DEVELOPMENT OF AN *IN VITRO* METHOD FOR SCREENING OF COMPOUNDS FOR μ OPIOID RECEPTOR ACTIVATION POTENCY

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Background and aim

Luminescence assay

The MOR is a G-protein coupled receptor (GPCR). In the canonical signaling pathway, following binding of a ligand, an inhibitory G protein is activated, which leads to a reduction in cAMP. This leads to membrane hyperpolarization, via a complex chain of signals, which in turn leads to reduced excitability of neurons. In this study, we used a transgenic cell line that enables fast, robust quantification of MOR activation through a modified mechanism (bioluminescence). CHO-K1 co-transfected with MOR, the G-protein Gα16 and apoaequorin were used (Revvity, Groningen, The Netherlands) . Upon MOR activation, Gα16 leads to a rapid increase in intracellular Ca2+. Apoaequorin subsequently binds Ca2+ and oxidizes a substrate (coelenterazine), resulting in the emission of light (460 nm), measured using a spectrophotometer (Figure 1).

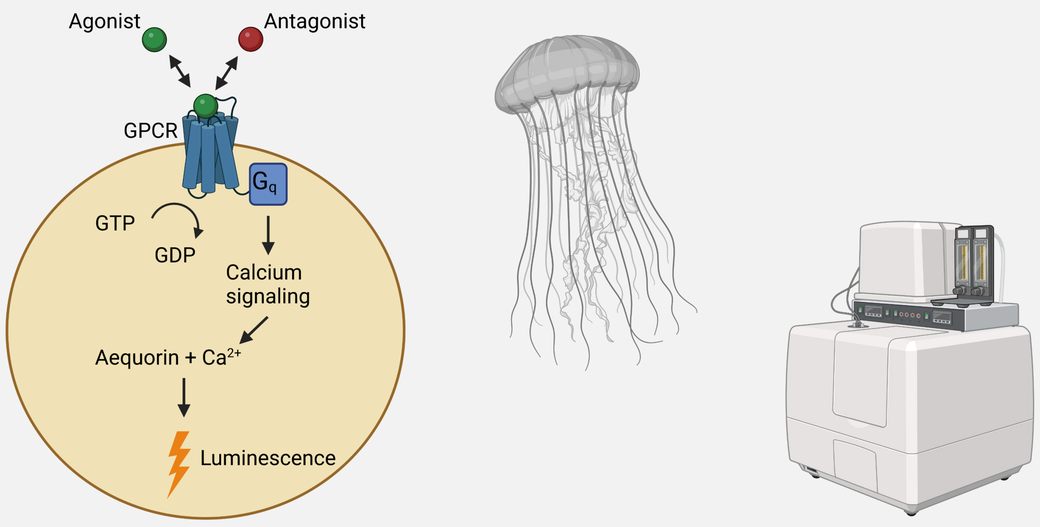


Figure 1. Schematic drawing of the double transfected CHO-K1 cell showing signaling pathway (left) and a picture of the Aequorea Victoria and the spectrophotometer (right)

Method development

The aim of the first experiments was to find the optimal conditions for the assay in terms of number of cells used per well, incubation time and luminescence read configuration (Figure 2). Digitonin was used as a positive control for the assay and [D-Ala2, N-Me-Phe4, Gly5-ol]-enkephalin (DAMGO), endomorphin I and II as reference agonists (Figure 3).

- Incubation of the CHO-K1 cells with coelenterazine for 4 hours
- Optimal cell density was determined to be 25,000 cells per well
- The kinetic mode was used to measure counts over 20 seconds in 10 ms intervals
- The luminescent signals measured were integrated (area under the curve, AUC), resulting in the concentration-effect curves
- Results were normalized relative to the positive control, digitonin (100 μM)

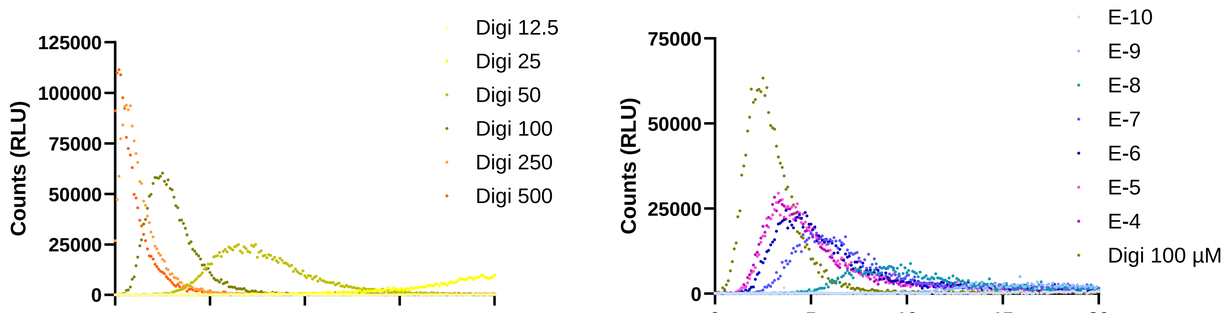
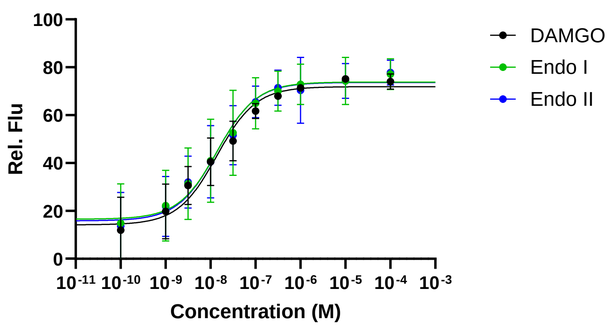


Figure 2 : The luminescent signal (460 nm) following addition of 25.000 cells to wells containing digitonin (left) or DAMGO (right). The cells were incubated with coelenterazine for 4 hours



Compound	EC_{50} (M)	Ref EC_{50} (M)
DAMGO	1.38E-8	3,3E-8
Endomorphin I	1.43E-8	1.2E-8
Endomorphin II	1.39E-8	NA

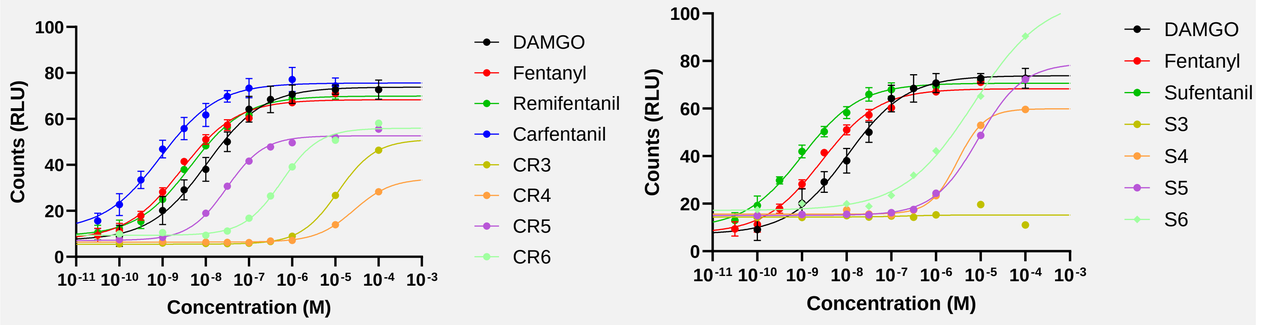
Figure 3 : Log concentration-effect of the reference agonists DAMGO, endomorphin I, and endomorphin II (left). The area under the curve (AUC) of the luminescent signal was calculated for each concentration and normalized relative to the positive control digitonin (100 μM). MOR activation potency (right) is reported as the half-maximal activation concentration (EC_{50}).

1. P.L. Feldman, et al P. L., James, M. K., Brackeen, M. F., Johnson, M. R. & Leighton, H. J. N-phenyl-N-(4-piperidinyl)amides useful as analgesics. US5019583A (1991).
2. Mathew, J. Process for the preparation of sufentanil derivatives by carbene addition/aminolysis of 4-piperidone. WO1995009152 (1995).
3. M.G. Feasel, F.S. Moran (2018). In vitro screening of opioid antagonist effectiveness. US army research, development and engineering command. Aberdeen Proving Ground, MD 21010-5424
4. Åstrand, Guerrieri, Vikingsson, Kronstrand, & Green. (2020). In vitro characterization of new psychoactive substances at the μ-opioid, CB1, 5HT1A, and 5-HT2A receptors—On-target receptor potency and efficacy, and off-target effects. Forensic Science International, 317, 110553.

Evaluation of (synthetic) opioid potency

In order to understand the risks of intermediates and by-products we evaluated the precursors that were produced during the 7-step synthesis routes for carfentanil and remifentanil[1] (CR1 t/m CR6) and for sufentanil[2] (S1 t/m S6) (Figure 4).

- CR5 and CR6 showed considerable receptor activity potency
- S4, S5 and S6 showed little receptor activity potency
- Purified end products exhibited high potencies showing a maximum response similar to DAMGO: carfentanil > sufentanil > fentanyl > remifentanil



Compound	EC_{50} (M)
DAMGO	1.05E-8
CR3	1.12E-5
CR4	2.06E-5
CR5	3.06E-8
CR6	6.05E-7
S4	3.02E-6
S5	8.81E-6
S6	6.99E-6
Fentanyl	2.75E-9
Sufentanil	1.02E-9
Remifentanil	4.47E-9
Carfentanil	9.10E-10

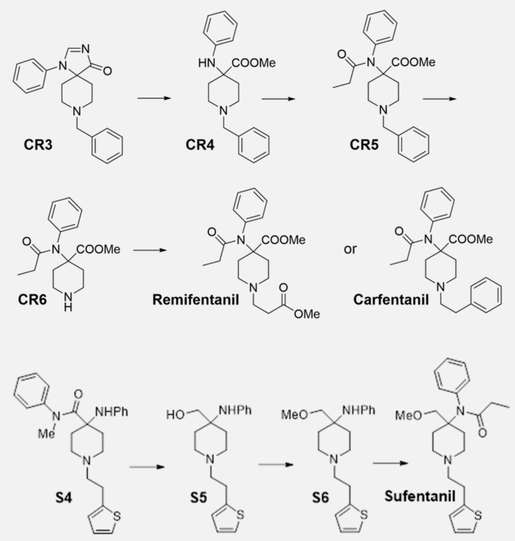
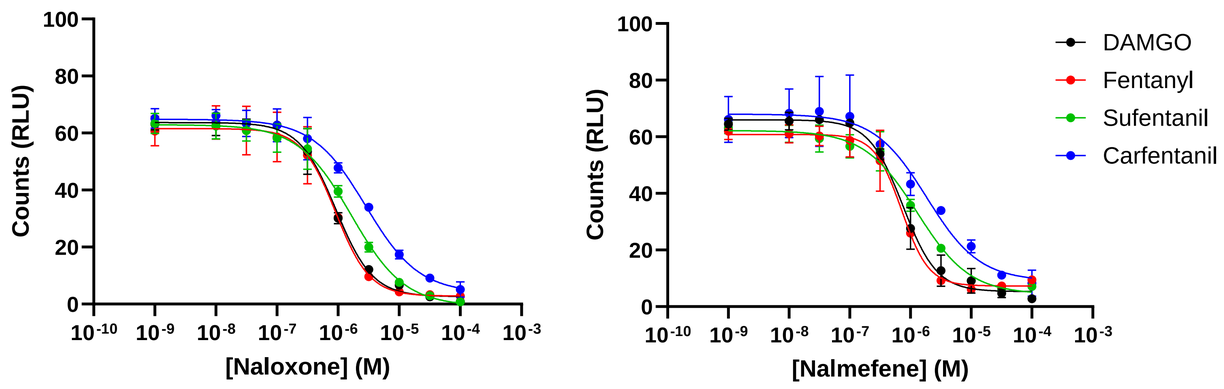


Figure 4 : Log concentration-effect of the reference agonist DAMGO, fentanyl and analogues and intermediates of synthesis routes. The area under the curve (AUC) was calculated for each concentration and normalized relative to the positive control digitonin (100 μM). MOR activation potency (bottom) is reported as the half-maximal activation concentration (EC_{50})

Evaluation of opioid antagonist effectiveness

Opioid overdoses are reversed with the opioid antagonist naloxone. However, naloxone may not be as effective against some of the fentanyl analogues[3]. In this study we also evaluate the effectiveness of the antagonists naloxone and nalmefene in competition with fentanyl and analogues. The antagonists were evaluated against an EC_{90} concentration of each of the agonists (Figure 5)

- Naloxone and nalmefene showed similar half-maximal inhibitory concentrations (IC_{50}), evaluated against fentanyl analogues
- Agonists that exhibited a higher potency required a higher antagonist concentration for effective inhibition: carfentanil > sufentanil > fentanyl



Compound EC_{90} (M)	Naloxone IC_{50} (M)	Nalmefene IC_{50} (M)
DAMGO (3.2E-7)	9.2E-7	7.5E-7
Fentanyl (1.0E-7)	9.1E-7	7.1E-7
Sufentanil (3.2E-8)	1.6E-6	1.3E-6
Carfentanil (3.2E-8)	2.8E-6	1.9E-6

Figure 5 : Log concentration-effect of antagonists naloxone and nalmefene in competition wit fentanyl and analogues (top). The area under the curve (AUC) was calculated for each concentration and normalized relative to the positive control digitonin (100 μM). MOR inhibition potency (bottom) of the antagonists is reported as the half-maximal inhibition concentration (IC_{50}).

Conclusions

- The developed method is suitable for the screening of MOR agonists, intermediates, by products as well competition with antagonists
- The EC_{50} values for the opioid analogues were in the same order of magnitude as fentanyl and correspond to those reported in the literature[4]
- Two precursors showed considerable MOR activation potency, consistent with their similarity in structure to their end product
- More naloxone or nalmefene was needed to counter the effects of sufentanil and carfentanil compared to fentanyl

Funding

This research was funded by the Netherlands Ministry of Defense (V2207 CBRN program).