An Opioid Agonist that Does Not Induce Mu Opioid Receptor – Arrestin Interactions or Receptor Internalization


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Running Title Page
Running Title: Herkinorin, a non-internalizing µOR agonist

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Nonstandard Abbreviations:
GPCR, G protein coupled receptor; GRK, GPCR kinase; βarr, beta-arrestin; µOR, mu opioid receptor (MOP); YFP, yellow fluorescent protein; GFP, green fluorescent protein; DAMGO ([D-Ala²,N-MePhe⁴,Gly-ol⁵]enkephalin); Herkinorin, ((2S,4aR,6aR,7R,9S,10aS,10bR)-9-(Benzoyloxy)-2-(3-furanyl)dodecahydro-6a,10b-dimethyl-4,10-dioxo-2H-naphtho-[2,1-c]pyran-7-carboxylic acid methyl ester)
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ABSTRACT

G protein-coupled receptor desensitization and trafficking are important regulators of opioid receptor signaling that can dictate overall drug responsiveness in vivo. Furthermore, different mu opioid receptor (µOR) ligands can lead to varying degrees of receptor regulation presumably due to distinct structural conformations conferred by agonist binding. For example, morphine binding produces a µOR with low affinity for β-arrestin proteins and limited receptor internalization whereas enkephalin analogs promote robust trafficking of both β-arrestins and the receptors. Here, we evaluate µOR trafficking in response to activation by a novel mu-selective agonist derived from the naturally occurring plant product, salvinorin A. Interestingly, this compound, termed herkinorin, does not promote the recruitment of β-arrestin-2 to the µOR and does not lead to receptor internalization. Moreover, while GRK overexpression can promote morphine-induced β-arrestin interactions and µOR internalization, such manipulations do not promote herkinorin-induced trafficking. Studies in mice have shown that β-arrestin-2 plays an important role in the development of morphine-induced tolerance, constipation and respiratory depression. Therefore, drugs that can activate the receptor without recruiting the arrestins may be a promising step in the development of opiate analgesics that distinguish between agonist activity and receptor regulation and may ultimately lead to therapeutics designed to provide pain relief without the adverse side effects normally associated with the opiate narcotics.
INTRODUCTION

G protein coupled receptor (GPCR) internalization as a means of receptor regulation has been associated with conditions as wide-ranging as opioid analgesia to opioid addiction (Alverez et al., 2001; Connor et al., 2004; Gainetdinov et al., 2004; Bohn et al., 2004a; Raehal and Bohn 2005). Agonist-induced, GPCR kinase (GRK)-mediated phosphorylation, subsequent β-arrestin protein binding, the assembly of clathrin coated vesicles and vesicular internalization describe a general paradigm for GPCR internalization (Shenoy and Lefkowitz 2003; Pierce and Lefkowitz 2001). The µ opioid receptor (µOR), however, has been shown to be differentially regulated depending on agonist occupancy. For example, while both morphine and etorphine are agonists at the µOR and can promote receptor desensitization and analgesic tolerance, morphine appears to be much less effective in promoting receptor phosphorylation, β-arrestin recruitment, and µOR internalization than etorphine (Zhang et al., 1998; Whistler and von Zastrow, 1998; Bohn et al., 2004b). Interestingly, each of these limitations can be overcome by overexpression of GRK2 in cell culture suggesting that the agonist occupancy promotes different receptor conformations that can determine overall receptor regulation in a GRK-dependent manner (Zhang et al., 1998; Bohn et al., 2004b).

The β-arrestin proteins, namely β-arrestin-1 (βarr1) and β-arrestin-2 (βarr2), play an important role in GPCR trafficking (Pierce and Lefkowitz 2001). While the morphine-bound µOR appears to be a poor substrate for βarr2 binding, a combination of both animal and cellular studies suggest that βarr2 is prominently involved in µOR regulation. Mice that lack βarr2 display enhanced and prolonged morphine analgesia with limited
development of tolerance (Bohn et al., 1999; 2000a; 2002; Przewlocka et al. 2002). Somewhat surprisingly, these animals are not as susceptible to the morphine-induced side effects including constipation and respiratory suppression (Raehal et al., 2005). Therefore the μOR-βarr2 interactions may represent a point at which agonist-directed signaling events diverge (Raehal and Bohn, 2005; Bruns et al., 2006).

Opioid agonists that do not induce receptor-βarr2 interactions or the subsequent receptor internalization may be promising candidates for dissociating the therapeutic effects from the unwanted side effects that usually confound opiate therapy. Recently, salvinorin A, a diterpene isolated from *Salvia divinorum*, was classified as a potent opioid receptor agonist (Ortega et al., 1992; Roth et al., 2002) that produces less receptor trafficking (Wang et al, 2005). Salvinorin A is selective for kappa opioid receptors (κOR) and acts as a potent and efficacious agonist in G protein coupling and adenylyl cyclase assays; however, it promotes 40-fold less efficient κOR internalization than what is seen by the typical kappa agonists including U50,488H (Roth et al., 2002; Chavkin et al., 2004; Wang et al, 2005). The chemical structure of the compound is unique in that it lacks a basic nitrogen atom, a property that is conserved among all other known opioid receptor agonists and thought to play a critical role in ligand binding. Together, these findings suggest that the κOR conformation induced by salvinorin A binding is conducive to G protein mediated signal transduction but resistant to internalization-mediated regulation.

Chemical derivatives of salvinorin A were recently described and here we report on one such derivative that has greater affinity for the μOR over the κOR (μ/κ = 0.13 from
This unique compound, termed, herkinorin ((2S,4aR,6aR,7R,9S,10aS,10bR)-9-(Benzoyloxy)-2-(3-furanyl)dodecahydro-6a,10b-dimethyl-4,10-dioxo-2H-naphtho-[2,1-c]pyran-7-carboxylic acid methyl ester, (Harding et al., 2005)) activates the µOR, leads to very little receptor phosphorylation, does not recruit βarr2 and does not lead to receptor internalization, even in the presence of overexpressed GRK2. Therefore, compounds based upon this structure may provide insight into the mechanisms of ligand-directed µOR regulation and may represent a step in the development of opiate analgesics that promote pain relief with limited adverse effects.

MATERIALS & METHODS:

**Drugs:** DAMGO (Tocris, Ellisville, MO), morphine sulfate, and naloxone (Sigma, St. Louis, MO) were prepared as 10mM stocks in phosphate buffered saline. Herkinorin (referred to as “analog 13” in Harding et al., 2005) was prepared in DMSO as a 10 mM stock. Dilutions were made directly into minimal essential media (without phenol red for confocal imaging). Herkinorin is subject to degradation; therefore, drugs were produced in small batches, protected from light and not stored in DMSO or extensively as a powder.

**Phospho-ERK1/2 immunoblot assay:** HEK-293 cells stably expressing an haemagglutinin (HA-N-terminus) tagged mouse µOR (~2 pmol/mg membrane protein) were assessed for agonist-induced ERK1/2 phosphorylation. Cells were serum starved at 37°C under 5% CO₂ for 30 min prior to drug treatment. Cells were stimulated for the
times indicated and cell lysates were prepared on ice in lysing buffer (20 mM Tris HCl pH 7.5, 150 mM NaCl, 2 mM EDTA, 0.1% SDS, 1% NP40, 0.25% deoxycholate, 1 mM sodium orthovanadate, 1 mM PMSF, 1 mM NaF, with Complete Mini, EDTA-free protease inhibitor cocktail tablet (Roche Diagnostics, Indianapolis, IN)). Protein levels were determined by the BioRad DC protein assay system (BioRad, Hercules, CA) and 20 µg protein per lane were resolved by 1-D gel electrophoresis on 10% Bis-Tris gels (BioRad or Invitrogen, Carlsbad, CA). Proteins were transferred to polyvinylidene fluoride (PVDF) membranes (Immobilon P, Millipore, Billerica, MA) and immunoblotted for phosphorylated ERK1/2 (p-ERK E-4: sc-7383, Santa Cruz Biotechnology, Inc., Santa Cruz, CA). Blots were stripped and blotted for total ERK1/2 levels (p44/42 MAP kinase Antibody, Cell Signaling Technology, Danvers, MA) which were used to normalize the overall phosphorylation of ERK1/2 between samples (Bohn et al., 2000b). Chemiluminescence was detected and quantified using the Kodak 2000R imaging system (Eastman Kodak Company, Rochester, NY) and GraphPad Prism software (GraphPad Software, Inc., San Diego, CA). Mouse embryonic fibroblasts expressing (MEF-WT) and lacking endogenous βarrestins 1 and 2 (MEF βarr1&2-KO) were kindly provided by Dr. Robert Lefkowitz (Kohout et al., 2001; Bohn et al., 2004b). The μOR expression in MEFs was obtained by using retroviral expression vectors. ERK activation studies were performed in parallel to trafficking studies to assure activity of the agonists.

**Phosphorylated μOR Immunoprecipitation:** HEK-293 cells stably expressing HA-tagged μOR were serum starved for 15 minutes and then treated for 10 minutes with
saline, DAMGO (1µM), Morphine (10µM), or Herkinorin (10µM). A mock transfection treated with DAMGO (1µM) was included as a control. Cells were washed on ice and then lysed with 250µl RIPA-P buffer (20mM Tris HCl pH8.0, 150mM NaCl, 2mM EDTA, 0.1% SDS, 1% NP-40, 0.25% deoxycholate, 1mM sodium orthovanadate, 1mM PMSF, 1mM NaF, and protease inhibitor cocktail (Roche Diagnostics, Indianapolis, IN). Cell lysates were collected and solubulized at 4°C for 1 hour. The insoluble fraction was removed with a 20,000 x g spin at 4°C for 30 minutes. Protein content was measured using Bio-Rad DC Protein Assay and samples were diluted to equal concentrations. Equal amounts of protein (700-1000µg) or buffer only (for “no protein” control) were incubated with 70µl of a 1:1 suspension of monoclonal anti-HA-agarose beads (Sigma, St. Louis, MO) overnight at 4°C with rotation. The immunoprecipitated complex was collected and washed per manufacturer’s instructions. Proteins were eluted from anti-HA-agarose in 30µl 4x XT Sample Buffer (BioRad, Hercules, CA) (62.5mM Tris-HCl, pH6.8, 25% glycerol, 2% SDS, 0.01% bromophenol blue) with 5% β-mercaptoethanol at 95°C for 4 minutes. Samples were resolved on 10% Bis-Tris XT Precast Gels (Bio-Rad) and transferred to PVDF membranes (Immobilon-P, Millipore, Billerica, MA). Membranes were incubated with a phospho-µOR antibody (1:500) that recognizes phosphorylated serine 375 of the mouse µOR (p-µOR Ser375, Cell Signaling, Danvers, MA). Chemiluminescence was visualized using a Kodak 2000R image station. Membranes were stripped and blotted with a primary antibody against the C-terminus of the µOR (1:1000) (Sigma, St. Louis, MO) to determine total levels of receptor per lane. Densitometry was assessed using the Kodak imaging software and p-µOR levels were normalized to the total receptor per lane as well as to the degree of stimulation compared
to saline-treated controls in each blot. Data were analyzed using GraphPad Prism software (GraphPad Software, Inc., San Diego, CA).

**Cross-linking and Co-Immunoprecipitation.** HEK-293 stably expressing the µOR tagged at the N-terminal with HA were used and the procedure is based on those described by Shenoy et al., (2006) and Gesty-Palmer et al., (2006). Cells were washed with PBS + 10 mM HEPES and then incubated in PBS-HEPES buffer for 20 minutes at 37°C. Cells were then treated with vehicle (0.1% DMSO), 1 µM DAMGO, or 10 µM Herkinorin for 5 minutes. The cross-linking reagent, dithiobis[succinimidylpropionate] (DSP, Pierce) was prepared in DMSO and administered drop-wise to the plates (2 mM final DSP concentration, at <10% DMSO); plates were then kept at room temperature for 20 minutes with constant agitation. The cross-linking reaction was stopped by the addition of 1 M Tris-HCl (pH 7.4) to give a final concentration of 50 mM. Cells generally came off of the plates with the agitation, so they were collected and centrifuged at 2000 rpm and then washed 4X in TBS. Cells were resuspended in lysing buffer (50 mM Tris-HCl, pH 7.4; 150 mM NaCl; 5 mM EDTA; 1% NP40; 1 mM Na-orthovanadate; 1mM PMSF; 1mM NaF and protease inhibitor pellet (1/10 mL, Roche)) and solubulized overnight at 4°C with rotation. Lysates were cleared by 12,000 rpm centrifugation and then immunoprecipitated with anti-HA conjugated agarose beads (Sigma) for 2 hours at 4°C with rotation. Proteins were eluted in SDS sample buffer (BioRad) with 5% 2-mercaptoethanol and 100 mM DTT by boiling for 10 min at 100°C. Proteins were resolved by SDS-PAGE under denaturing conditions and transferred to PVDF membranes. The A1CT antibody was kindly provided by Dr. Robert Lefkowitz and was
used to detect βarrestins 1&2 (Shenoy et al., 2006; Gesty-Palmer et al., 2006). Enhanced chemiluminescence was used to detect bands as described above. Controls included reprobing the blots for equal pull-down of µOR, lysates of HEK-293 cells transfected with HA-µOR for µOR immunoblotting and MEF-WT or MEF βarr1&r2-KO for βarrestin immunoblotting (Kohout et al., 2001).

**Cellular Trafficking.** HEK-293 cells were transiently transfected with combinations of the following cDNA as indicated in the figure legends: haemagglutinin (HA-N-terminus) tagged mouse µOR (10 µg cDNA); beta-arrestin2 tagged with green fluorescent protein (βarr2-GFP) (2 µg cDNA); mouse µOR tagged at the C-terminus with yellow fluorescent protein (µOR-YFP); and GRK2 (5 µg cDNA). In some cases, cells stably expressing the µOR were used and no differences were observed when compared to transiently transfected cells. Cell media was changed 10-20 minutes prior to addition of drug to serum-free and phenol red-free MEM. Cells were visualized using an Olympus confocal microscope with Green-Helium Neon and Argon Lasers. Multiple cells were recorded per dish following more than four separate transfection experiments; shown are representative cells. Transfection by electroporation and confocal imaging were performed as previously described (Bohn et al., 2004b).

**Immunofluorescence of Cell Surface Receptors:** Stably transfected HA-N-terminus tagged µOR-expressing HEK-293 cells were plated on a 96 well optical plate (collagen coated) and treated with either DAMGO or Herkinorin for the times indicated. Cells were washed 2X with MEM, fixed with 4% paraformaldehyde (PFA) room temperature
for 20 minutes and blocked in MEM + 5% goat serum for 1 hour. Cells were then incubated with an anti-HA antibody (monoclonal clone 12CA5, Roche Diagnostics, Indianapolis, IN) at 1:500 dilution in blocking buffer overnight at 4°C. Cells were washed 3X in blocking buffer and incubated for 1 h with the Alexafluor-488 goat anti-mouse secondary antibody (Molecular Probes, Eugene, OR) at room temperature.

Following 3 washes in phosphate buffered saline, immunofluorescence was assessed using a Fusion Plate Reader (Perkin Elmer, Boston, MA). Data are normalized to the controls in which no agonists were added. Non-specific secondary antibody interactions were subtracted from each point. Images were taken following the experiment using a 10X objective to assure that cells had remained uniformly attached; images were also taken of these cells. If cells came off the plate, the measurements were deleted for that well. Cells were also treated in parallel to this experiment in small confocal dishes. After 60 minutes of treatment, cells were processed in the same manner as above and immunofluorescent images were taken using a 40X objective via confocal microscopy.

**Biotinylated Receptor Internalization.** HEK-293 stably expressing the µOR tagged at the N-terminal with HA were used for cell surface biotinylation experiments based methods previously described (Cao et al., 1998). Cells were washed once with cold phosphate buffered saline (PBS) then sulfo-NHS-SS-biotin (Pierce, 600 µg/mL in PBS) was added to each dish and cells were incubated for 30 minutes at 4°C. Cells generally came off of the plates and were therefore collected, combined and spun down at 2,000 rpm for all subsequent washes. Cells were washed in Tris-buffered saline (TBS, 50 mM Tris-HCl 7.4pH, 150 mM NaCl) to quench unreacted biotin. Cells were divided into
identical aliquots to receive the following treatments. For drug treatment, cells were resuspended in MEM containing either vehicle (0.1% DMSO), 1 µM DAMGO or 10 µM herkinorin and incubated for 1 hour in a 37°C water bath. Following incubation, surface biotinylation was stripped away using glutathione stripping buffer (50 mM glutathione, 75 mM NaCl, 75 mM NaOH, 10% FBS, in H2O) for 15 minutes incubation on ice.

Glutathione is quenched with 15 minute incubation on ice in iodacatamide buffer (50 mM iodacatamide, 1% BSA in PBS (pH 7.4)). Cells were resuspended in Triton X-100 extraction buffer (TXB: 10mM Tris (pH7.5), 120 mM NaCl, 25 mM KCl, 1mM PMSF, 1 protease inhibitor pellet/ 10 mL (Roche, ), 1 mg/mL iodoacetamide, 0.5% Triton X-100) and solubulized on ice for 2 hours. Lysates were cleared by 12,000 rpm spin and then added to avidin-conjugated agarose beads (Pierce) for immunoprecipitation overnight at 4°C with rotation. Immunoprecipitates were centrifuged at 12,000 rpm and washed 3 times with TXB and once with PBS + 0.1% SDS. Immunoprecipitates were washed and proteins were eluted and resolved as described in the co-immunoprecipitation experiments. Immunoblots were performed using the µOR C-terminal directed antibody (Neuromics), donkey anti-rabbit secondary antibody and enhanced chemiluminescence detection as described above. Several controls were performed and they are as follows: 1) “100%” refers to total surface receptors and represents cells lysed directly following biotinylation and washes. 2) “Strip” refers to cells that have been biotinylated and then stripped; this serves as a control for the effectiveness of the glutathione stripping buffer. 3) Mock: Same as 100% but in cells that are stably mock transfected with empty vector (no µOR). 4) No protein refers to immunoprecipitation in the absence of cellular lysate.
**Statistics:** Statistical analyses were performed using the GraphPad Prism software (GraphPad Software, Inc., San Diego, CA) and the specific tests used are presented in the figure legends.

**RESULTS:**

The chemical synthesis of herkinorin from salvinorin A has been previously described; the chemical structures of herkinorin and salvinorin A are presented in Figure 1 for reference (Harding et al., 2005). Receptor binding assays revealed that Herkinorin has a greater affinity for μOR over κOR and very little affinity for the δOR; G protein coupling assays determined that Herkinorin preferentially activates the μOR (Harding et al., 2005).

In order to assess Herkinorin agonist activity in parallel with the current studies, we used the phosphorylation of the downstream MAP kinases (ERK1/ERK2) as an indicator of receptor activation. In Figure 2, we show that like other μOR agonists, such as the enkephalin analog, [D-Ala², N-MePhe⁴, Gly⁵-ol] enkephalin (DAMGO), herkinorin dose-dependently activates the MAP kinases, ERK1/2, in a μOR antagonist reversible manner as shown in Figure 2C (Belcheva et al., 1998). Similar results were also obtained using CHO cells expressing the human μOR (data not shown). A comparison of ERK phosphorylation between DAMGO and Herkinorin reveals comparable potencies, although there is a tendency for herkinorin to continue to activate ERK past the efficacy observed with DAMGO (Figure 2). In order to test whether β-arrestins are required to mediate μOR signaling to ERK we examined ERK1/2 phosphorylation in MEFs that lack both β-arrestin1 and β-arrestin2 and found that ERK1/2 could be activated in the absence of β-arrestins (Figure 2D)(Kohout et al., 2001; Bohn et al., 2004a).
Since agonist activation of many GPCRs leads to GRK-mediated receptor phosphorylation, β-arrestin recruitment and internalization, we compared herkinorin to other opiate agonists for their ability to regulate the µOR in HEK-293 cells, a model system routinely used to study opioid receptor regulation and trafficking. While both morphine and DAMGO activate the µOR, morphine is much less effective in promoting receptor phosphorylation (Figure 3) which is thought to underlie the decreased receptor trafficking that results from morphine binding (Whistler and von Zastrow 1998; Zhang et al., 1998; Bailey et al., 2003, Bohn et al., 2004b; Schulz et al., 2004; Koch et al 2005). Herkinorin resembles morphine in that it does not promote robust phosphorylation of the µOR at serine 375 (Figure 3).

Agonist-induced βarr2 translocation in response to morphine, DAMGO or herkinorin was assessed by confocal imaging of live cells. HEK-293 cells expressing both the µOR and a GFP tagged β-arrestin-2 (βarr2-GFP) were stimulated with agonist for the times indicated. Images were collected from the same plate of cells prior to and following drug treatment. Shown here are representative images at the indicated time points. The translocation of βarr2-GFP to the receptor is robust with DAMGO as compared to morphine (Figure 4A; Zhang et al., 1998; Bohn et al., 2004b). Herkinorin resembles morphine in that it does not appear to induce βarr2-GFP translocation to the µOR (Figure 4A). Cells were also observed at earlier (real time video every 30 seconds for first 10 minutes) and later time points (15, 20, 30, 60, 120 minutes) but no translocation was observed with morphine or herkinorin under either condition (data not shown). In order
to assess whether herkinorin could recruit endogenous βarrestins to the receptors, we stimulated the μOR expressing cells with drug, stabilized the βarrestin-receptor complex using a cross-linking reagent, immunoprecipitated the HA-tagged μOR and blotted for βarrestins using the A1CT βarrestin antibody (Shenoy et al., 2006; Gesty-Palmer et al., 2006). While DAMGO promoted μOR-βarrestin interactions, vehicle and herkinorin did not (Figure 4B).

Morphine can induce βarr2-GFP translocation when GRK2 is overexpressed in HEK-293 cells (Zhang et al., 1998; Bohn et al., 2004b). While this cellular manipulation is sufficient to promote morphine-induced translocation; the overexpression of GRK2 does not promote herkinorin-induced βarr2-GFP translocation to the μOR (Figure 4C). In order to be certain that the cells imaged had been transfected with GRK2, morphine was applied after herkinorin treatment and translocation was observed. Morphine does not normally induce βarr2-GFP translocation in the absence of GRK2 overexpression (Figure 4A, therefore, a positive response to morphine was taken as evidence that the cell was overexpressing GRK2. Conversely, a combination of morphine and herkinorin was not sufficient to induce translocation in cells that did not overexpress GRK2 (data not shown).

Since βarrestin-GPCR interactions precede receptor vesicular internalization, it is not surprising that DAMGO leads to robust receptor internalization, while morphine does not (Figure 5A, Whistler and von Zastrow 1998; Zhang et al., 1998; Bailey et al., 2003, Bohn et al., 2004b; Schulz et al., 2004; Koch et al 2005). HEK-293 cells expressing YFP-
tagged µOR were treated with each of the indicated opioids to assess receptor trafficking. Herkinorin, like morphine, does not promote µOR-YFP internalization in the HEK-293 cells (Figure 5A). The lack of herkinorin-induced receptor internalization is further demonstrated by immunolabelling the N-terminus of receptors in intact, non-permeabilized HEK-293 cells following drug treatment over 2 hours. While DAMGO treated cells show a loss of ~50% of cell surface receptors, the herkinorin treated cells maintain the same level of cell surface receptor expression over the 2 hour time period (Figure 5B,C). A cell surface protein biotinylation approach was also used to assess receptor internalization following drug treatment (Figure 5D) (Cao et al., 1998). DAMGO induced a significant increase in internalized receptors while vehicle and herkinorin did not.

The overexpression of GRK2 can promote morphine-induced µOR internalization as it does βarr2-GFP translocation (Figure 4C), suggesting that the agonist occupancy promotes a conformation that differs between DAMGO and morphine at the level of GRK-mediated phosphorylation of the agonist-bound receptor (Zhang et al., 1998; Bohn et al., 2004b). However, overexpression of GRK2 is insufficient to promote herkinorin-induced µOR internalization (Figure 6A) further demonstrating the differences between morphine and herkinorin with respect to agonist-induced µOR trafficking.

GPCR phosphorylation and internalization can be altered by changing the serine and threonine numbers in the C-terminal tail (Oakley et al, 2001). A naturally occurring splice variant of the mouse µOR, µOR1-D, differs from the µOR only in the C-terminal
sequence and has been shown to recruit βarr2-GFP and internalize following morphine treatment (L.M. Bohn, unpublished observations; Koch et al., 2001; Pan et al., 1999). The µOR1-D internalizes with morphine treatment while herkinorin fails to internalize this receptor variant (Figure 6B). Taken together, our findings demonstrate that unlike morphine, the herkinorin-bound µOR does not internalize or recruit βarr2-GFP in HEK-293 cells and this cannot be overcome by overexpressing GRK2 or by substituting the µOR1-D splice variant.

DISCUSSION:

Herkinorin is a µOR agonist that does not promote βarr2-GFP translocation or µOR internalization. It differs from morphine which can promote βarr2-GFP translocation and receptor internalization when GRK2 is overexpressed in the µOR-expressing HEK-293 cells. Herkinorin could be classified as a biased agonist of the µOR as it induces signaling through one pathway (MAP kinase) while it does not promote an interaction with another pathway (βarrestin translocation and receptor internalization). Agonists that can activate the receptor may be therapeutically useful considering that suppression of the βarrestin2-µOR interaction attenuates morphine antinociceptive tolerance and morphine-induced side effects in genetically modified mouse models (Bohn et al., 2000; Przewlocka et al. 2002; Raehal et al., 2005).

Nearly a decade ago, Burford et al. (1998) first described how morphine and DAMGO could be considered as “biased agonists” as both ligands could efficiently activate G proteins, yet only DAMGO induced robust µOR internalization in HEK-293 cells.
Biased agonism based on receptor signaling versus arrestin recruitment has also been described. Several recent reports from the Lefkowitz group have demonstrated that agonists can mediate distinct G protein- and arrestin-dependent downstream signaling (Gesty-Palmer et al., 2006; Shenoy et al., 2006). An earlier study by Kohout et al. (2004), describes a similar effect for CCL7 receptor activation by the two agonists-CCL19 and CCL21 wherein they show that while both agonists activate G protein mediated signaling, only CCL19 places the receptor into a conformation that can be phosphorylated and desensitized by βarrestins. Phosphorylation at the C-terminus was found to be important for the CCL19-mediated desensitization, and it was likely that both GRKs and protein kinase C (PKC) could play a role in the phosphorylation. The µOR is also phosphorylated by both GRKs and PKCs. Extensive work by the Chavkin group has indicated several potential residues by which the µOR can be phosphorylated by GRKs as well as by PKC and they have further shown that the phosphorylation profiles are tightly linked to agonist-specific regulation (Celver et al., 2001; Lowe et al., 2004; Celver et al., 2004). We have not evaluated the role of other protein kinases on regulation of the herkinorin-bound µOR, yet it is likely that some form of receptor regulation will occur. Further, our studies have examined phosphorylation of only one residue implicated in desensitization and it is likely that other phosphorylation sites in the receptor can impact receptor regulation. Further studies of this compound and its derivatives are ongoing.

To test for agonist activity of the compound in parallel with the trafficking studies, we employed a MAP kinase assay and found that Herkinorin could lead to phosphorylation of ERK1/2 in a dose-dependent, antagonist reversible manner (Figure 2). A recent report
by Wang et al. (2006) reports that β-arrestins can serve as a molecular switch in the activation of ERK via SRC mediated by α2 adrenergic receptor activation. In the absence of β-arrestins, they found that ERK phosphorylation persisted over time and that the phospho-ERK was predominantly localized to the nucleus at the later time points. Herkinorin treatment activates of ERK in a manner that appears to be more efficacious than DAMGO which may be due to a more persistent signal. Whether the altered ERK activation profile is due to a similar mechanism as those described for the α2 adrenergic receptors in the absence of β-arrestins remains to be determined. We have found that the µOR activates ERK in the absence of β-arrestins using mouse embryonic fibroblasts that lack endogenous β-arrestin1 and β-arrestin2 and these cells will be useful for further elucidating the contribution of the β-arrestins to the µOR activation of MAP kinase pathways. Moreover, the physiological significance of such molecular events downstream of µOR remain to be determined.

The implications of how opiates that can activate the receptor without recruiting β-arrestins will act in vivo remain to be seen. In studies using mice lacking β-arrestin-2, morphine produced enhanced and prolonged antinociception with very little morphine tolerance (Bohn et al., 1999, 2000, 2002). Therefore, opioid agonists that do not promote receptor–β-arrestin interactions may mimic the effect of blocking the β-arrestins and result in analgesics that do not promote tolerance. Moreover, agonists that activate the µOR without the recruitment of β-arrestins may have additional benefits besides preventing antinociceptive tolerance. Recently, morphine-induced side effects, including constipation and respiratory suppression, were shown to be greatly diminished in mice.
lacking βarr2 (Raehal et al., 2005). The mechanisms that determine how one µOR-mediated physiological response can be enhanced while another is suppressed due to the ablation of βarr2 is not known; however, these observations speak to the diverse roles that βarrestins may play in receptor signaling as well as regulation (Shenoy and Lefkowitz, 2003). If βarr2-opioid receptor interaction is required for opioid-induced constipation and respiratory suppression, then compounds that can activate the µOR and not recruit the βarrestins may be promising opioid analgesics with much fewer side effects (Bruns et al., 2006).

The question remains as to whether the βarrestin translocation and internalization profiles observed in the cellular system are preserved at the biological sites of action in vivo. This is an important consideration as morphine has been shown to promote efficient internalization profiles in certain neuronal populations while promoting no internalization in other neurons (Keith et al., 1996; Haberstock-Debic et al., 2003; 2005). Here we have used the HEK-293 cellular system to assess the herkinorin-induced µOR trafficking profiles since this model has been the most extensively used to study opioid receptor trafficking and should allow for the best overall comparison of herkinorin to other opioids. Ultimately, receptor trafficking induced by herkinorin and other opiates should be compared in neurons that mediate opiate-induced antinociceptive effects.

Salvinorin A is biologically active in vivo as it produces hallucinations by its actions at the κOR yet these effects are very short-lasting (Roth et al., 2002). Systemic injections of herkinorin into mice and rats do not produce analgesic responses; however, peripheral
injections do produce localized analgesia that can be blocked by the opioid antagonist, naloxone (T. E. Prisinzano and K. Tidgewell unpublished observations). This may be due to the fact that like salvinorin A, herkinorin is susceptible to degradation by esterase, or by other metabolic routes, in vivo and may not be readily available in the CNS. Moreover, poor solubility of the compound makes it difficult to administer high concentrations systemically. Further modifications of the herkinorin template are being generated with hopes of identifying a compound with greater stability and solubility than herkinorin.

The structure of herkinorin, as well as salvinorin A and its related compounds, is unique among opioid receptor ligands in that it lacks a basic nitrogen atom. This unique chemical backbone might provide the framework for the generation of new opioid agonists that take advantage of the differences in opioid receptor regulation induced by alterations in ligand-receptor interactions. Future agonists built on this novel chemical structure may shed light on the molecular determinants of opioid receptor signaling and may lead to the fine tuning of opioid analgesics by limiting adverse narcotic effects.

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FOOTNOTES

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FIGURE LEGENDS:

Figure 1. Chemical structures of salvinorin A and herkinorin.

Figure 2. Herkinorin promotes ERK1/2 phosphorylation. Herkinorin and DAMGO induce ERK1&2 phosphorylation in HEK-293 cells stably expressing an haemagglutinin (HA-N-terminus) tagged mouse µOR. A. Dose-response of Herkinorin and DAMGO activation of ERK. Stimulation was for 10 minutes at the concentrations indicated. The dose response comparison reveals a difference between DAMGO and Herkinorin as assessed by two-way ANOVA p<0.05 (n=2-6, n= 2 for 100 nM). Statistical legend: (a)p<0.05; (b)p<0.01; (c)p<0.0001 vs. vehicle control; or between groups indicated by a bar, Student’s t test. B. Time course of Herkinorin- and DAMGO- induced phosphorylation of ERK. Cells were treated with 10 µM Herkinorin or 1 µM DAMGO at the times indicated. Phopsho-ERK1/2 bands were analyzed by densitometry and normalized to total ERK levels (lower bands) and densitometry is presented as fold stimulation (mean ± SEM) over saline treated controls. The time course comparison reveals a difference between DAMGO and Herkinorin as assessed by two-way ANOVA p<0.05 (n=3-5); and a significant difference between the compounds at the 10 minute point. (b)p<0.01 Student’s t test, (n=3-5). C. Pretreatment with naloxone (10µM) during
the 30 min serum starvation blocked 1 µM herkinorin-induced ERK activation. **D.**

Activation of µOR in the absence of βarrestins (βarr1&2-KO MEFs) still leads to ERK1/2 phosphorylation. Immunoblots were performed as described above using lysates from MEFs.

**Figure 3. Mu opioid receptor phosphorylation at serine 375 following agonist treatment.** HEK-293 cells stably expressing µOR were treated with saline, 1 µM DAMGO, 10 µM Morphine, or 10 µM Herkinorin for 10 minutes. The receptor was immunoprecipitated from cell lysates using an anti-HA antibody-agarose bead complex. Representative western blots using antibodies that recognize the µOR phosphorylated at serine 375 (top) or the total µOR (C-terminal antibody) from the same blot (bottom) are shown. Densitometric analyses of 3 independent experiments performed in duplicate or triplicate were normalized to total receptor per lane and expressed as fold stimulation over saline control for each blot. Data are presented as the mean ± S.E.M. **p<0.001; #p<0.01 vs. DAMGO one-way ANOVA analysis of variance with Bonferroni's Multiple Comparison Test (n=4-6).**

**Figure 4. Agonist-induced βarrestin interactions with µOR in HEK-293 cells.**

HEK-293 cells transiently transfected with µOR and βarr2-GFP were imaged in real time following agonist treatment at room temperature. The cytosolic distribution of βarr2-GFP is shown in the untreated cells in the top left panels. A. βarr2-GFP translocation to µOR in HEK-293 cells. DAMGO (1µM) treatment leads to βarr2-GFP translocation within 5 min (white arrow- punctuate accumulation at membrane) while morphine (10 µM, 10
min) does not. Herkinorin (2 µM, 10 min) does not induce βarr2-GFP translocation. B. Co-immunoprecipitation of βarrestins and µOR following drug treatment. Cells were treated with vehicle (0.1% DMSO), 1 µM DAMGO, or 10 µM Herkinorin for 5 minutes. Cells were then cross-linked using a cell-permeable cross-linking reagent (DSP (Dithiobis[succinimidylpropionate])). Cell lysates were immunoprecipitated by anti-HA-conjugated agarose beads and proteins were resolved by SDS-PAGE under denaturing conditions. Blots (left): Immunoblotting was performed using the βarrestin antibody (A1CT); blots were stripped and then reprobed with the µOR antibody (Neuromics). Representative blots are shown. Mock refers to HEK cells transfected with empty vector. “No protein” contained no cell lysate in the immunoprecipitation. Antibody controls were performed on lysates run on the same gel as those shown for the immunoprecipitation. Densitometry (right): Densitometry was measured from a total of 3-4 samples of each treatment prepared on 2 separate days. Shown are the means ±SEM. **p<0.01 vs. WT or HERK, Student’s t test. C. βarr2-GFP translocation to µOR in HEK-293 cells overexpressing GRK2. Herkinorin does not promote βarr2-GFP translocation at 2µM after 10 min or at 100 µM after 30 min. The same cells were treated with morphine (10 µM, 10 min) and βarr2-GFP translocates demonstrating that these cells do overexpress GRK2 as morphine does not induce visible translocation otherwise.

Figure 5. Agonist-induced internalization of µOR-YFP in HEK-293 cells. A. HEK-293 cells were transiently transfected with µOR (2 µg cDNA) tagged at the C-terminus with yellow fluorescent protein (YFP). Cells were treated with the agonists
indicated. Internalization can be seen after DAMGO treatment as indicated by the appearance of donut-like intracellular vesicles (white arrow) and the disappearance of membrane receptor localization as seen in the basal panel. Herkinorin and morphine do not induce receptor internalization. **B.** Cell surface expression of receptor as determined by remaining N-terminal HA-immunoreactivity in *non-permeabilized* cells following drug treatment. DAMGO, but not herkinorin, leads to a loss of cell surface expression following 60-120 minutes of drug treatment. Two-way ANOVA analysis reveals that the curves differ (P<0.001) and that the DAMGO treated cells display less surface receptors at each time point as determined by Bonferroni post-hoc analysis (p<0.001 at each time point after 0). **C.** Immunocytochemistry of HA-MOR expression on cell surface following 60 minutes of agonist treatment. Top: 10X objective, images are taken directly from the 96 well plates imaged in 5B. Bottom: 40X objective images in parallel dishes. **D.** Internalization of receptors as determined by cell surface biotinylation assay.

*Blot (right)*: Cell surface proteins were biotinylated, treated with indicated drug or vehicle (Vehicle, 0.1% DMSO; 1 µM DAMGO or 10 µM herkinorin for one hour. Remaining surface proteins were then subjected to glutathione cleavage of biotin. Cells were then lysed and remaining biotinylated proteins were immunoprecipitated with avidin-conjugated agarose beads. Proteins were resolved via SDS-PAGE under reducing conditions. Immunoblotting was performed with the C-terminal specific μOR antibody (Neuromics, Edina, MN). 100% represents cells that were not stripped with glutathione. Strip represents remaining surface biotinylated μOR after glutathione treatment and without drug treatment. Mock represents DAMGO treated HEK-293 cells that were transfected with empty vector. No Prot represents no cell lysate present in the
immunoprecipitation. *Densitometry (left):* Densitometry was measured from a total of 5-7 samples of each treatment prepared on 3 separate days. ***p<0.001 vs. vehicle; DAMGO induced significantly more internalization than Herkinorin, p<0.05. Herkinorin did not differ from Vehicle p>0.1. Student’s *t* test.

**Figure 6. Receptor internalization under conditions where morphine will internalize µOR.**  
**A.** Agonist-induced µOR-YFP internalization in cells overexpressing GRK2. Experiments were performed as described in Figure 4A with the addition of co-transfecting 5 µg GRK2 cDNA. Morphine induces a redistribution of membrane associated µOR-YFP to intracellular vesicle (white arrows). Herkinorin does not.  
**B.** Agonist induced µOR1-D-GFP internalization. µOR1-D-GFP (2 µg cDNA) was transiently transfected into HEK-293 cells. Morphine treatment internalizes the receptor as indicated by the appearance of intracellular vesicles (white arrows) while herkinorin does not. Experiments were performed on at least 3 separate transfections; representative cells are shown.
Figure 1
Figure 2
Figure 3
Figure 4
Figure 6