-1,5,6,7-tetrahydro-4*H*-indazol-4-one **15** (53.3 mg, 44%) as a powder. ^{1}H NMR (CD_3OD, 400 MHz) δ 7.48 (s, 1H), 7.39 (d, *J* = 8.4 Hz, 1H), 7.28 (d, *J* = 8.4 Hz, 1H), 2.51 (s, 2H), 2.46 (s, 3H), 2.40 (s, 2H), 2.07 (s, 3H), 1.08 (s, 6H); HRMS calcd for $C_{17}H_{20}ClN_2O$ (M+H) 303.1264, found 303.1267.

5.2.16. 1-(2,4-Difluorophenyl)-3,6,6-trimethyl-1,5,6,7-tetrahydro-4*H*-indazol-4-one (16)

The reaction of 1-(2,4-difluorophenyl)hydrazine hydrochloride (72.2 mg, 0.40 mmol) with 2-acetyl-5,5-dimethyl-1,3-cyclohexanedione (72.9 mg, 0.40 mmol) in ethanol (1.2 mL) under reflux for 5 h, by a procedure similar to that for **20** using concd HCl (0.40 mL) instead of H_2SO_4, gave 1-(2,4-difluorophenyl)-3,6,6-trimethyl-1,5,6,7-tetrahydro-4*H*-indazol-4-one **16** (66.5 mg, 57%) as a powder. ^{1}H NMR (CD_3OD, 300 MHz) δ 7.64–7.56 (m, 1H), 7.34–7.27 (m, 1H), 7.20 (dd, *J* = 7.8, 7.8 Hz, 1H), 2.62 (s, 2H), 2.46 (s, 3H), 2.40 (s, 2H), 1.08 (s, 6H); HRMS calcd for $C_{16}H_{17}F_2N_2O$ (M+H) 291.1309, found 479.1360.

5.2.17. 1-(2,5-Dichlorophenyl)-3,6,6-trimethyl-1,5,6,7-tetrahydro-4*H*-indazol-4-one (17)

The reaction of 1-(2,5-chlorophenyl)hydrazine hydrochloride (85.4 mg, 0.40 mmol) with 2-acetyl-5,5-dimethyl-1,3-cyclohexanedione (72.9 mg, 0.40 mmol) in ethanol (1.2 mL) at room temperature overnight, by a procedure similar to that for **20** using concd HCl (0.40 mL) instead of H_2SO_4 and the crude product was purified by flash chromatography on silica gel eluting with EtOAc/hexane (4:1), gave 1-(2,5-dichlorophenyl)-3,6,6-trimethyl-1,5,6,7-tetrahydro-4*H*-indazol-4-one **17** (60.1 mg, 46%) as a powder. ^{1}H NMR (CD_3OD, 300 MHz) δ 7.69–7.60 (m, 3H), 2.57 (s, 2H), 2.46 (s, 3H), 2.40 (s, 2H), 1.09 (s, 6H); HRMS calcd for $C_{16}H_{17}Cl_2N_2O$ (M+H) 323.0718, found 323.0719.

5.2.18. 1-(2,4-Dichlorophenyl)-3,6,6-trimethyl-1,5,6,7-tetrahydro-4*H*-indazol-4-one (18)

The reaction of 1-(2,4-chlorophenyl)hydrazine hydrochloride (85.4 mg, 0.40 mmol) with 2-acetyl-5,5-dimethyl-1,3-cyclohexanedione (72.9 mg, 0.40 mmol) in ethanol (1.2 mL) under reflux for 5 h, by a procedure similar to that for **20** using concd HCl (0.40 mL) instead of H_2SO_4, gave 1-(2,4-dichlorophenyl)-3,6,6-trimethyl-1,5,6,7-tetrahydro-4*H*-indazol-4-one **18** (62.1 mg, 48%) as a powder. ^{1}H NMR ($CDCl_3$, 400 MHz) δ 7.58 (d, *J* = 2.0 Hz, 1H), 7.42–7.35 (m, 2H), 2.53 (s, 3H), 2.49 (s, 2H), 2.38 (s, 2H), 1.10 (s, 6H); HRMS calcd for $C_{16}H_{17}Cl_2N_2O$ (M+H) 323.0718, found 479.1360.

5.2.19. 1-(2,4-Dibromophenyl)-3,6,6-trimethyl-1,5,6,7-tetrahydro-4*H*-indazol-4-one (19)

The reaction of 1-(2,4-dibromophenyl)hydrazine hydrochloride **23a** (121.0 mg, 0.40 mmol) with 2-acetyl-5,5-dimethyl-1,3-cyclohexanedione (72.9 mg, 0.40 mmol), by a procedure similar to that for **20**, gave 1-(2,4-dibromophenyl)-3,6,6-trimethyl-1,5,6,7-tetrahydro-4*H*-indazol-4-one **19** (100.2 mg, 61%) as a powder. ^{1}H NMR (CD_3OD, 400 MHz) δ 8.07 (d, *J* = 2.0 Hz, 1H), 7.76 (dd, *J* = 2.0, 8.4 Hz, 1H), 7.44 (d, *J* = 8.4 Hz, 1H), 2.54 (s, 2H), 2.46 (s, 3H), 2.40 (s, 2H), 1.10 (s, 6H); HRMS calcd for $C_{16}H_{17}Br_2N_2O$ (M+H) 410.9708, found 410.9709.

5.2.20. 1-(2-Bromo-4-methylphenyl)-3,6,6-trimethyl-1,5,6,7-tetrahydro-4*H*-indazol-4-one (21)

The reaction of 1-(2-bromo-4-methylphenyl)hydrazine hydrochloride **23c** (95.0 mg, 0.40 mmol) with 2-acetyl-5,5-dimethyl-1,3-cyclohexanedione (72.9 mg, 0.40 mmol) in ethanol (1.2 mL) at room temperature overnight, by a procedure similar to that for **20**, gave 1-(2-bromo-4-methylphenyl)-3,6,6-trimethyl-1,5,6,7-tetrahydro-4*H*-indazol-4-one **21** (55.1 mg, 40%) as a powder. ^{1}H NMR (CD_3OD, 400 MHz) δ 7.67 (s, 1H), 7.37 (dd, *J* = 1.6, 1.6 Hz,

2H), 2.51 (s, 2H), 2.46 (s, 3H), 2.44 (s, 3H), 2.39 (s, 2H), 1.09 (s, 6H); HRMS calcd for $C_{17}H_{20}BrN_2O$ (M+H) 347.0759, found 347.0756.

5.2.21. 1-(2,4-Dichlorophenyl)-3-methyl-1,5,6,7-tetrahydro-4*H*-indazol-4-one (22)

The reaction of 1-(2,4-dichlorophenyl)hydrazine hydrochloride (85.4 mg, 0.40 mmol) with 2-acetyl -1,3-cyclohexanedione (61.7 mg, 0.40 mmol) in ethanol (1.2 mL) under reflux for 5 h, by a procedure similar to that for **20** using concd HCl (0.40 mL) instead of H_2SO_4, gave 1-(2,4-dichlorophenyl)-3-methyl-1,5,6,7-tetrahydro-4*H*-indazol-4-one **22** (49.9 mg, 42%) as a powder. ^{1}H NMR (CD_3OD, 300 MHz) δ 7.78 (d, J = 2.1 Hz, 1H), 7.59–7.52 (m, 2H), 2.88–2.69 (m, 2H), 2.56–2.49 (m, 2H), 2.46 (s, 2H), 2.37 (s, 1H), 2.19–2.12 (m, 2H); HRMS calcd for $C_{14}H_{13}Cl_2N_2O$ (M+H) 295.0405, found 295.0403.

5.3. Representative procedure for the preparation of phenylhydrazine hydrochlorides from anilines

A mixture of 2-bromo-4-chloroaniline (825.9 mg, 4.0 mmol) and concd HCl (1.6 mL) was cooled at 0 °C and treated with a solution of $NaNO_2$ (0.3 g, 4.4 mmol) in H_2O (1.5 mL) precooled to 0 °C. After 45 min, a solution of $SnCl_2$ (2.2 g, 11.5 mmol) in concd HCl (2.8 mL) was added and a precipitation was formed. The mixture was stirred at room temperature for 2 h. After filtration, the precipitation was collected and washed with water (7.0 mL) and ether (30.0 mL) to give 1-(2-bromo-4-chlorophenyl)hydrazine hydrochloride **23b** (446.7 mg, 43%) as a solid. ^{1}H NMR (DMSO, 300 MHz) δ 10.05 (br, 2H), 7.96 (br, 1H), 7.69 (d, J = 2.4 Hz, 1H), 7.46 (dd, J = 2.4, 8.7 Hz, 1H), 7.05 (d, J = 8.7 Hz, 1H).

5.3.1. 1-(2,4-Dibromophenyl)hydrazine hydrochloride (23a)

Preparation of 1-(2,4-dibromophenyl)hydrazine hydrochloride **23a** (0.5 g, 41%) from 2,4-dibromoaniline (1.0 g, 4.0 mmol) by a procedure similar to that for **23b**. ^{1}H NMR (DMSO, 400 MHz) δ 10.11 (br, 2H), 8.01 (br, 1H), 7.79 (d, J = 2.0 Hz, 1H), 7.88 (dd, J = 2.0, 8.8 Hz, 1H), 6.97 (d, J = 8.8 Hz, 1H).

5.3.2. 1-(2-Bromo-4-methylphenyl)hydrazine hydrochloride (23c)

Preparation of 1-(2-bromo-4-methylphenyl)hydrazine hydrochloride **23c** (311.5 mg, 33%) from 2-bromo-4-methylaniline (744.2 mg, 4.0 mmol) by a procedure similar to that for **23b**. ^{1}H NMR (DMSO, 300 MHz) δ 10.00 (br, 2H), 7.68 (br, 1H), 7.40 (s, 1H), 7.16 (d, J = 8.1 Hz, 1H), 6.95 (d, J = 8.1 Hz, 1H), 2.49 (s, 3H).

5.3.3. 5,5-Dimethyl-2-(trifluoroacetyl)cyclohexane-1,3-dione (25a)

To a solution of CDI (1.2 g, 7.1 mmol) in $CHCl_3$ (35.0 mL) was added a solution of trifluoroacetic acid (1.1 mL, 14.2 mmol) in $CHCl_3$ (53.0 mL) slowly. Then the mixture was treated with a solution of 5,5-dimethyl-1,3-cyclohexanedione (504.7 mg, 3.6 mmol) and imidazole (245.1 mg, 3.6 mmol) in $CHCl_3$ (35.0 mL), and stirred at room temperature for 45 min. After the reaction was finished, the mixture was washed with 1 N $HCl_{(aq)}$ (50.0 mL) and water (50.0 mL). The organic layer was dried over $MgSO_4$ and concentrated under reduced pressure. The residue was purified by Isco CombiFlash Companion column chromatography (silica gel, 0–100% EtOAc/hexane) to give compound **25a** (1.1 g, 77%) as a powder.

5.3.4. 2-(Cyclopropylcarbonyl)-5,5-dimethylcyclohexane-1,3-dione (25b)

To a solution of 5,5-dimethyl-1,3-cyclohexanedione (995.3 mg, 7.1 mmol), DMAP (256.6 mg, 2.1 mmol) and DIPEA (1.2 mL, 7.1 mmol) in CH_2Cl_2 (7.0 mL) was added a solution of cyclopropanecarbonyl chloride (0.65 mL, 7.1 mmol) in CH_2Cl_2 (1.8 mL) slowly. The reaction was refluxed for 2 h. After the reaction was finished, CH_2Cl_2 was removed and the residue was purified by Isco CombiFlash Companion column chromatography (silica gel, 0–30% EtOAc/hexane) to give compound **25b** (1.1 g, 77%) as a powder. ^{1}H NMR ($CDCl_3$, 400 MHz) δ 3.60–3.56 (m, 1H), 2.52 (s, 2H), 2.40 (s, 2H), 1.32–1.28 (m, 2H), 1.16–1.09 (m, 8H).

5.3.5. 1-(2,4-Dichlorophenyl)-6,6-dimethyl-3-(trifluoromethyl)-1,5,6,7-tetrahydro-4*H*-indazol-4-one (26)

The reaction of 1-(2,4-dichlorophenyl)hydrazine hydrochloride (85.4 mg, 0.40 mmol) with 5,5-dimethyl-2-(trifluoroacetyl)cyclohexane-1,3-dione **25a** (94.5 mg, 0.40 mmol) in ethanol (1.2 mL) under reflux for 5 h, by a procedure similar to that for **20**, gave 1-(2,4-dichlorophenyl)-6,6-dimethyl-3-(trifluoromethyl)-1,5,6,7-tetrahydro-4*H*-indazol-4-one **26** (71.5 mg, 47%) as a powder. ^{1}H NMR (CD_3OD, 400 MHz) δ 7.83 (d, J = 1.2 Hz, 1H), 7.61 (dd, J = 1.2, 1.2 Hz, 2H), 2.63 (s, 2H), 2.48 (s, 2H), 1.11 (s, 6H); HRMS calcd for $C_{16}H_{14}Cl_2F_3N_2O$ (M+H) 377.0435, found 377.0442.

5.3.6. 3-Cyclopropyl-1-(2,4-dichlorophenyl)-6,6-dimethyl-1,5,6,7-tetrahydro-4*H*-indazol-4-one (27)

The reaction of 1-(2,4-dichlorophenyl)hydrazine hydrochloride (234.9 mg, 1.1 mmol) with 2-(cyclopropylcarbonyl)-5,5-dimethylcyclohexane-1,3-dione **25b** (199.9 mg, 0.96 mmol) in ethanol (5.0 mL) at room temperature overnight, by a procedure similar to that for **20** using NaOH (44.0 mg, 1.1 mmol) instead of H_2SO_4, gave 3-cyclopropyl-1-(2,4-dichlorophenyl)-6,6-dimethyl-1,5,6,7-tetrahydro-4*H*-indazol-4-one **27** (36.2 mg, 11%) as a powder. ^{1}H NMR ($CDCl_3$, 400 MHz) δ 7.55 (d, J = 2.0 Hz, 1H), 7.39 (dd, J = 2.0, 8.4 Hz, 1H), 7.33 (d, J = 8.4 Hz, 1H), 2.62–2.58 (m, 1H), 2.45 (s, 2H), 2.40 (s, 2H), 1.10 (s, 6H), 1.03–1.00 (m, 4H); HRMS calcd for $C_{18}H_{19}Cl_2N_2O$ (M+H) 349.0874, found 349.0875.

5.3.7. 3,6,6-Trimethyl-1,5,6,7-tetrahydro-4*H*-indazol-4-one (28)

To a solution of 2-acetyl-5,5-dimethyl-1,3-cyclohexanedione (72.9 mg, 0.40 mmol) in THF (2.0 mL) was added hydrazine (0.80 mmol, 65% in H_2O). The reaction was stirred under reflux. After 4 h, THF was removed and the residue was washed with water (2.0 mL) and CH_2Cl_2 (1.0 mL) to give 3,6,6-trimethyl-1,5,6,7-tetrahydro-4*H*-indazol-4-one **28** (30.1 mg, 42%) as a powder. ^{1}H NMR (DMSO, 300 MHz) δ 2.59 (s, 2H), 2.33 (s, 3H), 2.21 (s, 2H), 0.98 (s, 6H); HRMS calcd for $C_{10}H_{15}N_2O$ (M+H) 179.1184.

5.3.8. 1-(2,4-Dibromophenyl)-3,6,6-trimethyl-4,5,6,7-tetrahydro-1*H*-indazol-4-ol (29)

A solution of tetrahydroindazol-4-one **19** (30.1 mg, 0.073 mmol) in CH_2Cl_2 (1.0 mL) and MeOH (0.30 mL) was treated with $NaBH_4$ (14.0 mg, 0.37 mmol), and stirred at room temperature overnight. The reaction mixture was quenched with satd $NH_4Cl_{(aq)}$ (1.0 mL) and extracted with CH_2Cl_2 (2.0 mL). The organic layer was dried over $MgSO_4$ and concentrated under reduced pressure. The residue was purified by Isco CombiFlash Companion column chromatography (silica gel, 0–70% EtOAc/hexane) to give 1-(2,4-dibromophenyl)-3,6,6-trimethyl-4,5,6,7-tetrahydro-1*H*-indazol-4-ol **29** (19.0 mg, 63%) as a powder. ^{1}H NMR (CD_3OD, 300 MHz) δ 8.00 (d, J = 1.8 Hz, 1H), 7.69 (dd, J = 1.8, 8.7 Hz, 1H), 7.31 (d, J = 8.7 Hz, 1H), 4.81 (dd, J = 7.8, 7.8 Hz, 1H), 2.34 (s, 3H), 2.29 (d, J = 15.9 Hz, 1H), 2.05 (d, J = 15.9 Hz, 1H), 1.90 (dd, J = 7.8, 13.2 Hz, 1H), 1.55 (dd, J = 7.8, 13.2 Hz, 1H), 1.09 (s, 3H), 0.96 (s, 3H); HRMS calcd for $C_{16}H_{19}Br_2N_2O$ (M+H) 412.9864, found 412.9864.

5.4. FLIPR® calcium assay

One day before the assay, CORNING® black with clear flat bottom 96-well assay plates were coated with a 0.1 mg/mL

Poly-L-Lysine solution. Chinese hamster ovary (CHO)-K1 cells, stably expressing hMOR and Gα15 (GenScript), were suspended in the F12 medium and plated at a density of $\sim 8 \times 10^4$ cells/well in 200 μL medium. Cells were incubated in a humidified atmosphere of 10% CO_2 at 37 °C overnight so as to reach an 80–90% confluent cell monolayer before assay. At the day of assay, 150 μL medium/well was removed from plate. To each well, 50 μL FLIPR® calcium assay reagent dissolved in 1× assay buffer (HBSS: KCl 5 mM, KH_2PO_4 0.3 mM, NaCl 138 mM, $NaHCO_3$ 4 mM, Na_2HPO_4 0.3 mM, d-glucose 5.6 mM, with additional 20 mM HEPES and 13 mM $CaCl_2$, pH 7.4), with 2.5 mM probenecid was added and the plate is incubated at 37 °C for 1 h. Compounds and other reagents were dissolved in the assay buffer. Using a FlexStationIII (Molecular Devices), the $[Ca^{2+}]_i$ fluorescence increases after robotic injections of compounds or other reagents were monitored every 1.52 s interval with excitation wavelength at 485 nm and with emission wavelength at 525 nm. The $[Ca^{2+}]_i$ fluorescence was measured up to 90 s after agonist injection. The fluorescence intensity from 6 to 12 wells of cells were averaged and the relative amount of $[Ca^{2+}]_i$ release was determined by integrating the AUC of the $[Ca^{2+}]_i$ fluorescence averages.

5.5. Competition radioligand binding assay

Briefly, HEK-MOR cells were washed twice and scraped off the culture plate with ice-cold phosphate-buffered saline (PBS), and pelleted by centrifugation at 1000*g*, 10 min, 4 °C. Cell pellets were homogenized in assay buffer (50 mM Tris–HCl, pH 7.5), at 4 °C. Cellular membrane pellet was collected by centrifuge at 24,000*g* for 30 min at 4 °C, and resuspended in assay buffer. For competition binding assay, cell membranes were incubated for 60 min in 100 μL of assay buffer, containing 5 nM [^{3}H]naloxone plus unlabeled compounds in concentrations ranging from 0.01 nM to 10 mM. The binding reaction was terminated by filtration over glass-fiber filters and washed in ice-cold assay buffer. Nonspecific binding was determined in the presence of 10 μM naloxone.

5.6. HTRF cAMP assay

We used Cisbio HTRF cAMP assay to measure [cAMP]. Briefly, 2000 HEK293 cells stably expressing opioid receptors (μ, δ, or κ) or parental cells were plated in 10 μL/well of DMEM in 96-well solid bottom white plates and 10 μL/well compound in HBSS in the presence with 1 μM forskolin and 100 μM IBMX. After 30 min incubation at room temperature, 10 μL/well of labeled d2-cAMP and 10 μL/well of anti-cAMP antibody (both diluted in lysis buffer) were added to each well. Two hours later, plates were measured using the FlexStationIII (Molecular Devices Corp.) with excitation at 330 nm and emissions of 620 nm and 665 nm.

5.7. Membrane potential assay

The mouse pituitary AtT-20 cells were transiently transfected with vehicle or a myc-tagged MOR plasmid using electroporation and seeded in 96-well plates. After 24 h, cells were serum-starved for 3 h to detect potassium conductance changes using a fluorometric imaging plate reader (FLIPR®) membrane potential assay according to the manufacturer's instructions (Molecular Devices). Briefly, serum-starved cells were treated with blue membrane potential dye for 0.5 h at 25 °C. Next, the cells were treated with vehicle or compounds, and their potassium conductances were measured using a FlexStation 3 benchtop multi-mode microplate reader (Molecular Devices).

5.8. Tail-flick test

Nociception of mice was evaluated by using a tail-flick instrument (Columbus Instruments). The intensity of the light was adjusted so that the averaged basal tail-flick latencies were 3–4 s. A cut-off time of 10 s was used as the maximum possible effect (MPE) to avoid tissue damage. A mouse that did not flick its tail within the cut-off time was considered as fully analgesic. The area under the time-response curve (AUC)[35] and the ED_{50} value was employed to evaluate the analgesic effect of the drug(s). The tail-flick latency was examined at 10, 20, 30, 40, 50, and 60 min after drug administration to plot time-response (test latency-basal latency) curve and the AUC value was calculated. The ED_{50} was determined by the up-and-down method.[36]

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Supplementary data

Supplementary data (the in vivo data of vehicle control and naloxone) associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.bmc.2014.07.012.

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21. We carried out the structure search by SciFinder 'exact structure search' for each of tetrahydroindazol-4-one analogs listed in this manuscript, 9 out of 26 analogs are available from commercial source (**1**, **2**, **4**, **5**, **6**, **7**, **9**, **18**, **28**), and 3 out of these 9 analogs have been reported in the literatures and performed biological assay or other applicable usage (**2**, **9**, **28**). Those 2 literatures are related to CDK inhibitors and NOS inhibitors, respectively. Most of the compounds discovered by us have never been reported in the literature, especially the most potent compounds in this manuscript, **19**, **20**, and **21**, belong to the new scaffold in MOR/KOR agonists.
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