Synthesis of a Delta Agonist-Mu Antagonist Ligand to Alter Ligand-receptor Interactions on Delta Opioid Receptors and Mu Opioid Receptors

Introduction

General Introduction

Since the mid 1970's, opioids have been known for their pain-relieving effects, due to their ability to act on receptors in the nervous system. However, since then, issues of tolerance and addiction to these drugs have been unveiled. As a result of the interactions between certain opioids and the receptors to which they bind, different pathways may be signaled which may lead to the analgesic (pain relieving) effects, behavioral consequences, or outcomes related to the addictive nature of these drugs (internalization of the receptors). The three receptors responsible for these pathways are the mu, kappa, and delta opioid receptors. These are all G protein-coupled receptors, meaning that when the receptor is activated it induces a signal which is sent to many intracellular effectors.[1] Extensive research has been conducted on the mu opioid receptors, which has revealed how opioid side effects of tolerance, dependence, and increased sensitivity to pain result from the interactions between the opioids and the mu receptor. The mu receptor has been linked to the addiction pathway,[1] therefore, through identifying agonists and antagonists that selectively bind to these receptors, inducing the desired conformations of the receptors, can allow for these pain relieving and addictive pathways to be better controlled, further preventing and lessening the undesired side effects. Through developing a combined delta agonist-mu antagonist, the pain relieving pathway can be favored over the addictive pathways. Some potential ethical issues include future in vivo studies conducted on mice, and clinical trials.

Goals

Successful completion of the proposed research will result in a new combined agonist-antagonist that will block the prominent pathway that leads to the addictive properties of opioids while enhancing the analgesic pathway, in order to promote pain relief. Furthermore, the additional proposed experiments will provide support that the newly synthesized ligand binds effectively to the delta opioid receptor and allow for visualization of the drug-induced receptor trafficking.

Background

The three opioid receptors that analgesic drugs interact with are mu, kappa, and delta. When opioids bind to the receptors, the effect is inhibitory meaning that it is subduing to the activity of the neurons which reduces the sensation of pain. With many current analgesic drugs, such as morphine, the main interaction is with the mu opioid receptor (MOR). With this interaction, a greater amount of morphine is required in order for pain relief, and the central dopamine reward pathway that leads to addiction is enhanced. The delta opioid receptor (DOR) has a role in analgesia as well as other neurological functions such as emotional responses, especially around antidepressive effects.[2] There are interactions between the mu and delta receptors, and there is evidence that the DOR may be a better target for pain relief than MOR. However, while there is extensive knowledge of mu receptors, less is known about delta, therefore, it would be a good target for testing, and from current studies, it has been found that DOR activation has assisted in pain alleviation. The delta receptor has been largely effective in dealing with chronic pain and depression.[3] While DORs may not be the most effective target for acute pain treatment, it has been shown that they are more effective in reducing chronic inflammatory or neuropathic pain.[4] Initially, it was thought that DOR mainly existed at

intracellular sites, but it has recently been found that there is a membrane localization of these receptors, and DOR is a flexible receptor that readily responds to agonists.

While all three opioid receptors share similar pathways, such as pain relief, there are selective ligands that can direct the receptors to favor one or more of the signaling pathways.[5] In research that has been done this far, DOR seems to be a promising target because it has not been seen to induce either addictive or aversive effects.[6]

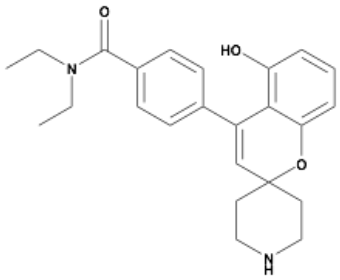
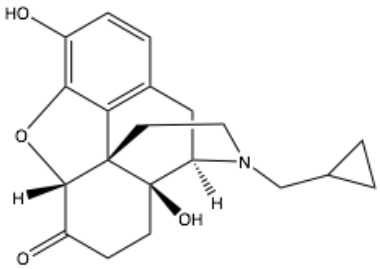
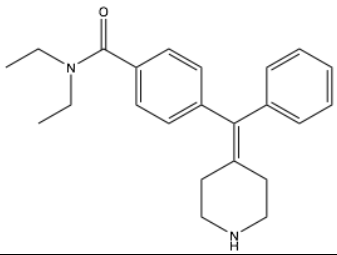
In the area of increasing the effectiveness of analgesic drugs, some different approaches have been taken. In the study completed by Gregory Corder, Vivianne Tawfik, Dong Wang, and Elizabeth Sypek, they determined that the co-administration of "methylnaltrexone bromide," which treats constipation, decreased morphine tolerance without diminishing the effects of pain control.[7] However, this involves the dosage of two different medications. While in the past some have tried to create an agonist for the MOR and an antagonist for the DOR, believing this would decrease tolerance and addiction while still having pain-relieving effects, using an agonist with the DOR and an antagonist with the MOR may actually be more effective due to a strong link between the mu receptor and opioid use leading to addiction, and it is that effect which needs to be dampened. Other research has focused on creating selective MOR agonists which target the pain relief pathway, not the addiction pathway, and this has lead to diminished side effects of respiratory suppression, dependence, tolerance and/or constipation. In another study, mutations of the MOR itself resulted in responsiveness to the antagonist naloxone while still having the agonistic effects of pain relief. The use of naloxone did not cause tolerance or dependence.[8]

In this research, the focus is on an agonist for the DOR and an antagonist for the MOR. Naltrexone is a mu opioid receptor antagonist that blocks the mu opioid receptors with negligible antagonism at the kappa and delta receptors. Previous research has shown that naltrexone is less mu receptor-selective[9] than naloxone, but it has a longer duration of action than naloxone.[10] Furthermore, it has been determined that naltrexone suppresses binding to the mu-opiate receptor but increases delta-opiate receptor activity in rat splenocytes.[11]

Delta opioid agonists range from high to low internalizing in their effect on the delta opioid receptors, depending on the structure of the agonist.[1] Studies have shown that both delta opioid agonists ARM-390 and ADL-5859 have similar binding affinities and signalling potencies as SNC80, another common delta opioid agonist, however, they have been identified to prevent receptor internalization unlike SNC80.[12] Therefore, the low internalizing agonists - ARM-390 and ADL-5859 - will be focused on in this research to ensure a maintained presence of the delta opioid receptors on the cell membranes.

Both ARM-390 and ADL-5859 exhibit a diethyl benzamide structural component, therefore, it is likely this component is essential in the binding of the agonist to the delta receptor (Table 1). This feature will be maintained in the proposed ligand that consists of a dual delta agonist and mu antagonist function in aim of maintaining the binding affinity for the delta receptor. Through maintaining this structural component, the intent is to ensure functional selectivity of this proposed ligand in favoring an active conformation of the delta opioid receptor, inducing the desired agonist response.[1]

Table 1. Structures of the delta agonists and mu antagonists as components of the proposed ligand

Delta Opioid Agonists	Mu Opioid Antagonist
ADL5859 	Naltrexone 
AR-M100390 	

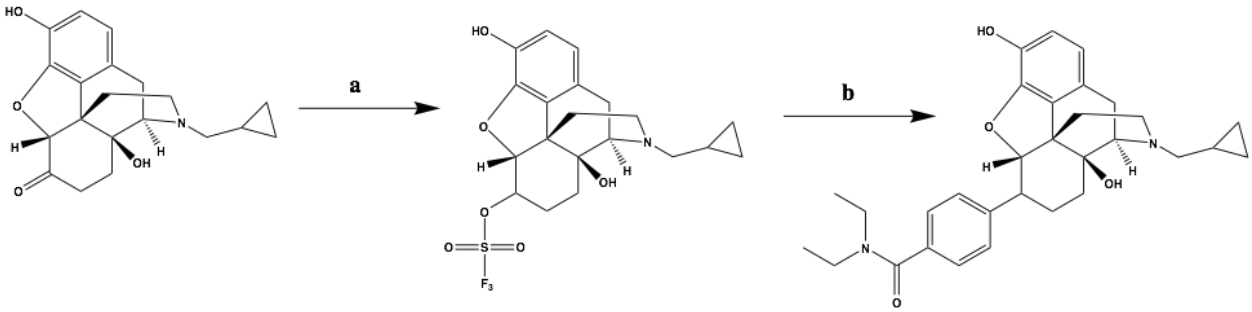
A problem that consistently arises in this area of research is that less is known about the features of delta opioid receptors, at cellular and molecular levels, compared to that know of the mu receptor. That which is known of the delta receptor features is very disjointed and unconnected to actual physiological and behavioral effects, which is where this topic of research needs future work. There have been several in-vitro studies, but studies in animals and humans are limited.[5]

Proposed Research

The first objective is to determine a way to focus on increasing the specificity of opioids to the delta receptor itself with the use of an agonist while decreasing tolerance without the co-administration of another medication. The next objective is to determine a reaction scheme that will allow the production of an agonist-antagonist that will bind to the delta receptor and allow the complete blockage of the mu receptor. Finally, the last objective is to characterize the agonist-antagonist produced and to quantify its affinity for the delta and mu opioid receptors. Successful completion of the proposed research will block the addiction pathway of the mu receptor leading to decreased addiction to opioids and increased pain relieving effects in the delta receptor.

In order to address the objectives, the synthesis of a mixed agonist-antagonist will be completed in order to increase the specificity of opioids to the delta opioid receptor while blocking the affinity to the mu opioid receptor. Various functional affinity and characterization studies will be completed in order to identify the ligand produced and quantitate its affinity.

Experiment 1:



Scheme 1. Synthesis of a dual delta agonist and mu antagonist ligand.

Scheme 1 was modified from a scheme proposed by Bourdonnec, et al. in 2008 in order to add the critical diethyl benzamide structural component at the location of the carbonyl on naltrexone.[13] In the first step of the scheme, the intermediate product is formed by a nucleophilic substitution with triflate and naltrexone. In the final step, triflate is substituted with diethyl benzamide to result in the target product.

Reagents and Conditions of Scheme 1:

a. A solution of Naltrexone in tetrahydrofuran at -78 degrees C under nitrogen atmosphere will be added dropwise to a 1.0 M solution of LiHMDS in THF (1.2 equiv). Reaction mixture will then be stirred for an hour at -78 °C. A solution of N-phenylbis(trifluoromethanesulphonimide) (1.2 equiv) in tetrahydrofuran will be added dropwise and warmed slowly to room temperature and stirring will continue for a further 12 h at room temperature. The mixture will then be poured into ice water and allowed to separate into two phases. The organic phase will then be washed with a 1N aqueous solution of hydrochloric acid, a 1 N aqueous solution of sodium hydroxide and brine, dried over sodium sulfate and filtered. The solvent will then be removed under vacuum and the tan oily residue will be used for the next step without further purification.

b. To the compound produced in Step 1 in dimethoxyethane (DME; 1.0 equiv), a 2 N aqueous solution of sodium carbonate (3.0 equiv), lithium chloride (3.0 equiv), 4- (N,N-diethylaminocarbonyl)phenylboronic acid) (1.1 equiv) and tetrakis(triphenylphosphine) palladium(0) (0.02 equiv) will be added. The mixture will then be refluxed for 10 h under nitrogen and then cooled to room temperature, filtered through a celite pad and washed with DME and water. Then, the mixture will be extracted with ethyl acetate and the organic layer will be washed with brine and dried over sodium sulfate. The crude product will then purified by chromatography (eluent: hexane/ethyl acetate mixtures of increasing polarity).

If the proposed reaction scheme fails to provide a mixed agonist-antagonist that results in the enhancement of the delta opioid receptor and the blockage of the mu opioid receptor, then additional alternative strategies will be examined. For instance, variations in the proposed reaction schemes can be made depending on whether it is the affinity for one or both receptors that is preventing the desired ligand-receptor interactions from occuring. Optimal reaction

conditions will be investigated based on the results of these initial conditions in order to determine a successful synthesis. Results will be analyzed by characterization methods, such as TLC monitoring, ^{1}H and ^{13}C NMR and mass spectrometry. Materials used in this experiment will all be purchased from Sigma-Aldrich.

Experiment 2:

Binding studies will be completed in order to obtain estimates of the affinity for the synthesized ligand for the delta and mu opioid receptors in order to determine if the scheme mentioned above will need to be modified. Kinetic and saturation experiments will be performed in order to quantify the binding of the ligand to the receptor(s). Kinetic experiments will allow for measurement of the binding concentrations of the ligands during an incremental series of time points.[14] In addition, the binding constant of the ligand for the receptor where the concentrations of the ligand are slowly increased will be measured in saturation experiments. The basic experimental protocol for the binding assays includes, the preparation of a solution containing the receptor(s) of choice which is then divided into aliquots. Furthermore, the ligand is then labeled with fluorescent and added to the aliquots in varying concentrations and allowed to incubate for a specific time and temperature. Finally, the data collected will be mathematically analyzed in order to quantify the estimates of the affinity through the calculation of rate and affinity constants.

Furthermore, functional affinity (KA values) will be determined to identify the affinity of the proposed ligand to the various conformations of the delta opioid receptor.[15] This will allow for any induced internalization due to the ligand to be identified.[15] In order to identify if the synthesized agonist-antagonist is a biased signal that supports analgesia and does not lead to undesired effects, computational tools will allow for the quantification of ligand-dependent signaling.[15] There are certain receptor ligands which can stabilize a specific conformation of the receptor that engages different signaling partners, which allows for desired analgesic responses. In order to support the proposed claim that the newly synthesized ligand will enhance the desired analgesic responses and block the undesired side effects, the ligand needs to first be evaluated to ensure that its response is contributing to analgesic efficacy.

Kenakin and Christopoulos in 2012[17], modified the operational model proposed by Black and Leff in 1983[16] in order to assess the conformational parameters and the maximal responses while allowing meaningful quantification of ligand-dependent bias independent of system and assay confounders. In the model, fractional response (E/Emax) at different agonist concentrations ([A]) can be calculated from the equation:

$$E/E\max = \tau^{n} \times [A]^{n} / (KA + [A])^{n} + \tau^{n} \times [A]^{n}$$

where E is drug effect, Emax is the maximal response allowed by the system and n describes the efficiency of the system to transduce receptor occupation into response. Kenakin and Christopoulos defined two ligand-related parameters: (i) *efficacy* (τ) of the agonist to couple receptor occupancy to a specific response and (ii) '*functional affinity*' (KA) of the ligand, which is defined as the tendency of the ligand to interact with the receptor state to mediate the desired response.[17]

Opioid receptors are part of the G-protein-coupled receptor (GPCR) superfamily. GPCRs have multiple different active conformations, and previous research indicates that certain agonists (ligands) acting at a certain receptor may produce different conformations which trigger

different effects, such as receptor trafficking and desensitization.[18] This proposed experiment will allow for the examination of the pain-relieving effects of the mixed agonist-antagonist ligand synthesized from delta opioid receptor agonists with similar binding and analgesic properties.

In order to complete binding assays, membrane preparations will need to be performed using COS cells which will be prepared the night before the assays are completed.[3] COS cells are monkey kidney fibroblasts and are important because they can be transfected (introduced) easily, in this case, with the delta opioid receptors. Opioid binding experiments will then be performed on the membrane preparations. For saturation experiments, 5–10 μg of membrane proteins will be diluted in 50 mM Tris-HCl, pH 7.4, in a final volume of 0.25–0.5 ml and incubated with variable concentrations of the two agonist-antagonists ligands for 1 h at 25 °C. For competition studies, membrane preparations will be incubated for 1 h at 25 °C with 0.5 nM of the synthesized ligands in the presence of other competing delta-opioid agonists at various concentrations. Ki, Bmax and Kd values will then be calculated in Microsoft Excel.

In addition, the [35S]GTPgammaS assay measures the level of G protein activation following agonist binding to the receptor. The assay allows for the potency, efficacy and affinity of the agonist to be determined, with the advantage that agonist measures are not subjected to amplification.[4] For each [35S]GTPγS binding assay, 5 μg of the membrane preparation protein will be used per well. Samples will be incubated with and without the mixed agonist-antagonists ligand, for 1 h at 25°C in assay buffer containing 50 mm Tris-HCl, pH 7.4, 3 mm MgCl2, 100 mm NaCl, 0.2 mm EGTA, 30 μm GDP and 0.1 nm [35S]GTPγS. Incubation will then be terminated by rapid filtration and washing in ice-cold buffer (50 mmTris-HCl, 5 mm MgCl2, 50 mm NaCl, pH 7.4).[19]

Future Research:

Previous research has determined that mouse models can be used to replace the endogenous delta opioid receptors with fluorescently-tagged delta opioid receptors.[20] The combination of fluorescent genetically encoded proteins allowed researchers to study gene expression patterns and migration in mice. Some researchers have been able to configure GFP (EGFP) in order to capture images of the G protein-coupled receptor in vivo. Mice were genetically modified in which the delta-opioid receptor was replaced by with DOR-EGFP. Then, real-time imaging using the confocal microscope was completed which allowed for the visualization of drug-induced receptor trafficking. The researchers were able to conclude that mice with internalized receptors were nonresponsive to additional agonist administration, since the receptors were no longer on the surface of the membrane and the agonist (ligand) could not bind.

Similar to the research mentioned above, after completing characterization procedures of the agonist-antagonist ligand, the proposed future plan is to use the delta-opioid receptor tagged with GFP. This will allow the visualization of drug-induced receptor trafficking which will further support the goal of this proposal, which is to enhancing ligand-receptor interactions on delta opioid receptors while decreasing these interactions in the mu opioid receptors in order to increase the specificity of opioids.

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