GLYCINE TRANSPORTER INHIBITORS IN MANAGEMENT OF NEUROPATHIC PAIN

Ph.D. Thesis

Amir Mohammadzadeh

Doctoral school of Pharmaceutical Sciences
Semmelweis University

Supervisor: Mahmoud Al-Khrasani, Pharm.D., Ph.D.

Official reviewers: Kornélia Tekes, Pharm.D., D.Sc.

Eszter Ducza, Pharm.D., Ph.D.

Head of the Complex Examination Committee:

Éva Szökő, Pharm.D., D.Sc.

Members of the Complex Examination Committee:

Péter Petschner, Pharm.D., Ph.D.

Gábor Pozsgai, M.D., Ph.D.

Budapest 2022

1.Introduction

The research to find better solutions for management of neuropathic pain is an ongoing process and yet most of compounds to cure this defect are insufficient or their benefits are limited by their adverse reactions. Pain signal transmits from peripheral primary afferent neurons (Aδ and C fibres) through the dorsal horns of spinal cord to secondary afferent fibres and then to higher centres such as reticular formation and thalamus and somatosensory cortex. However, this transmission is not a simple one-way passage, but spinal interneuronal interactions in lamina II and III modify the level and intensity of pain transmission. Spinal inhibition is mainly by GABA-ergic and glycinergic interneurons, while the excitation is by glutamergic interneurons. In healthy conditions there is a balance between inhibition and excitation. However, in neuropathic pain by reduction in glycinergic inhibition and increase in glutamergic excitation, this balance is lost in favour of the later, causing allodynia (pain sensation from innocuous stimulus) and hyperalgesia (pain sensation with higher intensity than its normal range).

In this regard, potentiation of glycinergic pathway by application of reversible and irreversible inhibitors of glycine transporter (GlyT) type 1 and 2 is a new approach to reduce the pain in research studies. Antinociceptive effects of many of these compounds have been shown in many studies. In regard to pain research the following irreversible selective GlyT-1 inhibitors have been thoroughly studied: NFPS (also known as ALX5407), Org-25935, bitopertin and N-ethylglycine. Irreversible GlyT-2 inhbitors such as ALX1393 and Org-25543 were also studied in different animal pain models for their analgesic effects. Examples of reversible GlyT-1 inhibitor is sarcosine (N-methyl glycine), and example of reversible GlyT-2 inhibitor is Narachidonylglycine (NAGly). On the other hand, observation of motor and respiratory side

effects from high doses have brought an objection for clinical use of these compounds. For example, treatment with high doses of 10-30 mg/kg (i.p.) of NFPS was associated with akathisia and respiratory depression in mice. Also, severe side effects such as convulsions and lethality have been associated with dose escallation of Org-25543 (20 mg/kg i.v.).

As shown by the above-mentioned studies, both GlyT-1 and 2 inhibitors display analgesic effect, however, to the best of our knowledge no study has examined the analgesic effect of a combination of irreversible GlyT-1 and GlyT-2 inhbitors. Therefore, the strategy based on the co-administration of sub-analgesic doses of these inhibitors might increase the analgesic effect with possible reduced occurrence of side effects.

2.Objectives

The aims of the study are as follows: **1)** Evaluation of analgesic effect of acute and chronic systemic administration of reversible GlyT-1 inhibitor (sarcosine 500 and 1000 mg/kg) in different pain models (acute thermal pain, acute inflammatory pain, and mono-neuropathic pain). **2)** Evaluation of anti-allodynic effects of acute and chronic systemic administration of irreversible GlyT-1 inhibitor (NFPS 1, 2, 4 mg/kg) and irreversible GlyT-2 inhibitor (Org-25543 2, 4 mg/kg), and the acute systemic co-administration of sub-analgesic doses of both GlyT-1 and 2 inhibitors (NFPS 1 mg/kg and Org-25543 2 mg/kg), in mono-neuropathic pain model. **3)** Examination of motor coordination and balance for all test compounds. **4)** Evaluation of glycine release in hippocampus tissue by sarcosine, NFPS and ACPPB (irreversible GlyT-1 inhibitor) **5)** Investigating the glycine content in cerebrospinal fluid (CSF) of animals treated by NFPS and Org-25543, or their combination, and spinal tissue **6)** Assessment of G-protein activity of sarcosine, NFPS and Org-25543

3.Methods

3.1. Animals

For experiments male Wistar rats (250-300 g) were used (for G-protein binding assay both male and female Wistar rats were used). Treatments for in vivo studies were subcutaneous (s.c.) in volume of 2.5 ml/kg. All housing and experiments were performed in accordance with the European Communities Council Directives (2010/63/EU), the Hungarian Act for the Protection of Animals in Research (XXVIII.tv. 32.§) and local animal care committee (PEI/001/276-4/2013 and PE/EA/619-8/2018).

3.2. Assessment of acute thermal pain

For the assessment of acute thermal pain tail-flick test was used. In these tests a beam of light was focused on the dorsal root of the tail and time latency (seconds) of tail-flick was noted (cut-off: 8s). Two doses of sarcosine (500 and 1000 mg/kg s.c.) were assessed after acute and chronic treatments.

$$\text{Analgesia (\%)} = \frac{\text{Latency after treatment} - \textit{Latency before treatment}}{\textit{Latency before treatment}} \text{x } 100$$

3.3. Assessment of acute inflammatory pain

For assessment of acute inflammatory pain formalin test was applied. In this test animals were treated with sarcosine (500 mg/kg s.c) and after a time lag (15' or 180'), formalin (2.5%, 20 µL i.pl) was injected into the right hind paws and immediately the number of nociceptive reactions were counted in each 5' for duration of 60'. Formalin test has 2 phases. Phase 1 is in the first 10' which is related to peripheral acute pain, and phase 2 is from 10' to 60' which is related to peripheral and central inflammatory pain. Both acute and chronic treatments were evaluated.

3.4. Assessment of mono-neuropathic pain

For the induction of the mono-neuropathic pain, partial sciatic nerve ligation (pSNL) was used. In this method animals undergone standard anesthesia with 60 mg/kg (i.p.) pentobarbital (2.5 ml/kg volume) and sciatic nerve of right legs were ligated partially. Sham group was without any nerve ligation. 14 days after operation the main experiment started for acute and chronic treatments. To assess mechanical allodynia, paw withdraw threshold (PWT) was measured in grams (g) by DPA (dynamic plantar aesthesiometer 37450; Ugo Basil, Italy). In this method, following 5' habituation, centres of paws were pressed by a metal filament of 0.5 mm diameter with a force rising from 1 to 50 g (cut-off). Each paw was measured three times in each evaluation and the averages were used for further analysis. Acute or chronic treatments (sarcosine 500 and 1000 mg/kg, NFPS 1, 2 and 4 mg/kg, Org-25543 2 and 4 mg/kg, co-administration of NFPS 1 mg/kg and Org-25543 2 mg/kg) were tested.

3.5. Assessment of motor coordination and balance

To assess motor coordination, rotarod test was used (rat rotarod, Model 7750; Ugo Basile). In this method male Wistar rats (170-250g) were located on the rotating cylinder and the fall-off time was noted in seconds (speed: 16 rpm, cut-off: 180 s). Training of animals was one day before test. All test compounds were investigated by this method.

3.6. Assessment of glycine efflux

Slices of the hippocampus were prepared from rat brains, loaded with [^{3}H]glycine and superfused with preheated (37^{o}C) and aerated (95% O2/5% CO2) Krebs-bicarbonate buffer at a rate of 1 ml/min. [^{3}H]glycine release was determined in 22 collected 3-min fractions and expressed as a fractional rate. GlyT-1 inhibitors sarcosine, NFPS and ACPPB were investigated in this experiment.

3.7. Assessment of glycine and L-glutamate content in cerebrospinal fluid (CSF) and spinal cord tissue

Evaluation of glycine and L-glutamate content was performed by capillary electrophoresis-laser induced fluorescence on CSF and spinal cord tissue (Lumbar 4-6). For sampling, animals with mono-neuropathic pain, after acute treatments with low doses of NFPS, Org-25543 or their combination or vehicle (based on the protocol of section 3.4.) were used. Sham group was considered as the control. Samples were deproteinized by acetonitrile and were centrifuged at 20000 g for 10 minutes at 4°C. Supernatants were diluted five times and they were derivatized with NBD-F in buffer pH 8.5 for 20 minutes at 65°C. 5 µM L-cysteic acid was the internal standard. Measurement was performed by a P/ACE MDQ Plus capillary electrophoresis system coupled with laser induced fluorescence detector equipped (SCIEX, Framingham, MA, USA).

3.8. Assessment of G-Protein activity

Spinal cord samples were homogenized in ice-cold TEM (50 mM Tris-HCl, 1 mM EGTA, 3 mM $MgCl_2$, 100 mM NaCl), centrifuged (18,000 rpm) for 20' at 4°C and supernatant removed and pellet was incubated at 37°C for 30' in a shaking water-bath then centrifugation was repeated and final pellet was poured in ice-cold TEM pH 7.4 buffer and kept at -80 °C The functional [^{35}S]GTPγS binding assay is when the GDP → GTP exchange of the $G_{\alpha i/o}$ protein is in the presence of a given radioactive, non-hydrolysable GTP analogue[^{35}S]GTPγS. Incubation of homogenates was at 30°C for 60' in TEM buffer with 20 MBq/0.05 ml [^{35}S]GTPγS (0.05 nM) and compounds in excess of GDP (30 µM) in a final volume of 1 mL. Termination of reaction was with rapid filtration under vacuum and washing with Tris-HCl pH 7.4 buffer through Whatman GF/B glass fibers. Measurements were with UltimaGold™ MV aqueous scintillation cocktail with Packard Tricarb 2300TR liquid scintillation counter.

3.9. Statistical analysis

Statistical analysis applied by GraphPad Prism 6.0 (GraphPad Software Inc., San Diego, CA, USA). Data were presented in ± SEM. For comparisons, vehicle treated group for in vivo studies, or data of sham group or basal activity for in vitro studies, were used as control. The differences were considered significant if P value was less than 0.05.

4.Conclusions

The result of our experiments can be summarised as it follows:

- Application of systemic sarcosine produced analgesia against acute thermal pain in rat tail-flick test, only after chronic treatment with the high dose (1000 mg/kg)
- Injection of acute or chronic sarcosine (500 mg/kg) systemically, failed to subside acute inflammatory pain in formalin test.
- Acute and chronic systemic administration of sarcosine (500 and 1000 mg/kg) subsided allodynia, however the analgesic efficacy of the lower dose was more pronounced.
- Sarcosine (a substrate GlyT-1 inhibitor) increased glycine efflux from rat hippocampus tissue while ACPPT and NFPS (none-substrate GlyT-1 inhibitors) did not show similar result.
- Rats treated with NFPS or Org-25543, irreversible GlyT-1 and 2 inhibitors respectively, showed anti-allodynic effect only in the higher acute doses (4 mg/kg). The chronic treatment with low dose of NFPS (1 mg/kg s.c.) also increased the baseline values of allodynia in neuropathic animals.
- Acute systemic co-administration of sub-analgesic doses of GlyT-1 and 2 inhibitors (NFPS 1 mg/kg and Org-25543 2 mg/kg) on neuropathic rats alleviated pain significantly.

- The test compounds (sarcosine, NFPS, Org-25543, combination of NFPS and Org-25543) and their corresponding vehicles, did not disturb motor coordination and balance of rats in rotarod test.
- Data from capillary electrophoresis measurement showed the elevation of L-glutamate in CSF samples from neuropathic animals, which was not changed by the combination of NFPS and Org-25543. On the other hand, only the combination of these two compounds raised the glycine level significantly compared to sham group. In addition, no changes were observed in glycine or L-glutamate content of spinal tissues of sham or neuropathic rats.
- Finally, based on our G-protein activity assay, we can exclude the G-protein coupled activation abilities of either of the applied compound.

In conclusion, systemic co-administration of sub-analgesic doses of GlyT-1 and 2 inhibitors, enhanced acute anti-allodynia in neuropathic rats, without any motor dysfunction. This combination increased the CSF glycine content significantly, which suggests the induction of glycinergic inhibition in spinal cord. The novel strategy of combination of sub-analgesic doses of GlyT-1 and 2 inhibitors by potentiation of efficacy and avoiding adverse reactions, might be a solution for future clinical application of these compounds in treatment of neuropathic pain.

5.Acknowledgements

I would like to thank my supervisor Dr. Mahmoud Al-Khrasani for his help and support to conduct this project and to Prof. Laszlo G. Harsing for his guidance. I thank Prof. Péter Ferdinandy, head of the Department of Pharmacology and Pharmacotherapy. I would like to thank Dr. Zoltán S. Zádori, Dr. Kornél Király, Dr. Pál Riba, Dr. Tamás Tábi, Dr. Péter P. Lakatos, Dr. Ferenc Zádor, Dr. Mihály Balogh, Dr. Dávid Árpád Karádi,

Ms. Anna Rita Galambos, Ms. Szilvia Barsi for their help and support. I would also like to thank Semmelweis University Doctoral School for the support and coverage of this research study.

6.Bibliography

Author's publications related to this study:

1. Mohammadzadeh A, Lakatos PP, Balogh M, Zádor F, Karádi DÁ, Zádori ZS, Király K, Galambos AR, Barsi S, Riba P, Benyhe S, Köles L, Tábi T, Szökő É, Harsing LG Jr, Al-Khrasani M. (2021) Pharmacological Evidence on Augmented Antiallodynia Following Systemic Co-Treatment with GlyT-1 and GlyT-2 Inhibitors in Rat Neuropathic Pain Model. Int J Mol Sci. 22(5):2479. **IF: 4,556**

DOI: https://doi.org/10.3390/ijms22052479

2. Al-Khrasani M, Mohammadzadeh A, Balogh M, Király K, Barsi S, Hajnal B, Köles L, Zádori ZS, Harsing LG Jr. (2019) Glycine transporter inhibitors: A new avenue for managing neuropathic pain. Brain Res Bull.152:143-158. **IF: 3,371**

DOI: https://doi.org/10.1016/j.brainresbull.2019.07.008

Author's publications unrelated to this study:

1. Zádor F, Mohammadzadeh A, Balogh M, Zádori ZS, Király K, Barsi S, Galambos AR, László SB, Hutka B, Váradi A, Hosztafi S, Riba P, Benyhe S, Fürst S, Al-Khrasani M. (2020) Comparisons of In Vivo and In Vitro Opioid Effects of Newly Synthesized 14-Methoxycodeine-6-*O*-sulfate and Codeine-6-*O*-sulfate. Molecules. 25(6):1370. **IF: 3,267**
DOI: https://doi.org/10.3390/molecules25061370

2. Balogh M, Zádor F, Zádori ZS, Shaqura M, Király K, Mohammadzadeh A, Varga B, Lázár B, Mousa SA, Hosztafi S, Riba P, Benyhe S, Gyires K, Schäfer M, Fürst S, Al-Khrasani M. (2019) Efficacy-Based Perspective to Overcome Reduced Opioid Analgesia of Advanced Painful Diabetic Neuropathy in Rats.Front Pharmacol.10:347. **IF: 4,225**
DOI: https://doi.org/10.3389/fphar.2019.00347

3. Fürst S, Zádori ZS, Zádor F, Király K, Balogh M, László SB, Hutka B, Mohammadzadeh A, Calabrese C, Galambos AR, Riba P, Romualdi P, Benyhe S, Timár J, Schmidhammer H, Spetea M, Al-Khrasani M. (2020) On the Role of Peripheral Sensory and Gut Mu Opioid Receptors: Peripheral Analgesia and Tolerance.Molecules, 25(11):2473.**IF: 3,267** DOI:https://doi.org/10.3390/molecules25112473

4. László SB, Lázár B, Brenner GB, Makkos A, Balogh M, Al-Khrasani M, Hutka B, Mohammadzadeh A, Kemény Á, László T, Scheich B, Szabados T, Kenyeres É, Giricz Z, Bencsik P, Varga ZV, Novák J, Helyes Z, Ferdinandy P, Gyires K, Zádori ZS. (2020) Chronic treatment with rofecoxib but not ischemic preconditioning of the myocardium ameliorates early intestinal damage following cardiac ischemia/reperfusion injury in rats. Biochem Pharmacol. 178:114099. **IF: 4,96** DOI: https://doi.org/10.1016/j.bcp.2020.114099

5. Lázár B, László SB, Hutka B, Tóth AS, Mohammadzadeh A, Berekméri E, Ágg B, Balogh M, Sajtos V, Király K, Al-Khrasani M, Földes A, Varga G, Makra N, Ostorházi E, Szabó D, Ligeti B, Kemény Á, Helyes Z, Ferdinandy P, Gyires K, Zádori ZS. A comprehensive time course and correlation analysis of indomethacin-induced inflammation, bile acid alterations and dysbiosis in the rat small intestine. Biochem Pharmacol. 2021 May 1.190:114590. **IF: 4,960**
DOI: https://doi.org/10.1016/j.bcp.2021.114590

The cumulative impact factor: 28,605