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A novel nanobody-based bio-assay using functional complementation of a split-nanoluciferase to monitor Mu-opioid receptor activation

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ABSTRACT: The Mu opioid receptor (MOR) has been the subject of intense research over the past decades, especially in the field of analgesic therapeutics. It is the primary target for both clinical and recreational opioids. Recently, camelid-derived nanobodies have received significant attention due to their applicability in stabilizing the crystal structure of activated MOR, via specific recognition of and binding to the active receptor conformation. In the present study, we developed and applied a novel bio-assay to monitor MOR activation, utilizing intracellular expression of one such nanobody, Nb39. The principle of functional complementation of a split-nanoluciferase was used to assess recruitment of Nb39 to MOR, following activation by a set of five synthetic opioids. The obtained pharmacological parameters - negative logarithm of EC_{50} (pEC_{50}, as a measure of potency) and maximal response provoked by a ligand (E_{max}, as a measure of efficacy; relative to hydromorphone) - were compared with those obtained using a G protein recruitment assay, in which a mini-Gi protein (engineered GTPase domain of Gαi subunit) is recruited to activated MOR. Similar EC_{50} but distinct E_{max} values were obtained with both bio-assays, with lower E_{max} values for the Nb-based bio-assay. Both bio-assays may assist to gain better insight into activation of the MOR.

KEYWORDS. New psychoactive substances, Mu opioid receptor, nanobody, bio-assay, functional complementation, nanoluciferase

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INTRODUCTION

The Mu opioid receptor (MOR) has been the subject of intense research over the past decades, especially in the field of analgesic therapeutics. This member of the G protein-coupled receptor (GPCR) family is the primary target for both clinical and recreational opioids [1]. As is the case for other GPCRs, the physiological effects of MOR are mediated by activation of heterotrimeric G proteins, for MOR this is primarily the adenylyl cyclase-inhibitory family of G proteins (Gi/o). MOR can also cause the recruitment of β-arrestins, which decouple the receptor from the G proteins [2].

Recently, nanobodies (Nbs) have gained popularity due to their application in stabilizing the active conformation of agonist-bound GPCRs, thus facilitating the acquisition of structural insights into GPCR activation [3-6]. As the name suggests, these Nbs are small, as they only correspond to the variable domain of heavy-chain antibodies, as opposed to conventional heterotetrameric antibodies. Yet, they possess the complete antigen-binding capacity of the parental antibody [4] and may serve as probes that specifically bind to the activated receptor, based on an intracellular conformational change caused by activation.

In order to understand the structural complex of MOR following receptor activation, Kobilka and colleagues [7] crystallized MOR bound to a morphinan agonist, BU72, in the presence of a G protein mimetic Nb. Amongst the several Nb clones that were generated were Nb33 and Nb39, which have been used in several studies [7-9]. Recently, Stoeber *et al.*, using live cell total internal fluorescence microscopy, compared the recruitment of mini-Gsi (chimeric Gs alpha with nine residues at the distal C-terminus replaced by corresponding residues from Gi alpha 1) and Nb33 to opioid receptors (MOR, Kappa opioid receptor (KOR)) in response to various opioids [10]. A robust and rapid recruitment of both intracellular molecules to both opioid receptors was observed following stimulation with their respective agonists, which was reversed upon addition of their respective antagonists [10]. Likewise, Gillis, Gondin and colleagues recently successfully applied these Nb33- and mini-Gsi proteins, fused to the Venus fluorescent protein, for characterizing the activation of MOR (fused to nanoluciferase) by a panel of ligands, using bioluminescence resonance energy transfer (BRET) [11]. Based on the above observations, these nanobodies (both Nb33 and Nb39) can be aptly referred to as “activation profile mimetics”.

With the above in mind, we have developed a novel, stable MOR reporter system, based on the recruitment of Nb39 to activated MOR by means of NanoLuc Binary Technology® (NanoBiT®) technology, a tool based on functional complementation of a split-nanoluciferase, designed to study protein-protein interactions. Given the relevance of *in vitro* characterization of newly emerging synthetic opioids in forensic toxicology [12-14], we applied, as a proof of principle, this novel MOR-Nb bio-assay on a set of five previously characterized synthetic opioids [13]. These comprised fentanyl and three of its analogues, as well as one non-fentanyl opioid from the benzamide family, U-47700. The results (pEC_{50} and E_{max}) of this new bio-assay were compared with those from another, recently

developed MOR-mini-Gi bio-assay, (also using the principle of functional complementation of a split-nanoluciferase) [13], as both events are a reflection of activated MOR. Both the Nb-and mini-Gi-based bio-assay rely on the coupling of an intracellular molecule (either mini-Gi or Nb39), fused to a small part of the split-nanoluciferase, to activated MOR, C-terminally fused to the complementary large part of the split-nanoluciferase, in the same cellular context. These bioluminescence-based bio-assays offer several advantages over (advanced) microscopy-based assays: they do not require sophisticated equipment (a standard luminometer suffices), and they are well-suited for high-throughput screening purposes and for pharmacological characterization of extensive sets of compounds. Moreover, in contrast to FRET/BRET assays, in which fusions with (a) larger fluorescent protein(s) are needed, the use of a split nanoluciferase allows a minimal increase in size of the partnering molecules [15].

METHODS

Chemicals and Reagents

The reference standards for the compounds fentanyl, acetylfentanyl, valerylfentanyl hydrochloride, butyrylfentanyl hydrochloride and U-47700 hydrochloride were purchased from Chiron Pharmasynth AS (Trondheim, Norway). Hydromorphone, poly-D-lysine and fetal bovine serum (FBS), chloroquine and puromycin were from Sigma-Aldrich (Steinheim, Germany). Anti-truncated nerve growth factor (dNGFR) antibody-coupled to allophycocyanin (APC) was purchased from Chromaprobe (Maryland Heights, MO, USA). Iscove's Modified Dulbecco's Medium (IMDM), a Calcium Phosphate Transfection kit, Dulbecco's Modified Eagle Medium (DMEM) + GlutaMAX™, OptiMEM® I Reduced Serum, trypsin, penicillin, streptomycin and amphotericin B were from Thermo Fisher Scientific (Pittsburg, PA, USA). FuGENE® HD, Nano-Glo® Live Cell substrate furimazine and Nano-Glo® dilution buffer were from Promega (Madison, WI, USA). Methanol and acetonitrile were from Biosolve Chemie (France). Human Embryonic Kidney (HEK) 293T cells (passage 20) were kindly provided by Prof. O. De Wever (Laboratory of Experimental Cancer Research, Department of Radiation Oncology and Experimental Cancer Research, Ghent University Hospital, Belgium).

Development of Nb39-NanoBiT® plasmid constructs

The NanoBiT® system from Promega was employed, using fusion constructs of MOR and Nb39 with split fragments of a modified nanoluciferase (NanoLuc). The generation of MOR-NanoBiT® fusion constructs has been described previously [16]. The different Nb39-NanoBiT® fusion constructs (Nb39-LgBiT, LgBiT-Nb39, Nb39-SmBiT and SmBiT-Nb39) were generated by standard cloning procedures (see **ESM S1**).

Selection of an optimal MOR-Nb39 reporter system

To study the recruitment of Nb39 to activated MOR, the optimal configuration of the MOR-NanoBiT®-Nb39 system had to be selected. This was achieved by transiently transfecting Human Embryonic kidney293T (HEK293T) cells with different combinations of MOR and Nb39, each fused to either Large BiT (LgBiT, large subunit of nanoluciferase) or Small BiT (SmBiT, small subunit of nanoluciferase). HEK293T cells were seeded in a 6-well plate at a density of 5 x 10^5 cells / well on the first day. On the next day, the cells were transiently transfected with distinct combinations of MOR and Nb39-fusion constructs using FUGENE® HD reagent as per the manufacturer's protocol. On the third day, the cells were detached with trypsin / EDTA and re-seeded on a white 96-well plate (poly-D-lysine coated) at a density of 5 x 10^4 cells / well and incubated overnight. On day four, i.e. on the day of the experiment, the cells were washed once with Opti-MEM® I Reduced Serum Medium and then 90 µL of this reduced serum medium was added. To this, 25 µL of the substrate, furimazine, which was prepared by 20-fold dilution with Nano-Glo® LCS dilution buffer, was added and the luminescence was monitored in a TriStar2 LB 942 multimode plate reader, controlled by ICE software (Berthold Technologies GmbH & Co., Bad Wildbad, Germany) until the signal stabilized. Next, 20 µl of a (6.75x) concentrated MOR agonist, hydromorphone (HM), in Opti-MEM® I or solvent control (Opti-MEM® I) was added and luminescence was monitored for 120 min.

Generation of a stable MOR-Nb39 reporter cell line by retroviral transduction

After selecting the optimal MOR-Nb39 reporter configuration, a stable cell line expressing MOR-LgBiT together with SmBiT-Nb39 was generated via retroviral transduction and cell sorting, following standard procedures, as described before (details in see **ESM Figure S1**).

Screening of synthetic opioids using MOR reporter assay

The performance of the newly developed MOR-Nb reporter assay was compared with that of a recently developed MOR-mini-Gi bio-assay (both in the context of stable HEK293T cell lines). The procedure for establishing the latter was described before [13]. For this comparison, we used a set of five synthetic opioids. The selection of these synthetic opioids was based on our previous work [13], wherein a panel of twenty-one synthetic opioids was tested on two platforms: coupling of mini-Gi and recruitment of β-arrestin-2 (βarr2) to MOR. Besides fentanyl and three analogues, also the benzamide U-47700 was taken along, as it was found to be a highly efficacious ligand in the mini-Gi assay [13].

HEK293T cells stably expressing MOR-LgBiT together with either SmBiT-Nb39 or SmBiT-miniGi fusion proteins were maintained in DMEM, supplemented with 10% FBS, 100 µg/mL streptomycin and 100 IU/l penicillin in a humidified environment (37°C, 5% CO_2). On day 1 of the experiment, the stable cells

were seeded at a density of 5 x 10^4 cells / well on a poly-D-lysine coated, white 96-well plate. The assay was performed as described above. Twenty µl of (6.75x) concentrated ligand solution prepared in Opti-MEM I Reduced Serum Medium was added, and the luminescence was measured for 2 hours. All the stock solutions were provided in methanol, except for that of butyrylfentanyl, which was in acetonitrile, and hence, 6.75x stock solutions were prepared in appropriate solvents. The final concentration of opioid in the well ranged from 10 pM - 10 µM. A solvent control (blank) with 0.5-5% methanol or acetonitrile in Opti-MEM® I was run in all experiments. HM was also run on every plate and used as a reference compound based on prior experience for the purpose of normalization between different plates as well as for comparison across different studies [13].

Data analysis

Statistical analysis and curve fitting for the normalized responses were performed using the GraphPad Prism software (San Diego, CA, USA). The absolute luminescence signals were first corrected for solvent control and inter-well variability. Concentration-response curves (used for calculation of the area under the curve; AUC) were normalized to the maximum response of the reference compound, HM, that was arbitrarily set to 100%. The data is expressed as mean ± standard deviation (SD) derived from three independent experiments, performed on different days and in duplicate. The pharmacological parameters E_{max} and EC_{50} were obtained by fitting a non-linear regression model (three parameter model) to the normalized responses. The same procedure was used for the calculation of pharmacological parameters E_{max} and EC_{50} from the first 16 minutes (ascending part of the curves, including the maximum).

For the selection of the optimal combination of Nb39 coupling to the activated receptor, the results are expressed as mean fold change ± standard deviation (SD), with eight replicates and statistical analysis by a two-tailed t-test.

Statistical analysis for the comparison of potencies (pEC_{50}) between MOR-mini-Gi and MOR-Nb39 for all compounds was performed by a two-tailed t-test.

RESULTS AND DISCUSSION

Development of a stable MOR-Nb39 reporter assay for real-time assessment of recruitment of Nb39 to activated MOR.

In this study, we set up a novel bio-assay by making use of split-nanoluciferase technology and Nb39 as a tool to assess MOR activation by synthetic opioids. The bio-assay's optimal configuration was evaluated by transiently transfecting different combinations of fusion constructs of MOR and Nb39 with the two fragments of a split-nanoluciferase (LgBiT and SmBiT) in HEK293T cells. As MOR is an integral membrane protein, it was tagged C-terminally with LgBiT or SmBiT [16], while the cytosolic Nb39 was either N- or C-terminally tagged. This resulted in four different combinations that were tested **(Figure 1)**. Addition of the MOR agonist hydromorphone (HM) (10 μM) yielded a clear signal for all combinations, indicating that tagging Nb39 did not hamper its recruitment to activated MOR. This can be explained by the fact that neither the N- nor C-terminus of Nb39 is engaging with MOR: the 6 key Nb39 residues that establish the contact with activated MOR lie in the Nb's framework region 3 and its complementarity determining region 2 and 3; the key residues of MOR that interact with Nb39 lie in the intracellular loop 2,3 and helix 8 [7]. As combining MOR-LgBiT with Nb39-SmBiT yielded the least fold change upon stimulation, this set-up was not further considered. From the other set-ups, which did not differ significantly in performance, we opted for MOR-LgBiT and SmBiT-Nb39 because this configuration most closely resembles the optimal combination chosen for the mini-Gi assay we previously established, in which MOR-LgBiT is combined with SmBiT-mini-Gi [13]. Since one of the aims of this study was to compare the read-out of the newly set up system with that of a readily existing system, we wanted to keep the number of variables as low as possible, to minimize any kind of artefact introduced due to e.g. differential tagging of MOR or the recruited cytosolic proteins. This is highly relevant as it is well-known that the set-up of a system may strongly govern the outcome [17]. Thus, in conclusion, as the cell line was the same (HEK293T), the receptor construct was the same (MOR-LgBiT), the assay principle was the same (functional complementation of a split nanoluciferase) and the read-out was the same (bioluminescence), the result should only be influenced by aspects related to the innate nature of the recruited intracellular molecule (either SmBiT-mini-Gi or SmBiT-Nb39) and its association with activated MOR.

Based on the above-mentioned results, a stable HEK293T cell line co-expressing MOR-LgBiT and SmBiT-Nb39 was generated by retroviral transduction. Periodically, the expression of both fusion proteins was monitored by flow cytometry by means of the co-expressed markers [Enhanced green fluorescent protein (EGFP) for MOR and truncated nerve growth factor receptor (dNGFR) for Nb39]. Importantly, expression of MOR-LgBiT (assessed via co-expressed EGFP) in the MOR-LgBiT + SmBiT-Nb39 cell line was similar to that in the MOR-LgBiT + SmBiT-mini-Gi cell line **(see ESM Figure S1)**.

Comparison of the MOR-mini-Gi and MOR-Nb39 bio-assays using a panel of synthetic opioids.

As a proof of principle, we evaluated a set of five synthetic opioids, including fentanyl, three designer fentanyls (acetylfentanyl, valerylfentanyl and butyrylfentanyl) and one non-fentanyl opioid (U-47700) from the benzamide family using both MOR reporter systems. These compounds were chosen because they display a variety of potencies and efficacies. All compounds yielded a sigmoidal concentration-response curve (although in the MOR-Nb39 assay no plateau was reached for valerylfentanyl). From these curves, the pharmacological parameters efficacy (by means of E_{max}) and potency (EC_{50}) were derived. The efficacies for all the compounds from both bio-assays were normalized to the E_{max} of HM, which was arbitrarily set to 100% **(Figure 2A, B, Table 1)**.

Comparison of efficacies (E_{max}) and potencies (pEC_{50}) for five synthetic opioids at the two platforms.

The parameters derived from the obtained activation profiles (pEC_{50} and E_{max}) at 2 h read-out were compared with those obtained using a similar assay to assess recruitment of G proteins to activated MOR, i.e. a previously established MOR-mini-Gi bio-assay. In theory, both bio-assays should monitor the same event, i.e. a conformational change in MOR following activation, thereby exposing residues that allow coupling of mini-Gi or are recognized by Nb39. We therefore assessed whether there was any difference using both platforms. When focusing on the efficacies of the compounds **(Figure 2C, Table 1)**, it is clear that, while the rank order of the test compounds was maintained, consistently lower E_{max} values were obtained in the Nb39-based assay. While in both bio-assays fentanyl and U-47700 were more efficacious than the reference agonist, HM, this was not the case for butyrylfentanyl and acetylfentanyl, which were only more efficacious than HM in the mini-Gi bio-assay. This would lead these compounds being scored as 'partial agonists' in the Nb-based assay. Also, the efficacy for valerylfentanyl, which already acted as a partial agonist when considering coupling of mini-Gi to MOR, was further reduced in the Nb39-based bio-assay (possibly because in the concentration-response curve a plateau was not reached). A panel of distinct functional assays exists for the measurement of activation of MOR (via assessing G protein-dependent signaling). While some assays monitor the event of nucleotide exchange, such as the GTPγ[^{35}S] binding assay, protein fragment complementation assays like NanoBiT® and bioluminescence/fluorescence energy transfer (BRET/FRET) techniques monitor real-time recruitment of cytosolic transducers to the receptor. The bio-assay platforms employed in this study are based on the recruitment of either mini-Gi or Nb39 to activated MOR, both of which are receptor proximal events. As a consequence, the issue of 'receptor reserve', a phenomenon in which only submaximal receptor occupancy is sufficient for the system to reach its maximal response, is less applicable here. In contrast, second messenger assays such as cAMP accumulation assays (measured via immunoassays, GloSensor™, etc.), calcium release (via AequoScreen®, calcium-sensitive fluorescent dyes) are accompanied by significant signal amplification, which may lead to a similar

maximal response by both full and partial agonists contributing to a 'ceiling effect' [17]. Assays which measure activation of G protein-coupled inwardly rectifying K^+ (GIRK) channels (via voltage clamp or voltage sensitive fluorescent dyes) are accompanied with limited levels of signal amplification [11]. In addition to issues related to receptor reserve, in assays which monitor further downstream events, signals that are not directly related to the receptor of interest may be picked up, giving rise to "off target effects".

When looking at the potencies **(Figure 2D, Table 1)**, all the synthetic opioids were equipotent in both bio-assays – the (non-significant) difference seen for valerylfentanyl is owing to the fact that for this compound the MOR-Nb39-based concentration-response curve did not reach a plateau. Thus, application of either of the bio-assays essentially yields the same conclusion regarding potency. This observation seems to differ from the findings by Stoeber *et al.*, who, using advanced fluorescence microscopy to assess the recruitment of mGsi and Nb33 to activated MOR and KOR, found that the concentration-response curve for mini-Gsi was consistently left-shifted as compared to that of Nb33 [10]. Several reasons may account for this apparent discrepancy, amongst which the use of different assay set-ups (with fluorescent probes being larger than nanoluciferase-based probes) and differential read-outs (fluorescence, as opposed to luminescence in our bio-assays) [17]. The most potent synthetic opioid was fentanyl, followed by butyrylfentanyl and U-47700, with acetylfentanyl being the least potent compound in both platforms. Acetylfentanyl was also found to be weakly potent in a GTPγ[^{35}S] assay by Hassanien *et al.* [12] which is in line with both our bio-assays, however, the advantage of our bio-assay lies in its ability to monitor the recruitment of mini-Gi/Nb39 in live cells, as opposed to membrane extracts in GTPγ[^{35}S] assay. Furthermore, to check whether the use of a partial-curve (corresponding to the ascending part of the curves, including the maximum) would lead to the same conclusions as use of the full curves (2 h measurement), we re-performed the entire data analysis, only using the RLU values obtained during the first 16 minutes. A comparison of the efficacies (E_{max}) and potencies (pEC_{50}) derived from the first 16 minutes to those obtained from the complete 2 h read-out at both the platforms for the five synthetic opioids revealed that the trends derived from the 2 h read-out were essentially recapitulated when using the data obtained from the 16-minute curves **(Figure 3, Table 2).**

CONCLUSION

The single domain antibody probes known as nanobodies are used for a variety of applications, ranging from therapeutic use to valuable *in vitro* biochemical tools [4, 18, 19]. Very recently, an extracellular nanobody was combined with NanoBiT technology to monitor ligand binding at the C-X-C chemokine receptor 4 (CXCR4) [20]. While the coupling of intracellularly expressed nanobodies to GPCRs has

already been assessed using advanced microscopy, FRET and BRET, to the best of our knowledge, this is the first report on a luminescent bio-assay based on the recruitment of an intracellularly expressed nanobody to an activated GPCR. Using as a proof of principle a set of five synthetic opioids, we not only demonstrated the applicability of a MOR-LgBiT / SmBiT-Nb39-based bio-assay, but also compared the derived pharmacological parameters efficacy (by means of E_{max}) and potency (pEC_{50}) with those obtained via a previously established MOR-mini-Gi bio-assay. Although the synthetic opioids were essentially scored equipotent in both bio-assays, the efficacy in the Nb-based assay was consistently lower. Whilst in future it would be interesting to characterize in-depth the molecular differences between the bio-assays deployed in the current study and those reported elsewhere, we can conclude that the MOR-LgBiT / SmBiT-Nb39-based bio-assay reported here can be used for studies aiming at structure-activity relationship determination of existing and emerging opioids.

DECLARATIONS

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Conflict of Interest

The authors declare that they have no conflict of interest.

Authors' Contributions

L.V. and C.S. designed the experiments. L.V. analyzed and prepared the manuscript. C.S. provided guidance, supervised the project, reviewed and edited the manuscript.

ADDITIONAL INFORMTION

Electronic Supporting Material (ESM)

ESM

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Figures

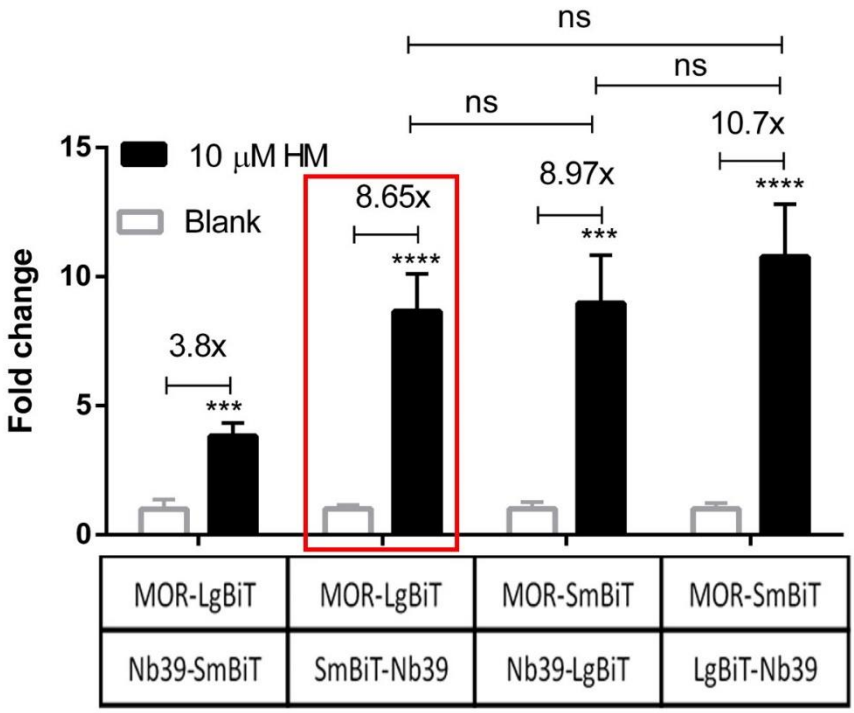

Figure 1. Selection of the optimal combination for the MOR-Nb39 bio-assay: Four distinct configurations were used to transiently transfect HEK293T cells: MOR-LgBiT + either Nb39-SmBiT or SmBiT-Nb39 and MOR-SmBiT + either Nb39-LgBiT or LgBiT-Nb39. Upon stimulation with 10 µM HM, the luminescence was measured for 120 min. The graphs show the average fold change ± SD of the stimulated conditions (filled bars) over the non-stimulated conditions (solvent controls) (open bars). The frame indicates the combination that was chosen for further experiments (n=8); *** $p \leq 0.001$, **** $p \leq 0.0001$, ns: not significant (two-tailed t-test).

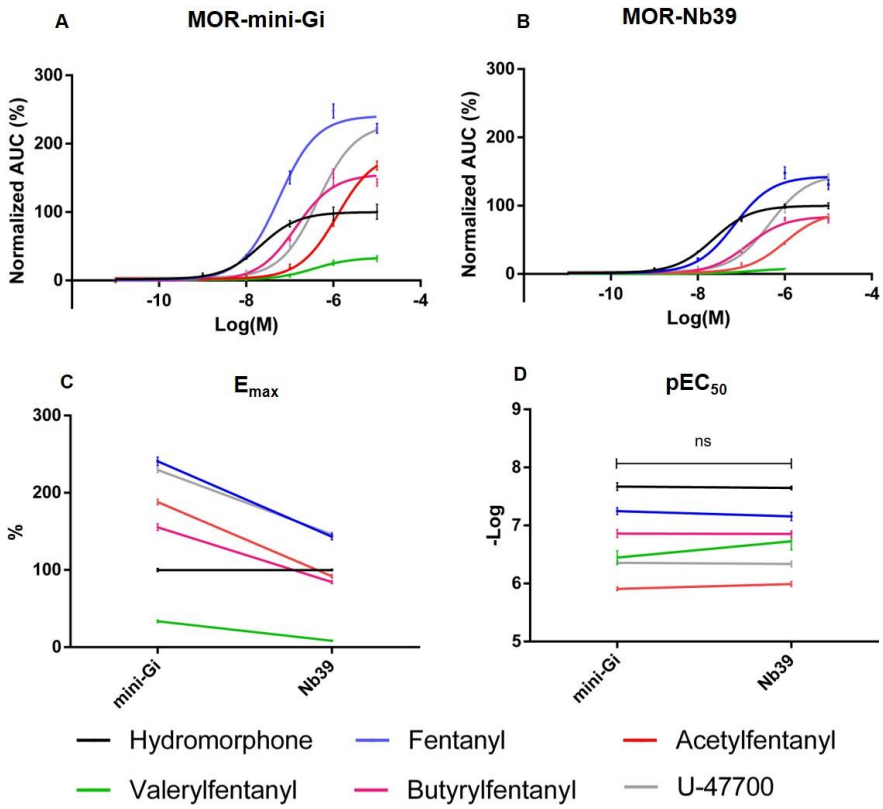

Figure 2. Sigmoidal concentration-response curves (A, B) and comparison of (C) efficacies (E_{max}), and (D) potencies (pEC_{50}) for five synthetic opioids in addition to the reference compound, HM. The curves (A) MOR-mini-Gi and (B) MOR-Nb39 are represented as AUC (± SD) from three independent experiments performed on different days after their normalization to the E_{max} of the reference agonist, HM. Likewise, (C) efficacies (E_{max}), and (D) potencies (pEC_{50}) (± SD) for five synthetic opioids relative to HM (arbitrarily set to 100%) are represented here. ns: not significant (two-tailed t-test).

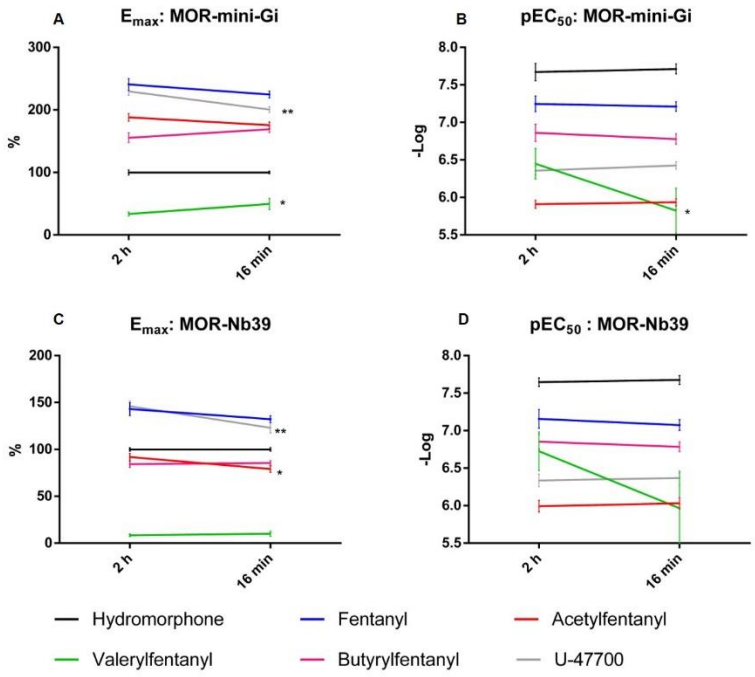

Figure 3: Comparison of efficacies (E_{max}), and potencies (pEC_{50}), derived from 2 h and the first 16 minutes of the read-out using the (A, B) MOR-mini-Gi and (C, D) MOR-Nb39 bio-assays, for five synthetic opioids relative to HM (arbitrarily set to 100%), * $p \leq 0.05$, ** $p \leq 0.01$, compounds marked (* or **) showed significance between 2 h and 16 min read-outs (two-tailed t-test).

Table

Table 1. Efficacies (E_{max}) and potencies (EC_{50}) of five synthetic opioids and the reference compound, HM, obtained using both reporter assays (MOR-mini-Gi and MOR-Nb39), with a 2 h read-out.

Compound	MOR-mini-Gi assay		MOR-Nb39 assay	
	E_{max} (%) ± SD	EC_{50} (nM) (95% CI)	E_{max} (%) ± SD	EC_{50} (nM) (95% CI)
Hydromorphone	99.97 ± 2.2	21.31 (15.40-29.49)	99.97 ± 1.1	22.55 (19.32-26.32)
Fentanyl	240.8 ± 5.4	56.57 (42.34-75.57)	143.0 ± 4.0	69.65 (49.09-98.83)
Acetylfentanyl	188.1 ± 3.6	1232 (1057-1436)	91.91 ± 2.3	1019 (822.50-1263.00)
Butyrylfentanyl	155.4 ± 4.4	137.60 (99.54-190.30)	84.25 ± 2.0	139.9 (106.70-183.40)
Valerylfentanyl*	33.50 ± 1.7	355.60 (201.90-626.50)	8.444 ± 0.8	187.7 (90.84-387.90)
U-47700	229.6 ± 3.6	439.90 (374.50-516.90)	146.0 ± 3.3	460.2 (365.10 - 580.10)
E_{max} represented here is relative to that of the reference agonist HM, which is arbitrarily set to 100%. *: no plateau was reached for valerylfentanyl in the MOR-Nb39 assay				

Table 2. Efficacies (E_{max}) and potencies (EC_{50}) of five synthetic opioids and the reference compound, HM, obtained using both reporter assays (MOR-mini-Gi and MOR-Nb39), calculated using only the data points of the ascending part (0-16 min) of the activation profiles.

Compound	MOR-mini-Gi assay		MOR-Nb39 assay	
	E_{max} (%) ± SD	EC_{50} (nM) (95% CI)	E_{max} (%) ± SD	EC_{50} (nM) (95% CI)
Hydromorphone	99.99 ± 1.3	19.39 (16.13-23.32)	99.98 ± 1.1	21.15 (17.90-24.99)
Fentanyl	224.6 ± 3.1	61.32 (51.51-73.00)	132.2 ± 2.2	84.07 (68.88-102.60)
Acetylfentanyl	175.5 ± 2.9	1157 (1011-1324)	79.82 ± 2.0	929.4 (751.70-1149.00)
Butyrylfentanyl	169 ± 2.9	166.2 (136.80-201.70)	85.59 ± 1.4	164 (136.20-197.40)
Valerylfentanyl	49.52 ± 5.1	1500 (648.60-3470.00)	10.12 ± 1.5	1082 (285.20-4107.00)
U-47700	200.5 ± 2.6	375.6 (327.40-431.00)	123.4 ± 3.2	427 (325.10-560.90)

E_{max} represented here is relative to that of the reference agonist HM, which is arbitrarily set to 100%. E_{max} and EC_{50} were calculated for the first 16 minutes (ascending part of the curves, including the maximum).