Synthesis and evaluation of a ligand targeting the μ and δ opioid receptors for drug delivery to lung cancer

Guo Li, Philip S. Low

PII: S0960-894X(16)30678-3
DOI: http://dx.doi.org/10.1016/j.bmcl.2016.06.067
Reference: BMCL 24020

To appear in: *Bioorganic & Medicinal Chemistry Letters*

Received Date: 3 April 2016
Revised Date: 24 June 2016
Accepted Date: 24 June 2016

Please cite this article as: Li, G., Low, P.S., Synthesis and evaluation of a ligand targeting the μ and δ opioid receptors for drug delivery to lung cancer, *Bioorganic & Medicinal Chemistry Letters* (2016), doi: http://dx.doi.org/10.1016/j.bmcl.2016.06.067

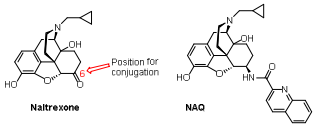
Graphical Abstract

To create your abstract, type over the instructions in the template box below.
Fonts or abstract dimensions should not be changed or altered.

Synthesis and evaluation of a ligand targeting the μ and δ opioid receptors for drug delivery to lung cancer

Li, G.; Low, P. S.

Leave this area blank for abstract info.



Bioorganic & Medicinal Chemistry Letters
journal homepage: www.elsevier.com

Synthesis and evaluation of a ligand targeting the μ and δ opioid receptors for drug delivery to lung cancer

Guo Li[a] and Philip S. Low[a*]

[a]*Department of Chemistry, Purdue University, 720 Clinic Drive, West Lafayette, 47907, USA.*

ARTICLE INFO

Article history:
Received
Revised
Accepted
Available online

Keywords:
lung cancer
small-molecule drug conjugate
naltrexone

ABSTRACT

A well-established approach to developing new imaging agents and treatments for cancer begins with the recognition of receptors that are overexpressed in cancer cells. Ideally, these same receptors would also be absent, or minimally expressed, in healthy tissue. The mu (μ) and delta (δ) opioid receptors (MOR and DOR respectively) match these criteria, with expression in cancer cells that is higher than primary lung epithelial cells. Naltrexone is a drug approved by the U.S. Food and Drug Administration (FDA) for treatment of alcohol dependence or prevention of relapse from opioid addiction. Since naltrexone binds with high affinity to both MOR and DOR, it was selected as the platform for development of novel ligands capable of delivering a cytotoxic payload to non-small cell lung cancer (NSCLC). This study outlines the synthesis of two ligands, with peptide or PEG linkers that were synthesized from 6-amino-naltrexone and conjugated with rhodamine dye or ^{99m}Tc for *in vitro* imaging, binding affinity or *in vivo* imaging and biodistribution studies. Transfected HEK cells were used as a model system for over-expression of the μ-opioid receptor (MOR) or the δ-opioid receptor (DOR). Naltrexone and naltrindole were used as competition for MOR and DOR respectively during the binding affinity studies. Mice bearing a xenograft of HEK cells transfected with μ (HEK-mu) or δ (HEK-delta) opioid receptors were the animal model used for PET imaging and *in vivo* biodistribution studies. Although the binding affinity studies were encouraging, the biodistribution data for the selected conjugates lacked sufficient specificity. These conjugates were abandoned from further development but information about their synthesis may be valuable to other laboratories working in this field.

Lung cancer is classified as one of two histopathological groups. Non-small cell lung cancer (NSCLC) is a heterogeneous disease that can present as adenocarcinoma, bronchioloalveolar carcinoma, squamous cell carcinoma, adenosquamous carcinoma, or large cell carcinoma and represents 80-85% of human lung cancer incidence.[1] The remaining 15-20% of cases are classified as small cell lung cancer (SCLC), which is differentiated by its higher proliferative and metastatic potential. Responsiveness to traditional forms of chemotherapy is another difference between the two types as illustrated by 5% survival rate over five years that is associated with SCLC compared to 14% for all forms of lung cancer in the same time span.[1]

Endogenous opioid peptides and their corresponding receptors are widely distributed throughout the body and exhibit regional differences in distribution. The three known categories of opioid receptors: μ-mu, δ-delta and κ-kappa are all members of the G-protein coupled receptor superfamily. All three opioid receptors are most highly expressed in the central nervous system, and can be also be found in peripheral tissues such as the heart, gastrointestinal tract, reproductive system, and immune system to varying degrees.[2,3] Both the μ-opioid receptor (MOR) and δ-opioid receptor (DOR) have been found in lung tissue, while the distribution of the κ-opioid receptor (KOR) is limited to pain neurons, spinal cord and specific regions of the brain.[4] While MOR is most closely associated with lung epithelial cells, DOR has also been identified in other cells found within lung tissue, such as nerve fibers within the bronchial submucosa, bronchial epithelial cells and alveolar macrophages.[5]

Exogenous opioids include drugs such as morphine, fentanyl and heroin that have a binding affinity for MOR two orders of magnitude greater than their affinity for the other opioid receptors.[6] Clinically relevant doses of morphine administered to nude mice with breast cancer xenografts resulted in increased tumor volumes, vascularization, total vessel length and branching, effects which were inhibited when naloxone, an MOR antagonist was co-administered and enhanced in the presence of opioid agonists.[7] The prevalence of MOR in the intestine links opioid peptide therapy to frequently-occurring side effects such as constipation.[6,7]

There are multiple associations between MOR and cancer. Endogenous opioids, such as β-endorphin, met-enkephalin and dynorphin A, are ligands of the μ-opioid receptor (MOR) and potent inhibitors of cell growth in both human and animal

tumors. Laboratory and animal studies have demonstrated a link between opioid receptor activation by the endogenous opioid met-enkephalin and repression of cell replication and growth during neoplasia, repression of angiogenesis and inhibition of wound healing (due to reduction in cell migration to wound site).

The MOR is also the target of exogenous opioids, such as morphine, fentanyl and heroin, which are administered clinically for pain management and anesthesia. MOR is overexpressed in human NSCLC, with a five-fold to ten-fold increase in expression in most of the cell lines representative of the disease, relative to expression in noncancerous cells.[6,8,9] When both normal and diseased tissue samples were collected from the lungs of patients with cytologically-confirmed NSCLC, the increase in MOR expression in the cancer cells was found to be approximately double that of healthy tissue. MOR also participates as a regulator of activation for the hypothalamic-pituitary-adrenal (HPA) axis. It is expressed in the paraventricular nucleus of the hypothalamus.

Lewis lung carcinoma will not form tumors when injected into MOR knockout mice and proliferates in vitro when morphine is introduced to cells derived from that form of cancer. Epidemiologic, retrospective and in vivo studies in mice have shown a connection between use of morphine for general anesthesia and a recurrence of cancer or an increase in angiogenesis.[6,7]

The δ-opioid receptor (DOR) has been found on cancerous cells in the lung, as well as normal cells including alveolar macrophages, bronchial glands, and on sensory nerve fibers within the bronchial epithelium.[5] High concentrations of DOR have also been identified in non-lung tissues such as spleen, intestine and skin. In functional studies, DOR agonists seem to modulate macrophage function such as cytokine release, chemokine production, chemotaxis, and phagocytic capacity.

The abundance of opioid receptors on lung cancer cells presents an opportunity for both diagnosis and treatment. After activation, MOR may undergo endocytosis and recycling which suggests that an MOR ligand carrying a cytotoxic drug could enter the cell and also that the receptor sites will be replenished to continue delivery of the conjugate until the cancer cell has been killed. Exploitation of an opioid antagonist for targeted drug delivery to lung cancer is based on (i) the previously mentioned over-expression of MOR in NSCLC and (ii) the ability of the μ-opioid receptor to avidly bind and internalize conjugates. The conjugates described in our paper are derivatives of naltrexone at the 6-position. We designed an amino acid linker for specific targeting of MOR and an aromatic linker for DOR with the intent of developing potential agents for imaging and treatment of lung cancer.

Naltrexone is a non-specific opioid antagonist with strong binding affinity for both the μ-opioid receptor (K_i = 0.26 nM) and δ-opioid receptor (K_i = 10.5 nM).[10] It is a marketed drug, approved for the treatment of alcohol addiction.[11] Another antagonist of interest is NAQ, which has a stronger binding affinity than naltrexone for both MOR (K_i = 0.11 nM) and DOR (K_i = 3.88 nM).[12,13] The structural relationship between Naltrexone and NAQ is shown in Figure 1.

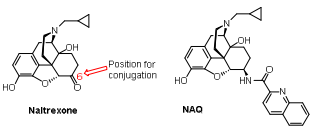
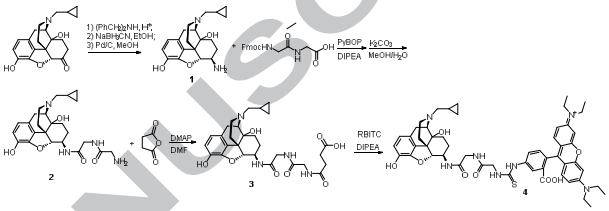


Figure 1. Structures of two opioid antagonists with strong binding affinity for both MOR and DOR

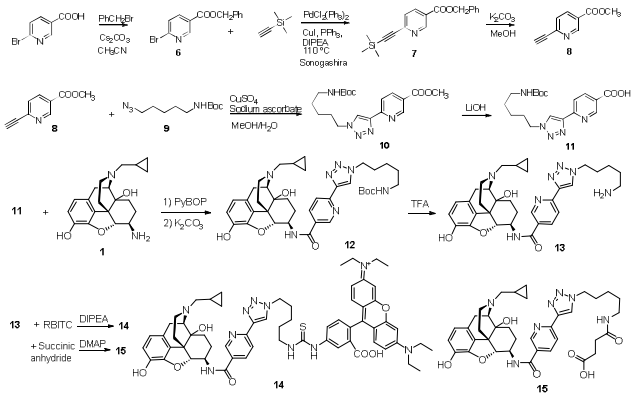
Multiple structure-activity relationship (SAR) studies of opioid antagonists have been published.[10,14] The crystal structure of the MOR receptor subtype was resolved by Manglik et al in 2012. That structure suggested that the orientation of structures attached to the 6-ketone group shown in Figure 1 would be pointing outside of the μ-opioid receptor binding pocket. The information provided by crystal structure was consistent with the results of a traditional SAR approach.[15]

It is noteworthy that naltrexone itself has been observed to influence various types of cancer. When mice were implanted with Lewis lung carcinoma cells and treated with a continuous infusion of naltrexone from implants, both the growth and metastasis were inhibited. Low, daily doses of naltrexone inhibited tumor growth in a nude mouse model of human squamous cell carcinoma by as much as 84%.[6] In the presence of naltrexone, growth of metastatic neuroblastoma in mouse was inhibited and survival rates increased.[16]



Scheme 1. Synthetic route for derivatization of naltrexone with rhodamine B

The synthesis of 6-β amino naltrexone (**1**) was achieved *via* a three step sequence in 58% yield (Scheme 1).[12,17] Then, amide coupling using PyBOP followed by Fmoc deprotection gave the amine derivative **2**. This smoothly underwent reaction with succinic anhydride to generate **3**, which in turn underwent reaction with rhodamine B isothiocyanate (RBITC) to yield rhodamine conjugate **4** (Scheme 1).



Scheme 2. Synthetic route for derivatization of compound **1** with a hetero-aromatic linker.

The intention of the next synthesis was to develop a conjugate with a hetero-aromatic linker that could be used with a DOR specific ligand. 2-bromo nicotinic acid benzyl ester **6** was first prepared by refluxing a mixture of cesium carbonate and benzyl bromide in acetonitrile. **6** then underwent Sonogashira cross coupling with ethynyltrimethylsilane, followed by protiodesilylation to give compound **8** in 68% yield over two steps.[18] Under the reaction conditions, potassium carbonate in methanol actually produced methyl ester **8** as the major product instead of the expected benzyl ester. The commercially available azide **9** underwent reaction with **8,** producing intermediate **10**, in

73% yield.[19] Following saponification of the ester, amide bond formation between **11** and **1** gave the Boc-protected amine **12**,

in and TFA deprotection, furnished primary amine **13** in 76% yield. Rhodamine conjugate **14** was then prepared I straightforward fashion by reaction between the amine **13** and rhodamine B isothiocyanate (RBITC) in DIPEA and THF. The acid intermediate **15** was also prepared from compound **13** by reaction with succinic anhydride and DAMP catalysis. Intermediate **15** and SSPS finished the synthesis of ^{99m}Tc chelating conjugate **16** with PEG linker.

Scheme 3. Solid phase synthesis of the ^{99m}Tc chelating conjugates **5** & **16**. The reagents and conditions used include the following: a) (i) 20% piperidine/DMF, rt, 10 min; (ii) Fmoc-Asp(OtBu)-OH, HBTU, HOBt, DIPEA, 3 h; b) (i) 20% piperidine/DMF, rt, 10 min; (ii) Fmoc-diaminopropionic (DAP) acid, HBTU, HOBt, DIPEA, 3 h; c) (i) 20% piperidine/DMF, rt, 10 min; (ii) Fmoc-NH-PEG$_3$-COOH, HBTU, HOBt, DIPEA, 3 h; d) (i) 20% piperidine/DMF, rt, 10 min; (ii) compound **3** or **15**, HBTU, HOBt, DIPEA, 6 h; f) TFA/H_2O/TIPS/EDT (92.5:2.5:2.5:2.5), 2 h.

The preparation of ^{99m}Tc chelating conjugates **5** and **16** were achieved with solid phase synthesis by adopting the Fmoc-Boc SPPS strategy and starting at Fmoc-Cys(Trt) Wang resin (Scheme 3 and supporting information). Compound **3** and **15** were reacted with linker in step d. The final technetium chelating conjugates were cleaved from the resin using a cocktail of 92.5% TFA/2.5 %TIPS /2.5% H_2O /2.5% 1,2-ethanedithiol. Using previously reported procedures,[20] these conjugates were formulated and chelated with ^{99m}Tc for use in the *in vitro* saturation binding assay and the *in vivo* biodistribution study. Naltrexone or Naltrindole was injected as competition for the MOR or DOR targets. Each mouse was then injected with 200 µCi (10 nmol) ^{99m}Tc conjugate by intravenous (tail vein) injection. After 4 hours, mice were sacrificed and photographed under both visible light and γ-emission camera. Organs and tissues were collected, weighed and the accumulated radioactivity quantitated. The uptake of ligand in each organ was calculated in %ID/gram unit by comparison with the value of a standard tube.

Rhodamine conjugate **4** accumulated in HEK-µ cells, while conjugate **14** exhibited uptake by HEK-δ cells. At 25 nM concentration, the uptake of conjugate **4** in HEK-µ was higher than the uptake in HEK-δ cells, suggesting more specificity for MOR (Figure1 in Supporting Information). Conjugate **14** demonstrated significant uptake at 10 nM for HEK-δ cells. Competition results, using a 100-fold higher concentration of the known highly selective DOR antagonist naltrindole, confirmed that uptake of conjugate **14** is DOR receptor mediated. After incubation for 1 h, the majority of the dye conjugates had internalized (Figure 2 in Supporting Information).. The observed endocytosis is consistent with literature reports and an indication of potential therapeutic application.

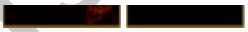


Figure 2. (a) The transfected cell line HEK-µ was treated with 25 nm of rhodamine conjugate **4** alone**,** or in competition with a 100-fold higher concentration of naltrexone. The confocal microscopy images confirm specific binding affinity for MOR. (b) The transfected cell line HEK-δ was treated with 25 nM of rhodamine conjugate **14** alone**,** or in competition with a 100-fold higher concentration of naltrindole. The confocal microscopy images confirm specific and competable binding affinity for DOR.

A biodistribution study was then performed to evaluate the distribution of the MOR and DOR conjugates *in vivo*. Gratifyingly, the MOR conjugate **5** shows specific and competable uptake in tumor, skin and intestine (Figure 3a). However, there is considerable nonspecific uptake in the kidney. The above data suggest that while conjugate **5** can target cells expressing MOR, any targeted cytotoxic therapeutic agent will also readily accumulate in the kidneys.

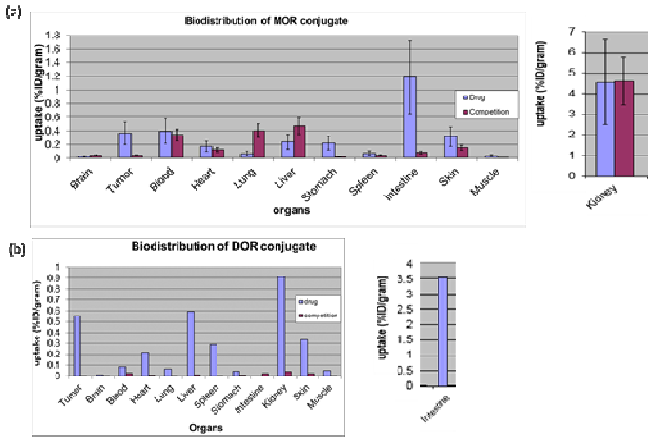


Figure 3. *In vivo* biodistribution studies of (a) MOR conjugate **5** and (b) DOR conjugate **16.**

As shown in Fig. 3b, conjugate **16** also exhibits specific uptake in the tumor, but also in the heart, liver, spleen, intestine kidney and skin. The highest specific uptake for conjugate **16** is the intestine,

consistent with published reports on the high expression of this receptor in that organ.[21-24]

The specificity of the conjugates described in this paper does not sufficiently differentiate tumors from MOR and DOR already present in healthy tissue, especially kidney and intestine. Alternate linkers might provide a different outcome during future investigations.

Acknowledgements

Funding for this research was provided by Endocyte Inc. The funding source was not involved in the study design, the conduct of the research, the writing of this report, or the decision to publish. Evaluations of conjugates by nuclear magnetic resonance were accomplished using the shared resources of the Purdue University Interdepartmental NMR Facility at the Purdue University Center for Cancer Research (PCCR). μ and δ opioid receptor transfected HEK cells (HEK-μ and HEK-δ respectively) were kindly provided by Professor Ping-Yee Law (Department of Pharmacology, University of Minnesota).

References and notes

1. Meuwissen R.; Berns A. *Genes Dev.* **2005**, *19*, 6, 643.
2. Mansour A.; Khachaturian H.; Lewis M. E.; Akil H.; Watson S. J. *Trends Neurosci* **1988**, 11, 7, 308
3. Wittert G.; Hope P.; Pyle D. *Biochem Biophys Res Commun* **1996**, 218, 3, 877.
4. Fine P.G.; Portenoy R.K. Chapter 2: The Endogenous Opioid System, in *A Clinical Guide to Opioid Analgesia*. McGraw Hill, **2004**, 9-15.
5. Krajnik M.; Schäfer M.; Sobanski P.; Kowalewski J.; Bloch-Boguslawska E.; Zylicz Z.; Mousa S. A. *Human Pathol.* **2010**, 41, 632.
6. Lennon F. E.; Moss J.; Singleton P. A. *Anesthesiology* **2012**, *116*, 4, 940.
7. Gupta K.; Kshirsagar S.; Chang L.; Schwartz R.; Law P. Y.; Yee D.; Hebbel R.P. *Cancer Res.* **2002**, *62*, 4491.
8. Mathew B.; Lennon F. E.; Siegler J.; Mirzapoiazova T.; Mambetsariev N.; Sammani S.; Gerhold L. M.; LaRiviere P. J.; Chen C. T.; Garcia J. G.; Salgia R.; Moss J.; Singleton P. A. *Anesth Analg* **2011**, 112, 3, 558.
9. Singleton P. A.; Mirzapoiazova T.; Hasina R.; Salgia R.; Moss J. *Br. J. Anaesthesia* **2014**, *113*, S1, 103.
10. Portoghese P. S.; Sultana M.; Takemori A. E. *J. Med. Chem.* **1990,** *33*, 6, 1714.
11. Vivitrol (Naltrexone) Medication Guide, approved by the U.S. Food and Drug Administration, **2010**, Alkermes, Inc. 852 Winter Street Waltham, MA 02451-1420
12. Li G.; Aschenbach L. C.; Chen J.; Cassidy M. P.; Stevens D. L.; Gabra B. H.; Selley D. E.; Dewey W. L.; Westkaemper R. B.; Zhang Y. *J. Med. Chem.* **2009,** *52*, 5, 1416.
13. Mitra P; Venitz J.; Yuan Y.; Zhang Y.; Gerk P. M. *Drug Metab Dispos,* **2011**, *39*,1589.
14. Portoghese P. S. *J. Med. Chem.* **1992**, *35*, 11, 1927.
15. Manglik A.; Kruse A. C.; Kobilka T. S.; Thian F. S.; Mathiesen J. M.; Sunahara R. K.; Pardo L.; Weis W. I.; Kobilka B. K.; Granier S. *Nature* **2012,** *485*, 321.
16. Zagon IS, McLaughlin PJ: *Cancer Lett* **1983**; *21*, 1, 89.
17. Sayre, L. M.; Portoghese, P. S. *J. Org. Chem.* **1980**, *45*, 16, 3366.
18. Wu B.; Barrios Sosa A. C. *Bioorg. Med. Chem. Lett*. **2006**, *16*, 3993.
19. Ehlers I.; Maity P.; Aubé J.; König B. *Eur .J. Org. Chem.* **2011**, 2474.
20. Reddy J. A.; Xu L. C.; Parker N.; Vetzel M.; Leamon C. P. *J. Nucl. Med.* **2004,** *45*, 857.
21. Pol O.; Palacio J.R.; Puig M.M. *J. Pharmacol. Exp. Ther.* **2003**, *306*, 455.
22. Holzer P. *Regul Peptides*, **2009**, *155*, 11.
23. Chan L.N. *Pract Gastroenterol***. 2008**, 37.
24. Fickel J.; Bagnol D.; Watsono S. J.; Akil H. *Mol. Brain Res.* **1997,** *46*, 1.

Supplementary Material

Supplementary material that may be helpful in the review process should be prepared and provided as a separate electronic file. That file can then be transformed into PDF format and submitted along with the manuscript and graphic files to the appropriate editorial office.