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# Distinct pharmacological properties of morphine metabolites at $G_i$-protein and β-arrestin signaling pathways activated by the human μ-opioid receptor

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ABSTRACT

Morphine and several other opioids are important drugs for the treatment of acute and chronic pain. Opioid-induced analgesia is predominantly mediated by the μ-opioid receptor (MOR). When administered to humans, complex metabolic pathways lead to generation of many metabolites, nine of which may be considered major metabolites. While the properties of the two main compounds, morphine-6-glucuronide and morphine-3-glucuronide, are well described, the activity of other morphine metabolites is largely unknown. Here we performed an extensive pharmacological characterization by comparing efficacies and potencies of morphine and its nine major metabolites for the two main signaling pathways engaged by the human MOR, which occur via $G_i$-protein activation and β-arrestins, respectively. We used radioligand binding studies and FRET-based methods to monitor MOR-mediated $G_i$-protein activation and β-arrestin recruitment in single intact 293T cells. This approach identified two major groups of morphine metabolites, which we classified into "strong" and "weak" receptor ligands. Strong partial agonists morphine, morphine-6-glucuronide, normorphine, morphine-6-sulfate, 6-acetylmorphine and 3-acetylmorphine showed efficacies in the nanomolar range, while the weak metabolites morphine-N-oxide, morphine-3-sulfate, morphine-3-glucuronide and pseudomorphine activated MOR pathways only in the micromolar range. Interestingly, three metabolites, normorphine, 6-acetylmorphine and morphine-6-glucuronide, had lower potencies for Gi-protein activation but higher potencies and efficacies for β-arrestin recruitment than morphine itself, suggesting that they are biased towards β-arrestin pathways.



## 1. Introduction

Morphine is an important alkaloid from opium poppy and serves as the prototypical analgesic drug. Morphine and some other opioids, for example 3,6-di-acetylmorphine (heroin) are also relevant for drug abuse. Analgesic effects of opioids are achieved largely via activation of μ-opioid receptors (MOR) [1,2]. MOR is a G-protein-coupled receptor (GPCR), which can activate several cellular signaling pathways. The first pathway is mediated by the activation of inhibitory pertussis-toxin sensitive G-proteins ($G_{i/o}$-proteins). The second pathway is induced by the recruitment of β-arrestins to the receptor, which leads to subsequent activation of other, so-called "non-classical" signaling cascades such as the mitogen activated protein kinase pathway [3,4].

When administered to humans, morphine and other opiate alkaloids undergo extensive biotransformation by various metabolic pathways. The predominant metabolic pathway involves conjugation with glucuronic acid, which leads to the major metabolites morphine-3-glucuronide and morphine-6-glucuronide [5]. Other metabolic pathways include conjugation with sulfonic acid, leading to morphine-6-sulfates and morphine-3-sulfates, acetylation to 3- and 6-acetylmorphines, oxidation to morphine-N-oxide and demethylation to normorphine [6,7] (Fig. 1). Among all known morphine metabolites, the pharmacological properties have been best studied for morphine-6-glucuronide and morphine-3-glucuronide, which are generally considered as active and inactive metabolites, respectively [8].

*Abbreviations:* DAMGO, [D-Ala$^2$, N-MePhe$^4$, Gly-ol]-enkephalin; FRET, fluorescence (or: Förster) resonance energy transfer; GPCR, G-protein-coupled receptor; GRK, G-protein-coupled-receptor kinase.

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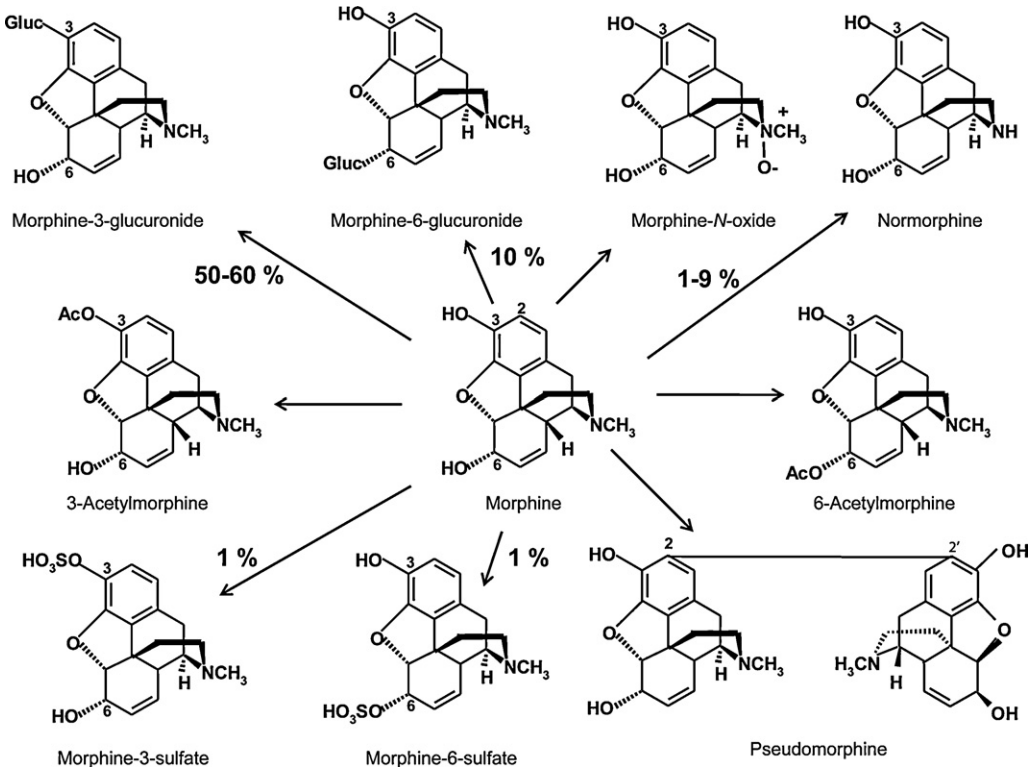


**Fig. 1.** Metabolic pathways by which morphine undergoes biotransformation in the human body. The majority of the alkaloid is conjugated with glucuronic acid to form morphine-3- and -6-glucuronides. The remainder can be modified to form other metabolites such as sulfate or acetyl esters, normorphine, morphine-N-oxide and pseudomorphine. Adapted from [7].

Much less is known about the activity of other metabolites, since no systematic pharmacological analysis has so far been performed, and only rare single reports on these compounds are available. Lack of information about opiate metabolites does not allow definitive conclusions about their roles in analgesia and in adverse effects of morphine [6,7].

To study the pharmacological properties of various ligands for G protein-coupled receptors, including MOR, we have developed a new real-time approach to monitor $G_i$-protein activation in intact cells by using biosensors which are based on Förster resonance energy transfer (FRET) [9–11]. In addition, we have developed another technique to monitor β-arrestin recruitment to various receptors by measuring FRET between a receptor and β-arrestin [12,13]. These approaches allow us to study pharmacological properties of different receptors and their ligands in single intact cells with high temporal and spatial resolution [14,15].

Here we used these FRET assays together with radioligand binding studies to perform a systematic analysis of the pharmacological properties of all major morphine metabolites at the human MOR. We found that morphine metabolites exert either strong or weak signaling effects, often comparable to morphine itself, and that some metabolites show a previously unrecognized β-arrestin-biased behavior and are more efficacious in recruiting β-arrestin to the receptor than morphine itself.

## 2. Materials and methods

### 2.1. Substances

Morphine, morphine-6-glucuronide, morphine-3-glucuronide and DAMGO ([D-Ala$^2$, N-MePhe$^4$, Gly-ol]-enkephalin) were purchased from Sigma–Aldrich (Deisenhofen, Germany). Normorphine, 3-acetylmorphine and 6-acetylmorphine were from Lipomed GmbH (Weil am Rhein, Germany).

Morphine-N-oxide, morphine-3-sulfate, morphine-6-sulfate and pseudomorphine were synthesized as follows. Morphine-N-oxide and pseudomorphine were prepared as described by Garrido et al. [16]. Morphine-3-sulfate was prepared via 6-acetylmorphine as previously described [17] and further sulfonated and deprotected by a procedure described by Mori et al. [18]. Morphine-6-sulfate was synthesized from morphine hydrochloride according to Preechagoon et al. [19]. Except where noted, all other chemicals were obtained from Sigma–Aldrich.

### 2.2. Plasmids

Human MOR-1A cDNA was obtained from imaGenes GmbH (Berlin, Germany) and subcloned into the pcDNA3 expression vector (Invitrogen) using HindIII and XbaI restriction sites. All fluorescent $G_i$-protein subunits used to monitor $G_i$ activation by FRET have previously been described [9,10]. Briefly, the rat PTX-insensitive $G_{\alpha i1}$-protein (C351I mutant) was labeled with enhanced yellow fluorescent protein (YFP) between positions 91 and 92. Human $G_{\gamma 2}$ was C-terminally tagged with enhanced cyan fluorescent protein (CFP), and the untagged human $G_{\beta 1}$ cDNA was co-expressed. For the FRET-based measurements of β-arrestin-2 recruitment, the plasmids encoding the human MOR-1A C-terminally tagged with CFP, bovine β-arrestin-2 C-terminally tagged with YFP, and untagged human G protein-coupled receptor kinase (GRK) 2 were used [12].

### 2.3. Cell culture and transfections

293T cells were maintained in Dulbecco's Modified Eagle's Medium (DMEM) supplemented with 10% fetal bovine serum, 2 mM L-glutamine, 100 units/ml penicillin and 0.1 mg/ml streptomycin (all from Pan Biotech GmbH, Aidenbach, Germany) at 37 °C and 7% $CO_2$. Cells were seeded onto poly-D-lysine (Sigma) coated

glass cover slips for FRET experiments. Transient transfections were performed with Effectene Transfection reagent (Quiagen, Hilden, Germany) according to the manufacturer's protocol, and FRET measurements were done 48 h after transfection.

To monitor $G_i$-protein activation, the following plasmids were transfected (μg DNA per 6-well plate): MOR 0.7, $G_{\alpha i1}$-YFP 0.8, $G_{\beta 1}$ 0.5, $G_{\gamma 2}$-CFP 0.2. To measure β-arrestin recruitment, cells were transfected with (μg DNA per 6-well plate): MOR-CFP 0.3, GRK2 0.2, β-arrestin-2-YFP 0.2). For receptor binding studies, 1.9 μg MOR plasmid were transfected per 90 mm cell-culture dish.

### 2.4. Receptor binding assays

MOR expressing 293T cells were homogenized in 5 mM Tris, 2 mM EDTA, pH 7.4. The cell homogenate was centrifuged for 10 min at 1700 × *g*, and the remaining supernatant was centrifuged for 30 min at 50,000 × *g*. The resulting pellet was resuspended in 50 mM of potassium phosphate buffer, pH 7.4, and aliquots were frozen in liquid nitrogen. 20 μg of membrane protein were mixed with 0.1–1 nM of $^3$[H]naloxone (American Radiolabeled Chemicals, Inc., St. Louis, USA), 100 μM GTP and various MOR ligand concentrations in 200 μl of 50 mM potassium phosphate buffer. Samples were incubated at room temperature for 3 h, then filtered through GF/F glass fiber filters (Millipore, Schwalbach, Germany) before liquid scintillation counting.

### 2.5. FRET measurements

FRET experiments were performed on a Zeiss Axiovert 200 inverted microscope with a PLAN-Neofluar oil immersion 100× objective and a dual emission photometric system as previously described [10]. Experiments were done at room temperature in a buffer containing 144 mM NaCl, 5.4 mM KCl, 2 mM $CaCl_2$, 1 mM $MgCl_2$, 10 mM HEPES, pH 7.3. Cells were excited using the monochromatic light from a Polychrome IV (Till Photonics, Gräfelfing, Germany) at 436 ± 10 nm and a frequency of 5 Hz. Emitted light was separated by the DCLP 505 beam splitter into two emission channels recorded at 480 ± 20 nm (CFP) and 535 ± 10 nm (FRET channel). The $F_{535}/F_{480}$ emission ratio was offline corrected for bleedthrough, direct YFP excitation and photobleaching using Origin software in order to obtain corrected $F_{YFP/CFP}$ ratios, which were used throughout the manuscript. Ratiometric traces were normalized to the initial ratio (set to 1).

## 3. Results

We analyzed the pharmacological properties of morphine and its nine major metabolites in three types of assays, namely radioligand binding studies, G-protein activation measurements and an assay to monitor β-arrestin recruitment to the opiate receptor. These experiments were designed to compare the action of morphine metabolites and their efficacies and potencies for two distinct signaling pathways engaged by human MOR.

### 3.1. Radioligand binding studies

Initially, we determined the affinities of morphine and its major metabolites (plus DAMGO as a reference agonist) for MOR in radioligand binding studies with cell membranes prepared from 293T cells expressing the receptor, using the antagonist [$^3$H]naloxone as the radioligand. Competition displacement curves for morphine and DAMGO (Fig. 2) showed $K_i$-values, which were comparable to published values for these ligands at the human MOR [20,21]. Next, we measured the $K_i$-values of the major morphine metabolites (see Fig. 1). The radioligand displacement curves (Fig. 2) revealed two groups of metabolites, which we classified into "strong" and "weak" ligands. The "strong" metabolites, normorphine, 3- and 6-acetylmorphine, morphine-6-glucuronide and -6-sulfate showed affinities in the nanomolar range, while the "weak" ligands, pseudomorphine, morphine-N-oxide, morphine-3-glucuronide and -3-sulfate, had micromolar $K_i$-values (Fig. 2 and Table 1).

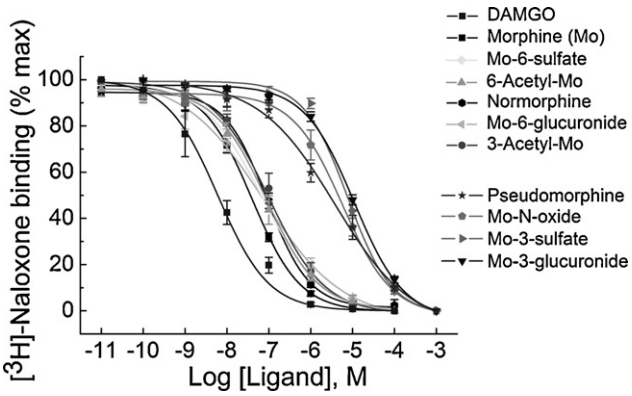


**Fig. 2.** Competition radioligand displacement curves for [$^3$H]-naloxone measured with 293T cell membranes expressing human MOR and treated with various ligands. Data are presented as means ± SE ($n$ = 4–8). Individual data points were fitted to a single binding site model, and the obtained $K_i$-values are presented in Table 1.

### 3.2. $G_i$-protein activation

Based on these affinity measurements we then set out to investigate the metabolites' functional effects. To monitor ligand-induced activation of inhibitory G-proteins, cells expressing human MOR and fluorescently labeled $G_i$-protein subunits were treated with the different morphine metabolites (Fig. 3A). In this assay, receptor activation leads to a rapid and reversible decrease of the FRET ratio, which reflects structural rearrangements between the G-protein subunits, and [9]. In MOR expressing cells, we observed robust decreases of the FRET ratio upon morphine treatment, which were even further enhanced by the full receptor agonist DAMGO, suggesting that morphine acts only as a strong partial agonist at MOR (Fig. 3B). Addition of naloxone on top of these ligands led to a complete, relatively slow, deactivation of G-proteins, confirming that our system can be used to monitor

**Table 1**
Pharmacological properties of various morphine metabolites at the human μ-opioid receptor (MOR) measured by radioligand binding and FRET assays. $EC_{50}$ values for $G_i$-protein activation and β-arrestin recruitment in the FRET-based assays show concentrations at which a half-maximal response for each individual substance was observed. Data from competitive displacement experiments using $^3$[H]-naloxone and MOR-expressing cell membranes are presented as $K_i$ values. All data are means ± SE calculated from three to seven independent experiments.

| Substance | Assay | | |
|---|---|---|---|
| | $G_i$ activation $EC_{50}$ (μM), means ± SE | β-Arrestin recruitment $EC_{50}$ (μM), means ± SE | Binding $K_i$ (μM), means ± SE |
| DAMGO | 0.046 ± 0.004 | 0.060 ± 0.002 | 0.005 ± 0.002 |
| Morphine | 0.015 ± 0.002 | 0.457 ± 0.157 | 0.022 ± 0.004 |
| Morphine-6-glucuronide | 0.037 ± 0.008 | 0.338 ± 0.062 | 0.063 ± 0.014 |
| Normorphine | 0.040 ± 0.007 | 0.267 ± 0.015 | 0.061 ± 0.013 |
| 3-Acetylmorphine | 0.057 ± 0.010 | 0.594 ± 0.019 | 0.098 ± 0.029 |
| Morphine-6-sulfate | 0.053 ± 0.005 | 0.196 ± 0.036 | 0.047 ± 0.004 |
| 6-Acetylmorphine | 0.100 ± 0.021 | 0.339 ± 0.023 | 0.054 ± 0.022 |
| Morphine-N-oxide | 2.2 ± 0.4 | 16.7 ± 1.3 | 3.4 ± 1.0 |
| Morphine-3-sulfate | 3.2 ± 0.5 | 20.1 ± 6.8 | 4.7 ± 1.1 |
| Morphine-3-glucuronide | 8.2 ± 1.4 | 23.7 ± 4.8 | 6.1 ± 0.4 |
| Pseudomorphine | 9.5 ± 0.6 | 53.6 ± 4.3 | 3.6 ± 1.8 |

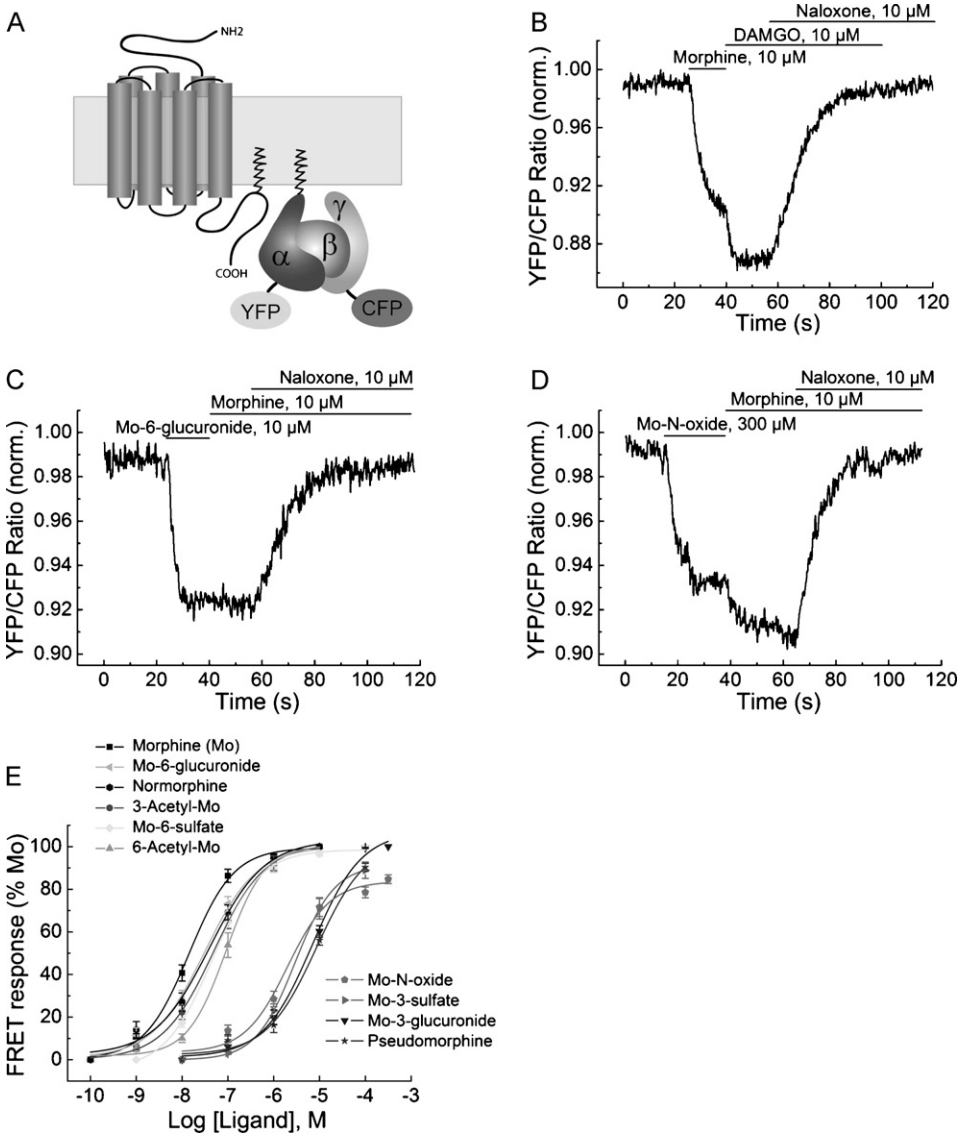


**Fig. 3.** Real-time monitoring of the morphine metabolite-induced effects on MOR-mediated $G_{i1}$-protein activation. (A) The FRET system for real-time monitoring of $G_{i1}$-protein activation in cells expressing MOR and fluorescently labeled G-protein subunits. FRET between CFP fused to the $\gamma_2$-subunit of the $\beta\gamma$-complex, and YFP on the $\alpha_{i1}$-subunit shows high basal levels, which decrease upon receptor-mediated activation. (B) Effects of saturating morphine (10 μM) and DAMGO (10 μM) concentrations on the FRET signal (presented as the normalized YFP/CFP ratio). Morphine acts as a partial agonist at the receptor and $G_i$-protein when compared to DAMGO. The MOR antagonist naloxone can fully block this FRET response. (C) Morphine-6-glucuronide (10 μM) produces a rapid FRET signal, which is comparable in its magnitude to that of morphine. (D) A weak metabolite morphine-N-oxide, even at high concentrations (300 μM), shows only a partial FRET signal. B–D are representative experiments ($n$ = 3–7). (E) Concentration–response dependencies for morphine and its nine major metabolites measured in the FRET assay reflecting MOR-mediated $G_i$-protein activation. Changes in FRET are normalized to the maximal response induced by 10 μM morphine. Data are presented as means ± SE ($n$ = 3–7).

specific pharmacological effects of receptor agonists, partial agonists and antagonists (Fig. 3B).

Next, we set out to compare FRET responses produced by morphine with those observed upon receptor activation with its various metabolites. Some of the metabolites, for example morphine-6-glucuronide, showed a strong G-protein activation signal, which had a similar amplitude as the response to morphine itself (Fig. 3C). Other metabolites, for example morphine-N-oxide, even at high concentrations, produced only partial effects, ranging from 70 to 90% of the morphine response (Fig. 3D).

To systematically analyze efficacies and potencies of all major morphine metabolites, we recorded concentration–response dependencies for the nine different compounds and compared them to morphine by normalizing their maximal FRET responses to the change of ratio evoked by the saturating morphine concentration of 10 μM. Again, these experiments identified the two groups of morphine metabolites that were seen in the radioligand binding experiments, i.e. the "strong" and "weak" ligands (Fig. 3E). The strong metabolites all showed not only potencies in the nanomolar range, but also efficacies at the MOR, which were comparable to morphine itself. These strong partial MOR agonists were morphine-6-glucuronide, normorphine, morphine-6-sulfate and both 3- and 6-acetylmorphine. The second group of "weak" metabolites had much lower potencies and slightly lower efficacies compared to morphine. These metabolites, morphine-N-oxide, morphine-3-sulfate and 3-glucuronide, as well as pseudomorphine, were able to activate $G_i$-proteins only in the micromolar concentration range, and their maximal effects ranged from 75 to 100% of those of morphine (Fig. 3E, Table 1).

### 3.3. β-Arrestin recruitment

To monitor the activity of the second important group of signaling pathways activated by MOR, we studied the effects of

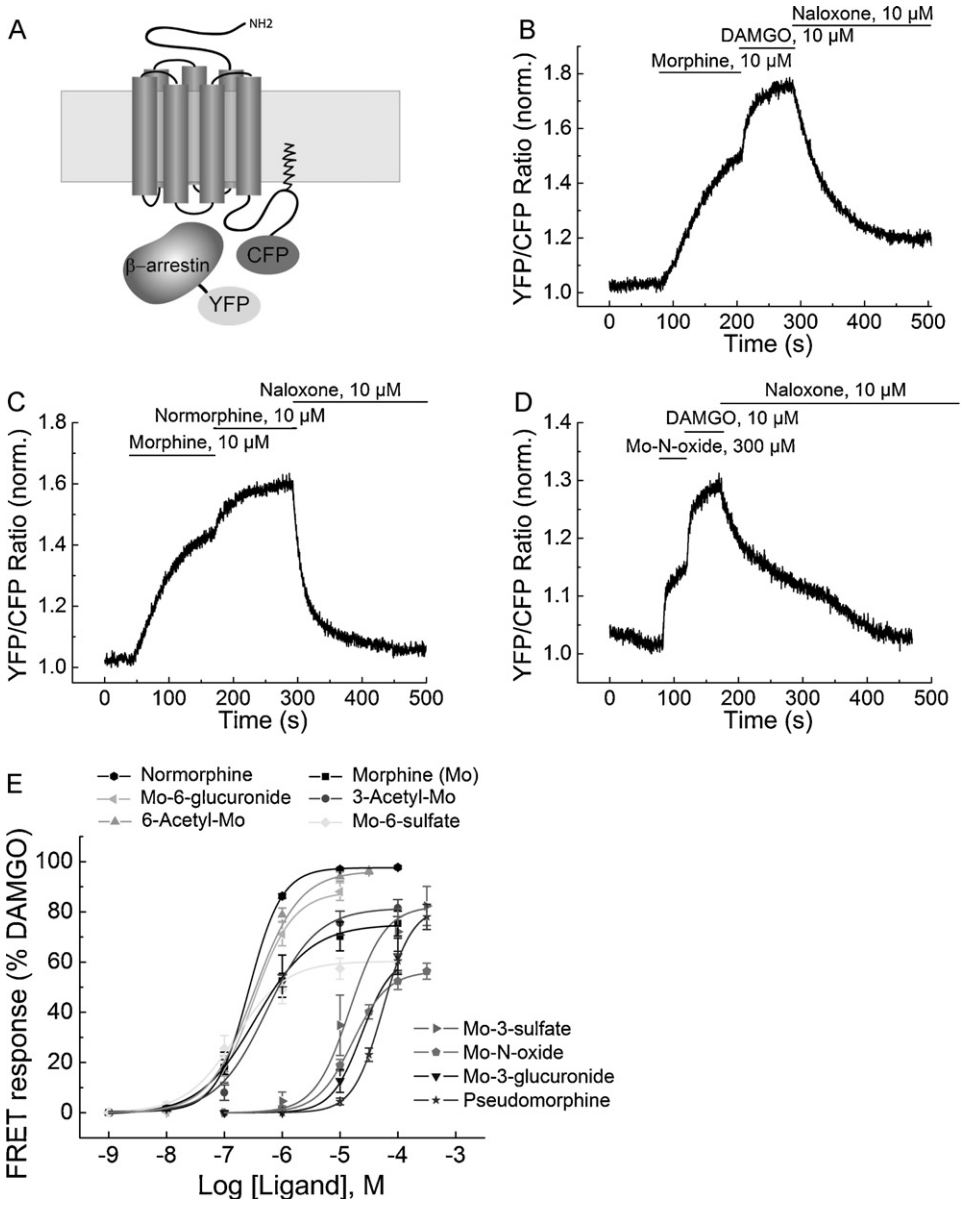


**Fig. 4.** Measurements of β-arrestin recruitment to the activated MOR by FRET. (A) The FRET system for real-time monitoring of receptor/β-arrestin interaction is based on using MOR, which is tagged with CFP on its C-terminus, and the YFP-tagged β-arrestin-2. Recruitment of β-arrestin-2-YFP to the activated receptor results in an increase of FRET. (B) Effects of the partial agonist morphine (10 μM), full agonist DAMGO (10 μM) and MOR antagonist naloxone (10 μM) on the FRET signal. (C) Normorphine (10 μM) induces a stronger β-arrestin recruitment compared to morphine (10 μM). (D) The weak metabolite morphine-N-oxide (300 μM) shows a partial effect on β-arrestin recruitment. B–D are representative experiments ($n$ = 4–7). (E) Concentration–response dependencies for DAMGO, morphine and its nine major metabolites for the MOR-mediated β-arrestin recruitment. Changes in FRET are normalized to the maximal response induced by 10 μM DAMGO. Data are presented as means ± SE ($n$ = 4–7).

morphine metabolites on the ligand-induced β-arrestin translocation to this receptor. We chose the previously well established FRET-based approach which relies on the use of C-terminally CFP-tagged MOR and C-terminally YFP-tagged β-arrestin-2 [12], which were transfected into 293T cells together with the receptor kinase GRK2 to assure full agonist-triggered MOR phosphorylation. Ligand-dependent MOR activation leads to GRK-mediated receptor phosphorylation and subsequent β-arrestin-YFP recruitment, which can be monitored as an increase of FRET (Fig. 4A). Again, morphine showed the typical response of a partial agonist, which was ~30% smaller than the change of FRET ratio evoked by the full MOR agonist DAMGO (Fig. 4B). Compatible with the need of prior GRK-mediated receptor phosphorylation [12], these responses were slower than those observed for $G_i$ (compare Figs. 3B and 4B; note the different time scales). Next, we continued to compare the FRET responses produced by morphine metabolites with their parent compound. Strikingly, we found that some of the "strong" metabolites, such as normorphine (Fig. 4C), morphine-6-glucuronide and 6-acetylmorphine, induced a significantly stronger β-arrestin recruitment than morphine itself. Therefore, we normalized all metabolite responses to the maximal FRET response produced by 10 μM of DAMGO and measured concentration–response dependencies for all nine morphine metabolites (Fig. 4D and E). As in the case of $G_i$-protein activation experiments, there were again the two groups of "strong" and "weak" partial agonists, which included the same members. Morphine-N-oxide and morphine-3-glucuronide showed lower potencies and significantly lower efficacies compared to DAMGO and morphine (Fig. 4D), while the other two weak metabolites were capable of translocating β-arrestin to the same extent as morphine, but at much higher concentrations (Fig. 4E and Table 1). Overall, the spread of efficacies was much greater in this assay than in the $G_i$-activation

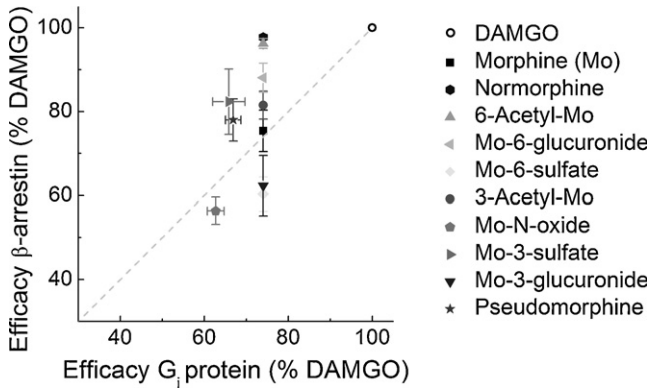


**Fig. 5.** Correlation of efficacies which various opioid ligands have at activating MOR-dependent G-protein and β-arrestin signaling. Data are taken from Figs. 3E and 4E and normalized to the maximal effects induced by 10 μM DAMGO. Several ligands elicit comparable effects on both pathways (e.g. DAMGO, morphine, morphine-N-oxide), while others more prominently activate β-arrestin signaling (e.g. normorphine, 6-acetylmorphine, morphine-6-glucuronide).

assay (compare Figs. 3E and 4E). Finally, we normalized the efficacies for all ligands measured in $G_i$- and β-arrestin assays to the maximal DAMGO response to graphically compare the extent of activation for each ligand and signaling pathway (Fig. 5).

## 4. Discussion

In this study, we performed a systematic analysis of the pharmacological properties of morphine metabolites at the human MOR. We analyzed the ability of these substances to bind to the receptor and to activate the two major independent downstream signaling pathways, which are activation of $G_i$-proteins and β-arrestin recruitment. The information obtained in these assays allows to predict, which of these metabolites might play a role in MOR signaling *in vivo*.

To analyze MOR activation, we used novel FRET-based approaches for real-time monitoring of $G_i$-protein activation and β-arrestin recruitment. These approaches provide pharmacological information at the single-cell level and directly at the initiating biochemical event. While the results of FRET measurements of $G_i$-protein activation generally match well with the data obtained in classical GTPγS binding studies [10], they provide fast kinetic readouts and can be done in intact cells, i.e. under much more physiological conditions. Likewise, measurements of β-arrestin recruitment by FRET allow visualization of receptor/β-arrestin interaction in real time. These novel assays thus reflect the pharmacodynamic properties of receptor ligands at their target intact cells, but without the pharmacokinetic constraints typical for *in vivo* experiments. For example, morphine-6-glucuronide, when directly injected into mice, has been shown to induce its behavioral effects much slower than morphine [22]. However, in our study, we observed similarly fast kinetics of $G_i$-protein activation as well as comparable efficacies and potencies for the two substances. The discrepancy between the results of the single-cell experiments and *in vivo* studies can be explained by differences in pharmacokinetics, which largely depend on the chemical structure and parameters such as hydrophobicity of the various metabolites. Indeed, it has been demonstrated that both morphine glucuronides are much slower at crossing the blood–brain barrier than morphine [23]. The single-cell pharmacological analyses presented in this study are capable of directly reporting dynamic properties of signaling at the level of target cells and therefore reflect the real pharmacodynamics of these compounds at their receptor.

There are two sources of morphine and its metabolites which are relevant for the human body. One is exogenous morphine and other analgesic opiates, which are administered for pain therapy or during opiate abuse. In this case, the circulating opiate concentrations can reach relatively high micromolar peak levels, which gradually decrease through metabolic pathways [22,24]. The second source of opiates in the human body is the endogenous morphine biosynthesis, which is capable of producing high nanomolar concentrations of morphine from endogenous biosynthetic precursors [25–27]. Much is known about the metabolic pathways which lead to morphine conjugation and biotransformation (see Fig. 1). 60–70% of exogenously administrated morphine is conjugated [5] to morphine-3-glucuronide (∼55%) and morphine-6-glucuronide (∼10%). After oral or parenteral morphine administration to patients, slightly higher plasma concentrations of morphine-6-glucuronide and ∼10-times higher concentration of morphine-3-glucuronide can be measured for up to 12 h [24]. Morphine-3-glucuronide is a very weak metabolite, whereas morphine-6-glucuronide can activate MOR almost as strongly as morphine. For morphine-3- and morphine-6-glucuronides we were able to confirm the previously observed differences [8] in their pharmacological properties. The former metabolite has been classified as a weak MOR ligand, while the latter glucuronide is a strong activator of MOR-induced signaling with potencies comparable to morphine.

Much less has been known about the pharmacological properties of other metabolites of morphine, which include normorphine, morphine-3- and morphine-6-sulfates, as well as 3- and 6-acetylmorphines. Normorphine is the N-demethylated metabolite of morphine which has been described as an active but slightly weaker opioid receptor ligand compared to morphine or morphine-6-glucuronide [28,29]. In our study, we confirmed this observation and showed that normorphine activates $G_i$-proteins at slightly higher concentrations than morphine. Morphine-6-sulfate, similarly to morphine-6-glucuronide, is a potent ligand, which requires ∼10–50-times lower parenteral dose to achieve an analgesic effect comparable to morphine [30]. In contrast, morphine-3-sulfate is a weaker metabolite, which has been described to have much lower binding affinity and lower analgesic potency [31,32]. In this study, we found that morphine-6-sulfate has binding and signaling properties similar to morphine, so that the analgesic potency of this metabolite cannot be explained by distinct pharmacodynamic properties but must be pharmacokinetic in origin. Morphine-3-sulfate, however, was much less potent and fell into the group of weak metabolites, which is compatible with earlier observations. The 3- and 6-acetylmorphines, which represent important heroin metabolites, have been reported to have similar or slightly less potent pharmacological effects than morphine [31] which was also the case in our single-cell experiments. Likewise, the weak analgesic morphine-N-oxide, which has been found to be 10–90 times less potent than morphine [33], showed almost 1000 times lower affinity and potencies in FRET assays.

Interestingly, we found that three metabolites, normorphine, 6-acetylmorphine and morphine-6-glucuronide, showed lower potencies for $G_i$-protein activation but higher potencies and efficacies for β-arrestin recruitment than morphine (compare Figs. 2E and 3E, see also Table 1 and Fig. 5). This finding suggests that such ligands might have a bias for β-arrestin over G-protein signaling. Although they induce weaker responses at G-proteins, these metabolites can activate β-arrestin signaling pathways much stronger–pathways, which have been mostly associated with the pharmacological effects of opioid peptides.

Based on the results of this study, one can predict whether various opioid ligands, especially morphine metabolites, can have pharmacological effects *in vivo*. In physiological settings, weak metabolites would not activate MOR, because they do not reach high micromolar concentrations, unless high opioid amounts have

been exogenously administrated. Several strong metabolites, such as normorphine and the main heroin metabolites 3- and 6-acetylmorphine, are much more relevant for receptor activation. Endogenously synthesized morphine and its strong metabolites might be pharmacologically relevant only if they are present at high nanomolar concentrations. In addition, normorphine and 6-acetylmorphine, together with morphine-6-glucuronide, can trigger previously unrecognized stronger activation of the β-arrestin signaling.

The most interesting aspect of this study is the observation that morphine metabolites show differential preference for the two major signaling pathways triggered by $G_i$-proteins and β-arrestins, respectively. A more detailed understanding of these two signaling pathways in eliciting the various pharmacological effects of morphine is certainly necessary before we might be able to exploit these differences for drug therapy. However, our data clearly show that there is such a potential for differential effects of morphine metabolites at the MOR.

## Acknowledgements

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