



Full Paper

Possible biased analgesic of hydromorphone through the G protein-over β -arrestin-mediated pathway: cAMP, CellKey™, and receptor internalization analyses



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ARTICLE INFO

Article history:

Received 3 April 2019

Received in revised form

6 June 2019

Accepted 10 June 2019

Available online 2 July 2019

Keywords:

μ -opioid receptor

Hydromorphone

Biased agonist

β -arrestin

G protein

ABSTRACT

Morphine, fentanyl, and oxycodone are widely used as analgesics, and recently hydromorphone has been approved in Japan. Although all of these are selective for μ -opioid receptors (MORs) and have similar structures, their analgesic potencies and adverse effects (AEs) are diverse. Recent molecular analyses of MOR signaling revealed that the G protein-mediated signaling pathway causes analgesic effects and the β -arrestin-mediated signaling pathway is responsible for AEs. We used several cell-based analyses that selectively measure cellular responses activated by either G protein- or β -arrestin-mediated pathways. GloSensor™ cAMP, CellKey™, and receptor internalization assays were performed with four different types of cells stably expressing differentially labelled MOR. EC₅₀ values measured by cAMP and CellKey™ assays had potencies in the order fentanyl \leq hydromorphone $<$ morphine \leq oxycodone, all also exhibiting full agonist responses. However, in the internalization assay, only fentanyl elicited a full agonist response. Hydromorphone had the strongest potency next to fentanyl; however, contribution of the β -arrestin-mediated pathway was small, suggesting that its effect could be biased toward the G protein-mediated pathway. Based on these properties, hydromorphone could be chosen as an effective analgesic.

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Peer review under responsibility of Japanese Pharmacological Society.

1. Introduction

For acute or chronic cancer pain, strong opioids such as morphine have been used as the last treatment choice in the WHO 3-step analgesic ladder.¹ However, opioids often cause adverse effects (AEs) and lead to economic burden² so that pharmacological profiles must be well understood³ to efficiently use them.

Opioids bind to opioid receptors (ORs, classified as μ , δ , or κ) that belong to the G protein-coupled receptors (GPCRs).⁴ Among them, μ -opioid receptors (MORs) play important roles in mechanisms of pain and the elucidation of AEs.³ Different signaling cascades are responsible for different responses to MOR, which have allowed us to separate analgesic from AE signaling.^{5–7} Such diverse effects are attributed to diverse cellular signaling including inhibition of adenylate cyclase to cause a variety of pharmacological effects.^{3,4,8}

Opioid-induced AEs include intolerance, toxicity, and dependence. With such AE, pain management is not always easy and requires a steep increase in analgesic dosage over time.^{2,9} To avoid this, a change to non-opioid or other opioid analgesics (opioid switching) is required in clinical situations. Following the concept of “biased agonism”, some agonists differentially activate signaling molecules downstream of GPCRs; based on which pathways these agonists activate, use of one opioid analgesic over the other could result in better clinical responses and represents novel strategies in pain management.^{4,10} Recent molecular analyses of the properties of MOR revealed two downstream pathways: one leading to mainly analgesic effects through a G protein-mediated and another through the β -arrestin-mediated pathway to cause unpleasant AEs such as respiratory depression.^{3,11,12} To further understand the mechanism, many experimental approaches have been used.¹³ Among opioids, morphine (MRP), oxycodone (OXY), and fentanyl (FEN) are widely used to alleviate moderate to severe pain, especially for cancer patients.¹⁴ and in 2017 hydromorphone (HDM) was approved as another strong opioid in Japan.¹⁵

HDM, a semi-synthetic morphine derivative first synthesized in 1924, differs only at its benzol ring having a keto-group instead of a hydroxy group and only one functional group is different between HDM and OXY (Fig. 1). As of now, HDM has been employed in 45 countries and regions alongside standard opioids.^{14,16,17} Despite this, the properties of HDM, particularly its effects on ORs are poorly clarified. In *in vitro* studies of cells overexpressing opioid μ , δ , and κ receptors, only three reports are available to compare the effects of HDM with other opioids.^{18–20} We therefore proposed to compare the properties and potency of HDM with other opioids using *in vitro* receptor assay systems, especially focusing on the biased signal mechanisms.^{3,10}

2. Materials and methods

2.1. Chemicals

The following reagents were used: D-Ala(2)-N-Me-Phe(4)-Gly(5)-enkephalin (DAMGO), forskolin, 3-isobutyl-1-methylxanthine (IBMX), Ro 20–1724 (Sigma–Aldrich, St. Louis, MO, USA); HaloTag[®] pH Sensor Ligand (Promega, Madison, WI,

USA); Hoechst 33342 (Dojinkagaku, Kumamoto, Japan); morphine hydrochloride (Takeda Pharmaceutical Co., Ltd., Tokyo, Japan); fentanyl (Janssen Pharmaceutical K. K., Tokyo, Japan); oxycodone hydrochloride hydrate (Shionogi & Co., Ltd., Osaka, Japan); and hydromorphone hydrochloride (Daiichi Sankyo Co., Ltd., Tokyo, Japan). Forskolin, IBMX, and Ro 20–1724 were diluted with dimethyl sulfoxide (DMSO) and other chemicals were diluted with water.

2.2. Construction of plasmids and generation of stable cell lines

Human MOR (hMOR) cDNA (NM_000914) with/without a stop codon was amplified from a Flexi ORF clone (Promega). The amplified hMOR fragment, N-terminal HaloTag[®] (Kazusa DNA Research Institute, Chiba, Japan) or Flag-fused hMOR were introduced into a pcDNA3.1 (+) vector (Life Technologies, Carlsbad, CA, USA). Additionally, a GFP coding sequence was inserted into the 3' terminus of the stop codon-deleted hMOR sequence. Human embryonic kidney 293 cells (HEK293, American Type Culture Collection, Manassas, VA, USA) stably expressing HaloTag[®] fused MOR (Halotag-MOR), Flag-fused MOR (Flag-MOR), or GFP fused MOR (GFP-MOR) were generated through transfection of plasmids using Lipofectamine (Life Technologies), and selected based on MOR activity measured using the CellKey[™] assay. pGloSensor[™] 22F plasmid (Promega) was transfected into hMOR-positive cells to generate a stable hMOR and pGloSensor[™] 22F (MOR/pGS22F) expressing cell line.

2.3. Cell culture

HEK293 cells (stably expressing Flag-, Halotag-, GFP-MOR or MOR/pGS22F) were cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum, 1% penicillin/streptomycin, and 700 μ g/mL G418 disulfate aqueous solution (Gibco, Palo Alto, CA, USA) to Flag-MOR and GFP-MOR, or 5 μ g/mL puromycin (InvivoGen, San Diego, CA, USA) to Halotag-MOR in a humidified atmosphere containing 5% CO₂ at 37 °C.

2.4. cAMP assay with GloSensor[™]

The GloSensor[™] cAMP assay was performed according to Meguro et al.²¹ Briefly, MOR/pGS22F cells were plated at 3.0×10^4 cells/well in poly-D-lysine-coated 96-well clear-bottomed plates (Corning, Corning, NY, USA). The next day, cells were equilibrated with diluted GloSensor[™] reagent (Promega) at RT for 2 h, and luminescence intensity was measured every 2.5 min for 40 min in the Synergy[™] H1 (BioTek

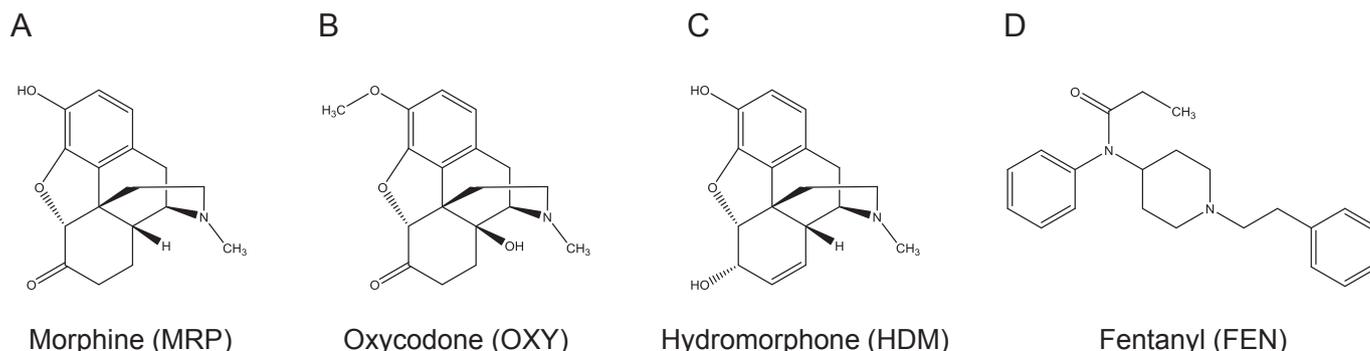


Fig. 1. Molecular structures of each opioid analgesic.

Instruments Inc., Winooski, VT, USA). Drugs were added and the luminescence intensity at the 10-min endpoint was defined as the baseline. Forskolin (3×10^{-6} M) was added at 10 min. Δ Luminescence intensity was calculated as the intensity of each point subtracted from baseline, and the area under the time- Δ luminescence intensity curve (AUC) was defined as the intracellular cAMP level (see Suppl. Fig. 2). AUC of each sample was subtracted from that of the negative control sample (Forskolin (3×10^{-6} M) alone). MOR responses were presented as percentage intracellular cAMP inhibition calculated by dividing the corrected AUC by data of standard sample (10^{-5} M MRP).

2.5. CellKey™ assay

The CellKey™ assay has been described previously.^{21,22} Briefly, cells were seeded at densities of 7.0×10^4 (Flag-MOR, Halotag-MOR), 5.0×10^4 (GFP-MOR), 3.0×10^4 cells/well (MOR/pGS22F) in CellKey™ poly-D-Lysine (Sigma Aldrich) coated 96-well microplates with an embedded electrode at the bottom of each well, and then incubated for 24 h.^{21,22} CellKey™ buffer composed of Hank's balanced salt solution (in mM: 1.3 CaCl₂·2H₂O, 0.81 MgSO₄, 5.4 KCl, 0.44 KH₂PO₄, 4.2 NaHCO₃, 136.9 NaCl, 0.34 Na₂HPO₄, and 5.6 D-glucose) containing 20 mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES) and 0.1% bovine serum albumin (BSA) was used. Cells were incubated for 30 min at 28 °C before assays. Changes in the impedance of an induced extracellular current (dZiec) in each well were measured every 10s for up to 30 min; taking the first 5 min as baseline, before drug treatment and dZiec measurement for 25 min. Magnitude of changes in dZiec values were defined as Δ Ziec (Suppl. Fig. 1). Δ Ziec values for each sample were corrected by that of the negative control sample. The standard sample was Δ Ziec by MRP reached peak at the minimum concentrations as indicated.

2.6. Receptor internalization assay with GFP-MOR

Cells expressing GFP-MOR were plated at 6.0×10^4 cells/well in 8-well chambered coverglasses (Thermo Fisher Scientific, Waltham, MA, USA) coated with polyethyleneimine, and then incubated for 24 h. Cells were washed with the internalization buffer (in mM; 10 HEPES, 140 NaCl, 5 KCl, 2 CaCl₂, 1 MgCl₂ and 10 D-glucose at pH7.4) and stained with 4 μ g/mL Hoechst 33342 for 10 min at 37 °C. Cells were then treated with opioid agonists or DAMGO and changes in localization of GFP-MOR and cell densities were captured by confocal microscopy (FLUOVIEW FV10i, OLYMPUS, Tokyo, Japan) every 30 min up to 180 min.

2.7. Internalization assay with Halotag-MOR

Internalization assay was performed with Halotag-MOR. Cells were stained with Hoechst 33342 for 15 min followed the pH sensor ligand at 0.5×10^{-6} M for 30 min (5% CO₂ at 37 °C) and washed once with the internalization buffer. Red spots in the cells were recorded by FLUOVIEW FV10i and counted using MetaMorph® 7.7 (Molecular Devices, San Jose, CA, USA). The density of "Spot count/cell" for each well before drug application was calculated and defined as 100% and data obtained every 30 min after opioid treatment. Graphs were made using GraphPad Prism 7 (GraphPad Software, La Jolla, CA, USA).

2.8. Statistical analysis and principles

Data are presented as means with S.E.M. for at least 3 independent experiments. Statistical analysis was performed using a

two-way analysis of variance (ANOVA) followed by the Tukey–Kramer test (GraphPad Prism 7). $P < 0.05$ was considered statistically significant.

All experiments were approved and performed in accordance with the Guide for Genetic Modification Safety Committee, National Cancer Center, Japan.

3. Results

3.1. Effects of each opioid agonist on intracellular cAMP levels measured with the GloSensor™ cAMP assay

We first measured intracellular cAMP levels as a result of activation of the G protein-mediated pathway using HEK293 cells expressing MOR/pGS22F.²³ We chose MRP, OXY, HDM and FEN as clinically used opioids (Fig. 1). All opioids caused a concentration-dependent decrease in cAMP levels (Fig. 2 and see Suppl. Fig. 1 for MRP and HDM). EC₅₀ values were the highest in FEN followed by HDM (FEN \leq HDM < MRP \leq OXY; Fig. 2 and Table 1) and some differences were clear between HDM and both OXY and MRP. In addition, there were significant differences in E_{max} values between HDM and both OXY and FEN (Fig. 2).

3.2. The potency and efficacy of each opioid agonist measured using CellKey™

We then used the CellKey™ system to measure whole cell MOR activity^{21,22} using four types of cells stably expressing each of Flag-MOR, GFP-MOR, Halotag-MOR or co-expressing MOR/pGS22F. All opioids elicited increases in cellular impedance in a concentration-dependent manner (Fig. 3: (A) Flag-MOR, (B) MOR/pGS22F, (C) GFP-MOR and (D) Halotag-MOR; and Suppl. Fig. 2: MRP and HDM). EC₅₀ values were FEN \leq HDM < MRP < OXY in all four cell types (Fig. 3 and Table 2). HDM was the second most potent agonist after FEN in terms of EC₅₀ values and showed different EC₅₀ compared to both OXY and MRP (Fig. 3 and Table 2). Although each opioid behaved as an almost full-agonist in the MOR assay, E_{max} was significantly different between HDM and other opioids, specifically OXY and FEN (Flag-MOR) and OXY and FEN (MOR-pGS22F), with no difference among four opioids in GFP-MOR or with FEN alone (Halotag-MOR) (Fig. 2 and Table 2). Although there were slight differences in EC₅₀ depending on the opioid, HDM was the second most potent agonist (Figs. 2 and 3 and Tables 1 and 2).

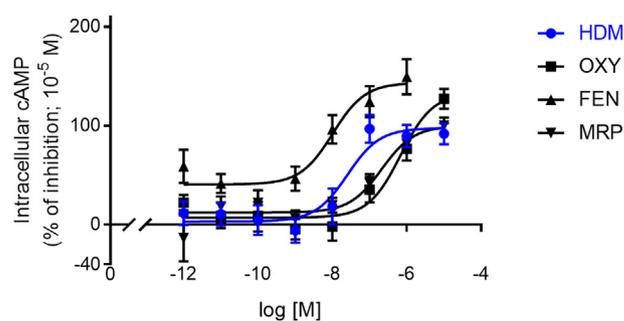


Fig. 2. Changes in cAMP levels induced by several opioids in HEK293 cells co-expressing μ -opioid receptor (MOR)/pGS22F. Intracellular cAMP levels in cells treated with various concentrations of morphine (MRP), oxycodone (OXY), hydromorphone (HDM), or fentanyl (FEN) were measured with the GloSensor™ cAMP assay. Concentration-response curves of the four opioids (MRP, OXY, HDM, and FEN) were described by calculating intracellular cAMP levels relative to data obtained at 10^{-5} M MRP. All data are presented as means \pm S.E.M. for 3 independent experiments ($n = 6-12$).

Table 1
E_{max} and EC₅₀ of each opioid with the GloSensor™ cAMP assay.

| | Log EC ₅₀ (M) | E _{max} (%) |
|-----|------------------------------|-------------------------------|
| HDM | -7.60 ± 0.29 | 97.67 ± 9.40 |
| OXY | -6.17 ± 0.22**** | 133.40 ± 14.71** |
| FEN | -7.99 ± 0.28 ^{n.s.} | 143.40 ± 11.86*** |
| MRP | -6.68 ± 0.24** | 100.00 ± 9.15 ^{n.s.} |

E_{max} (means ± S.E.M.) and log EC₅₀ (means ± S.E.M.) were calculated according to the results of Fig. 2.

p < 0.01, *p < 0.001, and ****p < 0.0001 versus HDM. n.s., not significant.

3.3. Internalization of MORs induced by each opioid in cells stably expressing GFP-MOR

Internalization is a process thought to be activated by the β-arrestin-mediated signaling pathway.³ To measure MOR internalization from cell surface to cytosol, cells stably expressing GFP-MOR were evaluated. GFP-MOR was localized at the cell surface before stimulation (Fig. 4). After treating the cells for 30 min, all opioid-treated cells showed internalization of GFP-MOR, whereas vehicle-treated cells did not (Fig. 4). Particularly, FEN and DAMGO elicited robust MOR internalization within 30 min, which persisted

until 60 min and after 180 min, internalized MOR seemed to be concentrated and formed large pits. MRP, OXY, and HDM caused less internalization of GFP-MOR compared to FEN and DAMGO (Fig. 4).

3.4. Internalization of MORs by each opioid in cells stably expressing Halotag-MOR

Although there was a certain tendency that some opioids caused receptor internalization with the cells expressing GFP-MOR as shown in Fig. 4, qualification by counting receptor numbers seemed to be subjective. Alternatively, we used cells stably expressing Halotag-MOR previously stained the HaloTag® with pH sensor ligand to quantify the internalization induced by each opioid. Halotag-MOR bound to the pH sensor ligand shifts from non-fluorescent to red in local acidic conditions, manifesting as red spots²⁴ (Suppl. Fig. 3). Upon application, FEN and DAMGO induced the significant appearance of red spots, possibly localized to the endoplasmic reticulum where pH is low. The increases in numbers of FEN- and DAMGO-induced red spots were time-dependent until 150 min (Fig. 5B). With OXY, MRP and HDM, there was also a time-dependent accumulation of red spots in

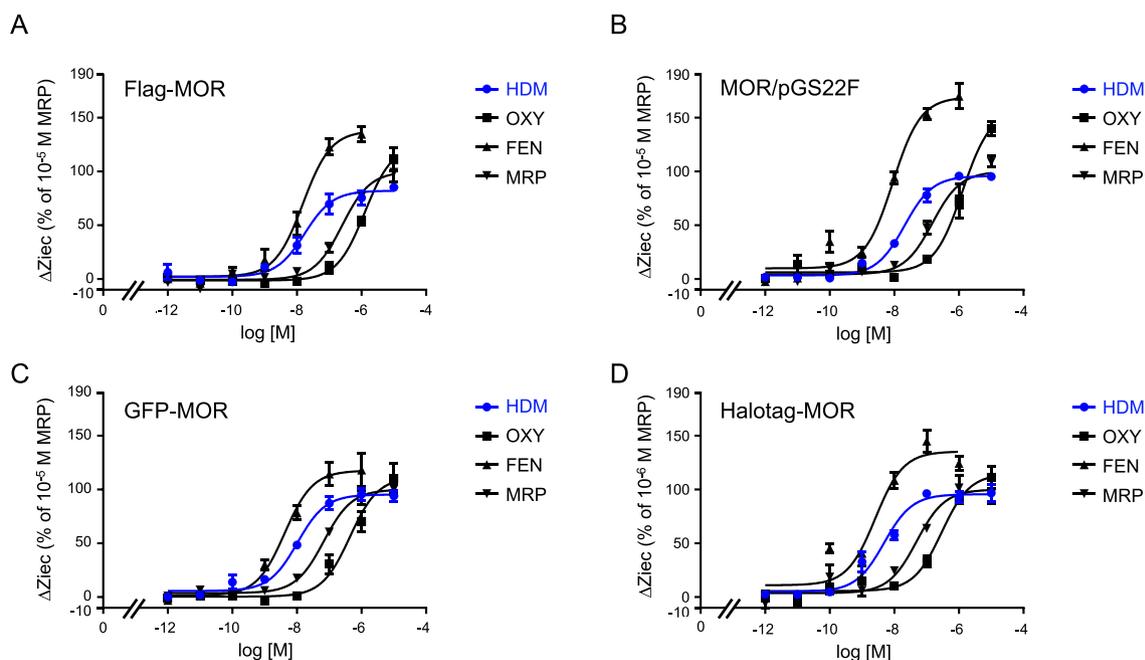


Fig. 3. Effects of several opioid analgesics on μ-opioid receptor (MOR) activity measured by the CellKey™ system in four types of cells expressing differentially tagged MORs. Potencies of each agonist [morphine (MRP), oxycodone (OXY), hydromorphone (HDM), or fentanyl (FEN)] were measured by the CellKey™ system. Cells were treated with various concentrations of opioids at the indicated concentrations. Concentration-response curves of the four opioids were described by calculating ΔZieac relative to data obtained at 10⁻⁵ M (A-C) and 10⁻⁶ M (D) MRP. The four types of MOR-expressing HEK293 cells were: Flag-tagged MOR (A), MOR co-expressed with pGS22F (B), GFP-tagged MOR (C), and Halotag-MOR (D). All points are presented as means ± S.E.M. for 3 or 4 independent experiments (n = 6–14).

Table 2
E_{max} and EC₅₀ of each opioid with the CellKey™ assay in four cells types.

| | Flag-MOR | | MOR/pGS22F | | GFP-MOR | | Halotag-MOR | |
|-----|------------------------------|------------------------------|------------------------------|------------------------------|------------------------------|------------------------------|------------------------------|------------------------------|
| | Log EC ₅₀ (M) | E _{max} (%) | Log EC ₅₀ (M) | E _{max} (%) | Log EC ₅₀ (M) | E _{max} (%) | Log EC ₅₀ (M) | E _{max} (%) |
| HDM | -7.76 ± 0.14 | 82.2 ± 3.38 | -7.69 ± 0.08 | 95.8 ± 2.63 | -7.98 ± 0.10 | 95.4 ± 2.98 | -8.32 ± 0.13 | 95.7 ± 3.50 |
| OXY | -5.90 ± 0.09**** | 125.4 ± 7.17**** | -5.89 ± 0.08**** | 157.1 ± 7.46**** | -6.36 ± 0.14**** | 111.4 ± 7.71 ^{n.s.} | -6.54 ± 0.15**** | 114.6 ± 7.40 ^{n.s.} |
| FEN | -7.82 ± 0.12 ^{n.s.} | 137.9 ± 5.23**** | -8.05 ± 0.10 ^{n.s.} | 169.2 ± 6.46**** | -8.38 ± 0.14 ^{n.s.} | 117.6 ± 5.69 ^{n.s.} | -8.59 ± 0.14 ^{n.s.} | 135.4 ± 5.69*** |
| MRP | -6.60 ± 0.11**** | 100.0 ± 4.73 ^{n.s.} | -6.82 ± 0.15** | 100.0 ± 6.26 ^{n.s.} | -7.17 ± 0.08** | 100.0 ± 2.98 ^{n.s.} | -7.31 ± 0.16*** | 100.0 ± 5.75 ^{n.s.} |

E_{max} (means ± S.E.M.) and log EC₅₀ (means ± S.E.M.) were calculated according to the results of Fig. 3.

p < 0.01, *p < 0.001, and ****p < 0.0001 versus HDM. n.s., not significant.

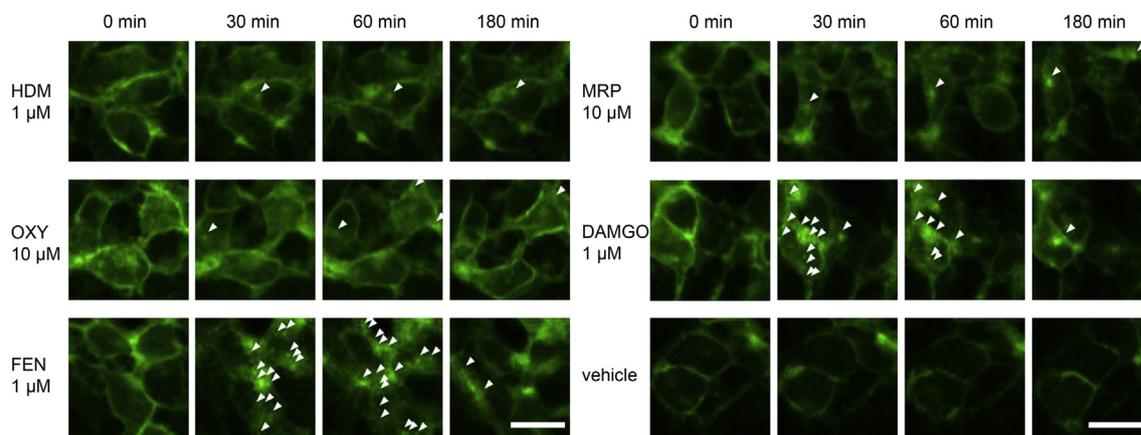


Fig. 4. Internalization of μ -opioid receptors (MORs) induced by several opioids in HEK293 cells expressing GFP-MOR. Internalization of GFP-tagged MOR was detected by confocal microscopy in HEK293 cells stably expressing GFP-MOR at four time points: 0, 30, 60, and 180 min, after treatment with morphine (MRP), oxycodone (OXY), hydromorphone (HDM), or fentanyl (FEN) or D-Ala(2)-N-Me-Phe(4)-Gly-ol(5)-enkephalin (DAMGO) at the indicated concentrations. Arrow heads indicate flocculated MOR in the cells. Scale bar = 20 μ m.

cells; however, no statistically significant difference was observed as vehicle-treated cells (Fig. 5B). FEN caused a concentration-dependent increase in red spots in the cells and significant at 10^{-6} M FEN (Fig. 5C). Similar results were obtained with the case of DAMGO (data not shown).

4. Discussion

Activation of MOR by several opioids involves two downstream pathways: one leads to mainly analgesic effects through a G protein-mediated signaling pathway and the other may cause AEs through a

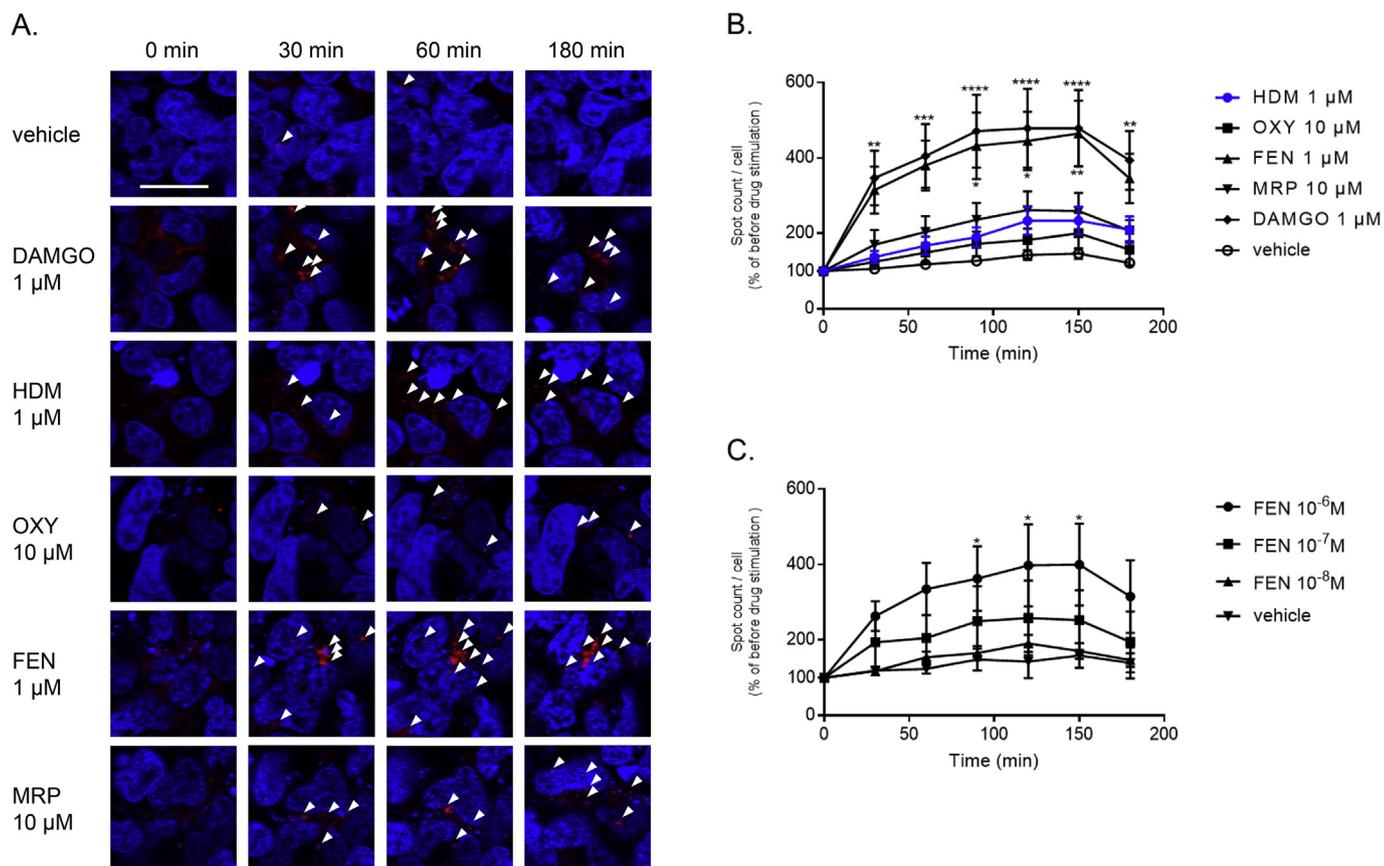


Fig. 5. Internalization of μ -opioid receptors (MORs) induced by several opioids in HEK293 cells stably expressing Halotag-MOR. HEK293 cells stably expressing Halotag-MOR were treated with the indicated opioids or a vehicle up to 180 min and were observed at the indicated time points. To quantify internalization levels, red spots in the cells were counted and calculated as spot counts/cell (%). (A) Representative images at the indicated time points in opioid-treated cells. (B) Time courses describing Halotag-MOR internalization after treatment with each opioid. (C) Time course of internalized Halotag-MOR after treatment with various concentrations of fentanyl (FEN; 10^{-8} to 10^{-6} M). All data are presented as means \pm S.E.M. ($n = 5-23$). * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$ and **** $p < 0.0001$ versus vehicle. All data are presented as means \pm S.E.M. for 5–23 independent experiments. * $p < 0.05$ and ** $p < 0.01$ versus vehicle. Scale bar = 20 μ m.

β -arrestin-mediated signaling pathway.^{3,11,12} Here, we evaluated the induction of these pathways by opioids including HDM using several experimental approaches. We found HDM to be biased toward the G protein-mediated signaling pathway. EC_{50} values measured by cAMP activity were $FEN \leq HDM < MRP \leq OXY$, indicating that HDM has higher analgesic potency next to FEN. Internalization of MOR by activation of the β -arrestin-mediated signaling pathway only occurred in the case of FEN, suggesting that HDM could be a biased agonist with a higher potency than MRP or OXY.

HDM, produced in 1924, has been used worldwide, but in Japan, it was approved for clinical use only in 2017.¹⁵ Molecular characterization of HDM using *in vitro* systems has not been documented. However, Gharagozlou and colleagues have measured some properties of HDM in cells expressing mouse μ , δ , and κ opioid receptors to compare its effects with other opioids.^{18–20} They showed the rank order cAMP inhibition potencies by MOR activation was $HDM > FEN > MRP$.¹⁸ Our results are different: $FEN \geq HDM > MRP \geq OXY$, which could be due to the inter-species difference between OR clones, and/or cells expressing ORs (Chinese hamster ovary [CHO] cells in theirs and HEK293 cells in ours).

In the present study, we determined MOR activities with the CellKey™ system to measure GPCR-mediated whole cell activity.^{21,22} The CellKey™ assay can detect changes in cellular shape and volume using cellular dielectric spectroscopy, an emergent technology that measures whole-cell responses in a label-free format.²⁵ In our assay, the results of CellKey™ as well as cAMP assay seemed to be similar, suggesting that both assays mainly detected G protein-mediated signals. An earlier report demonstrated that results from cAMP and CellKey™ assay in CHO cells expressing dopamine D₂₅ receptors had well correlated with D₂₅ agonist profiles.²⁵ Further, we previously showed MOR-mediated signaling by opioids and oxytocin with cAMP and CellKey™ assay almost identical.²¹ These results suggest that data with the CellKey™ and cAMP assay are representative of G protein-mediated signaling, at least in the case of MOR.

We compared the potency and efficacy of each opioid with four types of cells expressing MOR with the CellKey™ assay. Our results showed that the E_{max} and EC_{50} ratio of several opioids differed slightly but not significant among cell types. Previous experiments with different tag-expressed δ opioid receptors demonstrated that there were no significant differences between cell types.²⁶ Our results with cells in different tag-added MOR were not so different, indicating that data obtained with the cells can be compared even across the different measurement methods used in this study.

Internalization is a process through β -arrestin activation followed by binding of the receptors and β -arrestin.^{3,11,27} For internalization assay, we used two different methods, first to confirm that GFP-MOR upon activation internalized from cell surface to the cytosol, and then to quantitatively measure Halotag-MOR internalized into cells. Cells expressing Halotag-MOR can be stained with several types of “Halotag ligands” including for multicolor fluorescence imaging or detecting red spots at low pH in case of the pH sensor-Halotag ligand.²⁴ With the former method, GFP-MOR internalized were observed from just beneath the membrane, however, it might be difficult to count numbers of GFP-MOR objectively. With the latter method, Halotag-MOR pre-stained with the pH-sensor ligand can be measured by counting red spots, however, we may count internalized MOR only in the acidic endoplasmic reticulum.²⁴ With these two methods, nonetheless, we showed that FEN and DAMGO caused robust internalization.

Accumulating evidence indicates that agonists for MOR that activate only the G protein-signaling pathway are likely to cause less AEs.^{3,11,28–30} We previously reported that another opioid methadone is a β -arrestin-biased MOR agonist by comparing the above two signaling pathway profiles.³¹ We also reported that FEN

but not MRP, if treated repeatedly, caused the rapid development of tolerance resulting in antihyperalgesic effects in a murine neuropathic pain model,³² probably due to FEN-induced activation of β -arrestin-mediated pathways. Taking our previous and present results into account, HDM would be classified as a G protein-biased signaling agonist.

TRV130 has been reported to exhibit G protein-mediated signaling over β -arrestin-mediated signaling and showed significantly attenuated AEs while maintaining its analgesic potency.²⁹ Further, a synthetic analgesic SR-17018 activated G protein-mediated but not β -arrestin-mediated signaling *in vitro* and caused analgesic effects with less AEs *in vivo*.¹¹ A recent review regarding the discovery of novel opioids indicated that biased G protein-mediated drugs are attractive candidates as MOR agonists with lower AEs.³⁰

In the clinic, the order of analgesic potency determined by opioid conversion ratio is assumed to be $FEN < HDM < OXY \leq MRP$.³³ Our results are slightly different, with the order of potency being $FEN \leq HDM < MRP < OXY$. Intravenous or intramuscular administration potency, however, has been reported as $FEN < HDM < MRP < OXY$, which is well correlated with ours.^{34,35} In clinical use, a single opioid affects many signaling cascades that modulate analgesic potency (e.g. OXY also affects κ -OR³⁶), and such situation should also be considered.

According to the E_{max} values, the efficacy rates of MOR activation was $FEN \geq OXY \geq MRP \geq HDM$ except in the CellKey™ assay using GFP-MOR cells *in vitro* in our system. However, within maximal clinical concentration levels for each opioid, HDM is actually not a weak agonist. The maximum plasma concentration of each opioid (C_{max}) is as follows: FEN, 6.84×10^{-9} M (100 μ g/h transdermal)³⁷; OXY 2.60×10^{-7} M (40 mg in an immediate release capsule)³⁸; MRP 7.75×10^{-8} M (30 mg in an immediate release tablet)³⁹ and HDM 2.93×10^{-8} M (8 mg in an immediate release tablet).⁴⁰ Within these concentrations, the efficacy of maximal response of HDM is second to FEN (Figs. 2 and 3). In terms of E_{max} below maximal clinical concentrations and EC_{50} , HDM is indicated as a biased agonist with a potent analgesic effect.

A meta-analysis study for comparison of clinical effects of HDM and MRP conducted in 2011⁴¹ showed that HDM had some advantages over MRP regarding analgesia. Further, side effects in patients with renal failure or during acute analgesia titration were lower with HDM than with MRP.⁴¹ However, this was based on limited evidence and authors pointed out the need for further confirmation. A recent meta-analysis on opioid switching data with MRP, OXY, FEN, and HDM indicated that data of opioid switching among them has little scientific basis and that further research-based investigation is needed.³³ In this context, further basic and clinical studies regarding the properties of FEN, MRP, OXY, and HDM are required.

In conclusion, we demonstrated that HDM has biased analgesic properties. Comparison of MOR activities induced by several opioids in the study showed that HDM could be a useful analgesic that has a higher analgesic potency next to FEN and activates the β -arrestin-mediated signaling pathway to a lesser extent.

Conflict of interest

Yasuhito Uezono received financial support from Daiichi Sankyo Co. Ltd.

Acknowledgments

This work was supported by JSPS KAKENHI Grant Numbers JP15K08686, JP15K10522, JP15K08215, JP16K08568, JP18K07404 and JP18K08858; the National Cancer Center Research and

Development Fund (29-seeds-5); a grant from the Nakatomi Foundation; and a grant from Daiichi Sankyo Co., Ltd.

Appendix A. Supplementary data

Supplementary data to this article can be found online at <https://doi.org/10.1016/j.jpshs.2019.06.005>.

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