Summery of Ph.D. thesis

Comparative biochemical and pharmacological investigations of various newly developed opioid receptor ligands

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INTRODUCTION

Opioid system and pain

Morphine is a prototype opioid agonist binding to MOR and is still the most frequently used drug in pain medication. Beside pain relief and analgesia, it has serious side effects including decreased respiratory effort, low blood pressure and it also has a high potential for addiction and abuse. Therefore, it is very important to find new ligands with higher affinity, selectivity and stability to get more effective drugs to decrease the side effects.

An opioid epidemic is the overuse or misuse of addictive opioid drugs. It has significant medical, social and economic consequences, including overdose deaths. The proclamation of "The Opioid Crisis" by the U.S. Surgeon General began with the over-prescription of opioids in the 1990s, which are the most prescribed class of medications in the United States. Opioids administrated postoperatively or for pain management are one of the leading causes of opioid misuse, where approximately 6% of people continued opioid use after surgery or trauma. In 2017 fentanyl was the most common synthetic opioid, it was also listed by the World Health Organization as an essential drug in the form of fentanyl patches for cancer pain. Yet in 2016, the illegally used fentanyl and its analogues caused the most common overdose deaths in the United States, accounting for more than 20,000, around half of opioid-related deaths.

The three primary symptoms (which may not always be present together) for opioid toxicity are: respiratory depression, generally accompanied by depressed consciousness and miosis. Naloxone therapy is the standard treatment for opioid toxicity. Opioids that require larger doses of naloxone: codeine, methadone, diphenoxylate, propoxyphene, pentazocine, butorphanol, nalbuphine. Naloxone can precipitate withdrawal symptoms including: anxiety, irritability, and restlessness; gooseflesh; hot and cold sweats; muscle-, bone- and joint aches; tremor; nausea, vomitting and diarrhea; increased resting pulse rate.

Opioids combined with non-opioid drugs are another way to treat moderate to severe pain decreasing the risk of tolerance and dependence (*e.g.* Percodan: oxycodone/aspirin, Percocet: oxycodone/paracetamol, Vicodin: hydrocodone/paracetamol, Vicoprofen: hydrocodone/ibuprofen).

KYNA and pain

Different NMDA receptor sites were examined in the rat model of formalin-induced facial pain. The results showed that the nociceptive behavioural responses were effectively reduced by the NR2 subunits, which means that these subunits have importance in inflammatory pain diseases. Intracisternal KYNA attenuates formalin-induced nociception in animals together with antagonist activity at the glycine binding site of NMDA receptor, which is associated with analgesic properties in rats. At the peripheral sites, KYNA decreases the nociceptive behaviour in the tail flick- and hot plate tests. Administration of kyn and probenecid together with KYNA analogues inhibits NMDA receptors in animal models of trigeminal activation and sensitization. Noteworthy, KYNA and its analogues are able to act on second-order neurons, decreasing mechanical allodynia and pain sensitivity in different animal pain models.

The activation of GPR35 by KYNA, leads to hyperpolarization in the cell, which decreases glutamate and pro-inflammatory substance release from the glial and immune cells, another way of the analgesic effect of KYNA in inflammatory models. The agonist KYNA and zaprinast could inhibit the AC of DRG in an inflammatory pain model through GPR35. Probenecid injection amplified the effects of the two agonists, zaprinast was more effective in lower concentration compared to KYNA. These results suggest that the GPR35 could be a promising and novel pharmacological target for the inflammatory pain reduction.

Both kynurenine and KYNA binds to HACR3, which is then able to promote the generation of immune-suppressive T cells that support cancer development. The question is whether the KYNA-induced activation of HCAR3 and the effects, such as elevated kynurenine/KYNA levels can increase the risk of developing malignancies. Or the anti-inflammatory actions of kynurenines due to a controlled activation of HACR3 may be used for the treatment of different immune disorders. This can be another approach for the management of cancer related pain.

Bentley analogues

Natural morphine alkaloids (*e.g.* morphine, codeine, thebaine, neopine, oripavine) can be converted into a variety of pharmacologically more advantageous compounds, such as the so called nal-compounds (naloxone, naltrexone, nalbuphine) and the ring-C bridged derivatives (6,14-ethenomorphinans or Bentley-compounds, *e.g.* etorphine (**9**), buprenorphine, diprenorphine). In this study nine previously synthesized orvinol and thevinol-type MOR-selective ligands were examined (compounds **1e**, **1f**, **2a**, **2b**, **2d**, **4**, **5**, **7**, **8** (3-methoxyetorphine)). 6-*O*-desmethyl-dihydroethorphine (**2c**) is a new compound synthesized for this study.

A number of structure-activity relationship studies dealing with thevinol and orvinol derivatives are available, but the biochemical and pharmacological properties of our target compounds have not been reported except for **8**. The aim of present study was to compare the receptor binding properties and the MOR, DOR and KOR selectivity of some Bentley compounds in rat and guinea pig brain membrane preparations. The ligands were also investigated in [^{35}S]GTPγS functional binding assays to examine G-protein activation *via* opioid receptors. The effect of the investigated derivatives was observed *in vivo* nociceptive tests.

The presence or absence of specific functional groups in the orvinol and thevinol derivatives can not be straightforwardly related to their pharmacological profiles. As an example, the 17-*N*-substituent serves as an acknowledged pharmacological switch between agonists and antagonists being methyl or cyclopropylmethyl (CPM), respectively. However, it is highly ambiguous within this class of opioids, regarding that 17-*N*-CPM derivative can be full agonist as well which may be a consequence of the bigger size of these opiates resulting in a more complex interaction pattern with the receptor. According to this, it seems plausible to investigate the interacting residues or atoms of the receptor leading to the specific response, *i.e.* pharmacological feature.

Oligopeptides

Considering the presence of kyn residue in natural peptide sequences and the important role exerted by both kynurenines in the CNS, the aim was to investigate the biological consequences of the insertion of these residues in opioid pharmacophore sequences. Kyn could be used in place of phenylalanine (Phe), considering its aromatic side chain, whereas KYNA could be used as a C-terminus to mimic an additional aromatic residue. The synthesis and biological screening of six novel kynurenines containing peptides were performed, aiming to investigate the modifications imposed by the presence of kyn and KYNA on the biological properties of known endogenous and synthetic opioid peptides *in vivo* and *in vitro*. Peptide **KA1** retains the DAMGO primary sequence, but the OH terminal group is esterified by KYNA. Peptides **K2** and **K3** are EM-2 analogues in which the Phe residues in positions 3 and 4 have been replaced with kyn and kyn C-terminal amides, respectively. Peptides **K4–K6** are enkephalin-like peptides containing kyn in position 5, bearing as C-terminal the methyl ester, acid, and amide group, respectively. The novel chemical entities were prepared following solution phase peptide synthesis and were obtained as TFA salts in good overall yields and excellent purities.

AIMS OF THE STUDY

Opioid ligands still play inevitably role in the MOR-mediated central and peripheral antinociception, although their use can be very dangerous. The aim of this study is to find new ligands, which have higher maximal efficacy at lower concentration than the frequently used morphine to decrease the risk of side effects (such as dependence and tolerance) of analgesic drugs. For this purpose, 9 known and 1 new Bentley analogues, and 6 newly synthesized oligopeptides (DAMGO, EM-2 and enkephalin-like peptides containing kynurenines) are described in this thesis to find out after comparison which direction of synthesis is more expedient.

The objectives of the study presented in the thesis were the following:

- To measure the binding affinity of the 10 Bentley compounds towards MOR, DOR and KOR and the 6 new oligopeptides towards MOR, DOR, KOR and NMDA receptor in competition binding assay in rat and guinea pig brain membranes.
- To examine G-protein activation and opioid receptor mediation of the ligands in functional [^{35}S]GTPγS binding.
- To analyse the antiallodynic effects of the Bentley analogues and the oligopeptides in vivo test using inflammation models.
- To compare the final results of biochemical and pharmacological experiments of the examined opioid ligands.

METHODS

Competition binding experiments

The radioligand competition binding experiment is a type of binding assay, where radioactive ligand is applied (in most cases tritium labelled) in fixed concentrations in the presence of increasing concentrations of an unlabelled ligand. If the unlabelled ligand has specificity towards the receptor as the radioligand, The value of binding affinity (K_i) can be received about the applied unlabelled ligand.

Functional [^{35}S]GTPγS binding experiments

In the [^{35}S]GTPγS binding assays the ligand mediated G-protein activation is monitored, namely the GDP → GTP exchange of G_α, in the presence of an agonist ligand in increasing concentration. The nucleotide exchange is detected by a non-hydrolysable, radioactive GTP analogue called [^{35}S]GTPγS. Two values can be obtained from the experiments: E_{max} value (maximum efficacy) and logEC_{50} value (ligand potency) describe the agonist activity of the ligands.

In vivo nociceptive studies

Bentley analogues were examined using male Wistar rats. Osteoarthritis was induced by injecting MIA (1 mg/30 mL) into the tibiotarsal joint of the right hind leg on two consecutive days.

Oligopeptides were examined using CD-1 male mice. In the formalin test, the injection of a dilute solution of formalin (1%, 20 μL/paw) into the dorsal surface of the mouse hind paw evoked biphasic nociceptive behavioural responses.

SUMMARY OF THE RESULTS

Bentley analogues

In vitro competition binding experiments all derivatives showed low subnanomolar affinity to MOR. For DOR the ligands showed comparable binding affinities than the selective DOR agonist Ile^{5,6}-deltorphin II peptide ligand except **8** (K_i > 3000 nM). In the KOR binding assays the analogues still displayed nanomolar affinities.

In G-protein activity measurements compound **1f**, **2a**, **2b** had antagonistic; **1e**, **2c**, **8** had partial agonistic and **2d**, **4**, **5**, **7** had full agonistic effects.

Ligands were examined in G-protein activation tests in rat brain membrane, the selectivity could not be observed as the receptor selective antagonists such as Cyp, NTI, nor-BNI and the selective agonists such as DAMGO, Ile^{5,6}-deltorphine II, U-69,593 are not able to inhibit the effects of the extremely potent Bentley analogues.

In vivo tests in osteoarthritis inflammation model the thevinol derivatives showed a significant antiallodynic effect, while orvinol derivatives, except for **2c**, did not display this effect.

Oligopeptides

In competition binding assays the KYNA-containing peptide, **KA1** bound selectively to the MOR with a low K_i value and high selectivity ratio, the other oligopeptides also showed selectivity to MOR, except **K3**, which bound to MOR and DOR with similar affinity.

In the G-protein activation tests the EM-2 containing compounds, **K2** and **K3** stimulated G-protein with low efficacy, compound **KA1**, **K4**, **K5** behaved as full agonists, while **K6** had efficacy and potency higher than those of the reference compound DAMGO.

In functional binding assays all oligopeptides were inhibited by Cyp (MOR) and NTI (DOR) in rat brain membrane. In guinea pig brain membrane **K4** and **K6** stimulated G-protein, the efficacy of **K4** was inhibited by nor-BNI, while the effect of **K6** was not.

K6 exhibited a strong antinociceptive effect in formalin test.

LIST OF PUBLICATIONS

This thesis is based on the following publications:

I. **Edina Szűcs**, János Marton, Zoltán Szabó, Sándor Hosztafi, Gabriella Kékesi, Gábor Tuboly, László Bánki, Gyöngyi Horváth, Pál T. Szabó, Csaba Tömböly, Zsuzsanna Varga, Sándor Benyhe, Ferenc Ötvös (2020) **Synthesis, biochemical, pharmacological characterization and in silico profile modelling of highly potent opioid orvinol and thevinol derivatives.** EUR. J. MED. CHEM., 191:1121-45.

(5.572 impact factor, Q1)

II. **Edina Szűcs**, Azzurra Stefanucci, Marilisa Pia Dimmito, Ferenc Zádor, Stefano Pieretti, Gokhan Zengin, László Vécsei, Sándor Benyhe, Adriano Mollica (2020) **Discovery of Kynurenines containing oligopeptides as potent opioid receptor agonists.** BIOMOLECULES, 10:1-18.

(4.082 impact factor, Q1)

Sum of the impact factors related to the thesis: 9.654

Other thematic publications not directly connected to this thesis:

1. **Szűcs, E.**, Büki, A., Kékesi, G., Horváth, G., Benyhe, S. (2016) **Mu-Opioid (MOP) receptor mediated G-protein signaling is impaired in specific brain regions in a rat model of schizophrenia.** NEUROSCI. LETTERS, 619: 29-33.

2. Monti, L., Stefanucci, A., Pieretti, S., Marzoli, F., Fidanza, L., Mollica, A., Mirzaie, S., Carradori, S., De Petrocellis, L., Schiano Moriello, A., Benyhe, S., Zádor, F., **Szűcs, E.**, Ötvös, F., Erdei, A.I., Samavati, R., Dvorácskó, S., Tömböly, C., Novellino, E. (2016) **Evaluation of the analgesic effect of 4-anilidopiperidine scaffold containing ureas and carbamates.** J. ENZYME INHIB. MED. CHEM., 31: 1638-47.

3. **Szűcs, E.**, Dvorácskó, S., Tömböly, C., Büki, A., Kékesi, G., Horváth, G., Benyhe, S. (2016) **Decreased CB receptor binding and cannabinoid signaling in three brain regions of a rat model of schizophrenia.** NEUROSCI. LETTERS, 633: 87-93.

4. Mollica, A., Pelliccia, S., Famiglini, V., Stefanucci, A., Macedonio, G., Chiavaroli, A., Orlando, G., Brunetti, L., Ferrante, C., Pieretti, S., Novellino, E., Benyhe, S., Zador, F., Erdei, I.A., **Szűcs, E.**, Samavati, R., Dvorácskó, S., Tömböly, C., Ragno, R., Patsilinakos, A., Silvestri, R. (2017) **Exploring the first Rimonabant analog-opioid peptide hybrid compound, as bivalent ligand for CB1 and opioid receptors.** J. ENZYME INHIB. MED. CHEM., 32: 444-451.

5. Zádor F, Balogh M, Váradi A, Zádori S Z, Király K, **Szűcs E**, Varga B, Lázár B, Hosztafi S, Riba P, Benyhe, S, Fürst S, Al-Khrasani M. (2017) **14-O-Methylmorphine: A Novel selective mu-opioid receptor agonist with high efficacy and affinity.** EUR. J. PHARMACOL., 814: 264-273.

6. Samavati, R., Zádor, F., **Szűcs, E.**, Tuka, B., Martos, D., Veres, G., Gáspár, R., Mándity, I.M., Fülöp, F., Vécsei, L., Benyhe, S., Borsodi, A. (2017) **Kynurenic acid and its analogue can alter the opioid receptor G-protein signaling after acute treatment via NMDA receptor in rat cortex and striatum.** J. NEUROL. SCI., 376: 63-70.

7. Stefanucci, A., Novellino, E., Mirzaie, S., Macedonio, G., Pieretti, S., Minosi, P., **Szűcs, E.**, Erdei, A.I., Zádor, F., Benyhe, S., Mollica, A. (2017) **Opioid receptor activity and analgesic potency of DPDPE peptide analogues containing a xylene bridge.** AMER. CHEM. SOC. MED. CHEM. LETTERS, 8: 449-454.

8. Stefanucci A, Carotenuto A, Macedonio G, Novellino E, Pieretti S, Marzoli F, **Szűcs E**, Erdei AI, Zádor F, Benyhe, S., Mollica A. (2017) **Cyclic biphalin analogues incorporating a xylene bridge: Synthesis, characterization, and biological profile.** AMER. CHEM. SOC. MED. CHEM. LETTERS, 8: 858-863.

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13. Piotr F. J. Lipiński, **Edina Szűcs**, Małgorzata Jarończyk, Piotr Kosson, Sándor Benyhe, Aleksandra Misicka, Ján Cz. Dobrowolski, Joanna Sadlej (2019) **Affinity of fentanyl and its derivatives for the σ1 receptor.** MED. CHEM. COMM., 10: 1187 –1191.

14. Ferenc Zádor, Gábor Nagy-Grócz, Gabriella Kékesi, Szabolcs Dvorácskó, **Edina Szűcs**, Csaba Tömböly, Gyöngyi Horváth, Sándor Benyhe, László Vécsei (2019) **Kynurenines and the Endocannabinoid System in Schizophrenia: Common Points and Potential Interactions.** MOLECULES, 24: 3709.

15. Ferenc Zádor, Gábor Nagy-Grócz, Szabolcs Dvorácskó, Zsuzsanna Bohár, Edina Katalin Cseh, Dénes Zádori, Árpád Párdutz, **Edina Szűcs**, Csaba Tömböly, Anna Borsodi, Sándor Benyhe, László Vécsei (2020) **Long-term systemic administration of kynurenic acid brain region specifically elevates the abundance of functional CB1 receptors in rats.** NEUROCHEM. INT., 138:1047-52.

16. **Edina Szűcs**, Eszter Ducza, Alexandra Büki, Gabriella Kékesi, Sándor Benyhe, Gyöngyi Horváth (2020) **Characterization of dopamine D2 receptor binding, expression and signaling in different brain regions of control and schizophrenia-model Wisket rats.** BRAIN RESEARCH, 1748:147-74.

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