TOLPERISONE-PREGABALIN-BASED APPROACH FOR NEUROPATHIC PAIN MANAGEMENT AND MORPHINE TOLERANCE

Ph.D. thesis

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1. Introduction

Neuropathic pain (NP) is a debilitating disease, and its treatment has not been fully solved thus far. The International Association for the Study of Pain (IASP) defined NP as pain resulting from damage or diseases affecting the somatosensory nervous system at the peripheral or central nervous system (CNS) or both. NP presents with a variety of symptoms, including two distinct manifestations: allodynia and hyperalgesia. According to estimates, the incidence of NP varies between 7% and 10% worldwide. However, this prevalence rises to approximately 20% to 30% in diabetic patients. NP treatment is challenging due to complex symptoms, poor outcomes, and difficult treatment decisions. Developing non-addictive novel medications and treatment approaches or repurposing existing medications with adequate analgesia of fast onset and tolerable side effects is a profound challenge in pain research. Using multimodal analgesia containing two or more drugs at sub-analgesic doses may increase efficacy and decrease side effects compared to using single-drug therapy. Voltage-gated sodium channels (VGSCs) and voltage-gated calcium channels (VGCCs), specifically the $\alpha_2\delta$ hosting subtype, have been studied extensively in relation to NP. The current treatment approach for NP includes topical and systemic medications targeting VGSCs and systemic medications targeting VGCCs, which are considered third and first-line options, respectively. Repurposing drugs with mechanisms based on the inhibition of VGSCs alone or alongside VGCCs is of interest in the context of managing NP. Thus, our choice fell on tolperisone (TOLP), a centrally acting skeletal muscle relaxant known for its significant blocking effect on VGSCs. In the present scenario, TOLP or pregabalin (PGB), as VGSC or VGCC blockers, respectively, were selected for the purpose of this study to investigate their impact against mono-neuropathic pain (mono-NP) when administered either alone or in combination. Other drugs, such as duloxetine (DUL) and carbamazepine (CBZ), were assessed similarly. The current management of polyneuropathic pain, specifically the diabetic type, has not provided a satisfactory outcome. Since VGCC blockers are among the first-line treatments, promising combinations based on multiple targets may provide satisfactory pain management.

Opioid analgesic tolerance is a barrier to continuing opioid treatment in the context of long-term treatment. To circumvent it requires dose escalation, which is associated with worsening opioid analgesics' side effects. Previous studies have shown that VGSC or

VGCC blockers could delay the development of morphine (Morph) antinociceptive tolerance. This provides justification for assessing the impact of TOLP and PGB on the development of opioid antinociceptive tolerance.

2. Objectives

1. To evaluate the anti-tactile allodynic effects of acute and long-term oral treatment with TOLP, PGB, CBZ, and DUL in rats with mono-NP induced by partial sciatic nerve ligation (pSNL).
2. To evaluate the acute anti-tactile allodynic effects of sub-analgesic doses of oral TOLP combined with oral PGB, oral DUL, or subcutaneous Morph in rats with mono-NP induced by pSNL.
3. To further investigate the acute antiallodynic effects of the promising combination in a rat model of type 1 diabetes-induced polyneuropathic pain (induced by streptozotocin).
4. To assess the impact of TOLP versus PGB on Morph antinociceptive tolerance in the rat tail-flick test.
5. To assess the impact of the promising combination (TOLP/PGB) on motor function and gastrointestinal (GI) transit in rats.
6. To decipher how the potential combination and its constituent drugs produce their antiallodynic effects, the following measurements were taken:
    - 6.a. Quantifying glutamate content in cerebrospinal fluid (CSF) of rats with mono-NP and treated with TOLP, PGB, vehicle, or TOLP/PGB combination.
    - 6.b. Evaluation of the effect of TOLP, PGB, or their combination on glutamate release from rat brain synaptosomes induced by 4-aminopyridine.
    - 6.c. Determining μ opioid receptors (MOR) protein levels in spinal cord tissue from diabetic neuropathic rats by Western blot.
    - 6.d. Determining D-serine and glycine levels in CSF samples from tolerant rats by capillary electrophoresis.
    - 6.e. Assessing the efficacy (E_{max}) of the TOLP, PGB, and reference compounds (DAMGO, a highly MOR selective peptide, and morph) in vitro in the mouse vas deferens (MVD) assay.
    - 6.f. Assessing the effect of TOLP and PGB or Morph combinations in MVD.

6.g. Assessing the impact of TOLP or PGB on MVD developing tolerance to Morph.

3. Methods

3.1. Partial Sciatic Nerve Ligation

Male Wistar rats (120-150 g) were used to evoke a mono-NP model by the pSNL. The development of tactile allodynia was assessed using the dynamic plantar aesthesiometer (DPA). Next, the acute (at day 7) and chronic effects (on days 14 and 21) of PGB and TOLP (both at 25, 50, and 100 mg/kg), CBZ (16.25, 32.5, and 65 mg/kg), and DUL (10 and 20 mg/kg) on tactile allodynia were assessed. On another set of animals, the acute antiallodynic effect of the PGB and TOLP (both at 25, 50, and 100 mg/kg), CBZ (16.25, 32.5, and 65 mg/kg), and DUL (10 and 20 mg/kg) as well as TOLP/PGB combination (both at 25 mg/kg), the TOLP/DUL combination (25 mg/kg + 20 mg/kg), and TOLP/Morph combination (25 mg/kg + 3.22 mg/kg), on tactile allodynia were assessed at day 14 post-operation. The CSF glutamate content was determined by capillary electrophoresis. In addition, the effect of the tested drugs on glutamate release from rat brain synaptosomes was also assessed.

3.2. Animal Model of Type 1 Diabetes-Induced Polyneuropathic Pain

Type 1 diabetes was induced by intraperitoneal injection of a single large dose of streptozotocin (60 mg/kg). After 9 weeks, diabetic neuropathic rats were selected and received either the vehicle or the drugs (25 mg/kg PGB, 25 mg/kg TOLP, or their combination) to assess their acute antiallodynic effect measured at 60 and 120 min post single oral administration. Then, the impact of the tested drugs on the spinal cord MOR protein level was assessed by Western blot analysis.

3.3. Morph Antinociceptive-Tolerance Model

Male Wistar rats weighing 170-200 g were used to evaluate the effect of co-administering TOLP (oral, 100 mg/kg, twice daily, 10 days) or PGB (oral, 100 mg/kg, twice daily, 10 days) on the development of the Morph antinociceptive tolerance (s.c., 10 mg/kg Morph alone was used as a positive control). Then, D-serine and glycine content in CSF samples were evaluated by capillary electrophoresis.

3.4. Isolated Mouse Vas Deferens Assay

In brief, MVD experiments used 35–45 g male NMRI mice of 6–10 weeks of age. Vasa deferentia were separated, taken out of their sheaths, and put in 5 ml organ baths containing Krebs solution aerated with a mixture of 95% O_2+ 5% CO_2 right away. They were then suspended between two electrodes; the lower one is straight, while the upper one is ring-shaped. MVD experiments were used to assess the E_{max} of the TOLP and PGB compared to the reference compounds, DAMGO and Morph. Next, the effect of TOLP and PGB or Morph combinations was tested. Finally, the impact of TOLP or PGB on MVD developing tolerance to Morph was also assessed.

3.5. Motor Function Test in Naïve Rats

The rat rotarod test was used to assess the effect of TOLP (100 and 150 mg/kg), PGB (25, 50, and 100 mg/kg), and the TOLP/PGB combination (both at 25 mg/kg), or vehicle, at 60 and 120 min after acute oral treatment in naïve male Wistar rats. The fall-off time, or the latency time, was used to indicate motor coordination.

3.6. Determination of Gastrointestinal Peristalsis in Naïve Rats

The charcoal meal test was used to assess the effect of TOLP (25 and 50 mg/kg), PGB (25 and 50 mg/kg), or the TOLP/PGB combination, both at 25 mg/kg, on GI transit in naïve male Wistar rats. An oral charcoal suspension was prepared (10% charcoal in 5% gum Arabic) and given via oral gavage 30 min post-oral drug administration in a volume of 2 mL/animal. After another 30 minutes, animals were sacrificed, and the whole small intestines were taken. Then, the charcoal travel distance was measured and compared with the whole small intestinal length.

3.7. Statistical Analysis

Data were statistically analyzed using GraphPad Prism 8.0 Software (San Diego, CA, USA). Values were presented as mean ± standard error of means (S.E.M.). Data were analyzed by one-way or two-way ANOVA followed by Dunnett's or Tukey's post-hoc test for multiple comparisons. Data were analyzed by unpaired t-test for Figure 9, panels (a-c, e). Significant differences were considered if $p < 0.05$. ROUT analysis was done to find outliers, with a Q value = 0.5%.

4. Results

4.1. Chronic Oral TOLP and PGB Treatments Reduce Tactile Allodynia in Rats with Mononeuropathic Pain

On day 7 after the operation, oral TOLP, PGB, DUL, and CBZ failed to alleviate the developed tactile allodynia in rats with mono-NP evoked by pSNL. Oral TOLP (100 mg/kg) and PGB (50 mg/kg) produce significant anti-tactile allodynic effects only after 2 weeks of chronic treatment in the same NP rat model. However, oral DUL and CBZ failed to produce anti-tactile allodynic effects either after acute or chronic treatment.

4.2. Acute Oral Co-Administration of TOLP and PGB Alleviates Tactile Allodynia Post-pSNL in Rats on Day 14, Unlike DUL or Morph.

Surprisingly, acute oral co-administration of TOLP with PGB but not with DUL or Morph alleviates tactile allodynia evoked by pSNL in rats on day 14 post-operation. In addition, in this series of experiments, rats with mono-NP displayed an elevation in the CSF glutamate content. Acute administration of per os TOLP, PGB, or their combination significantly decreased the elevated glutamate content. Furthermore, TOLP per se, or TOLP/PGB combination, inhibited the 4-aminopyridine-induced glutamate release from rat brain synaptosomes.

4.3. The Impact of TOLP and PGB Combination on an Animal Model of Type 1 Diabetes-Induced Polyneuropathic Pain

Nine weeks after streptozotocin injection and the establishment of allodynia, the tested drugs (TOLP and PGB) at 25 mg/kg and their combination were evaluated for their anti-tactile allodynic effects following acute oral administration. Of the given treatments, only PGB treatment significantly alleviated the tactile allodynia 120 min post-administration. Moreover, only acute treatment with PGB resulted in a significant elevation of spinal cord MOR protein levels in rats with peripheral diabetic polyneuropathy..

4.4. The Impact of PGB or TOLP on Delaying the Development of Morph Antinociceptive Tolerance

PGB, in contrast to TOLP, significantly delays the development of Morph antinociceptive tolerance when administered concurrently, as evidenced in the rat tail-flick assay. In the CSF samples from rats treated with PGB and Morph, the level of D-serine was

significantly decreased compared to the level of D-serine obtained from the vehicle-treated group. On the other hand, the level of glycine did not change after the aforementioned treatments.

4.5. PGB and TOLP Attenuate the Developed Morph Tolerance in Isolated Mouse Vas Deferens

TOLP, PGB, Morph, or DAMGO, in a concentration-dependent manner, inhibited the electrically evoked MVD smooth muscle contractions. The measured E_{max} for TOLP, PGB, Morph, or DAMGO was 84.85, 36.57, 74.01, or 91.06 %, respectively. TOLP and PGB combination produces a more potent inhibitory effect than TOLP or PGB per se in isolated MVD. With regard to Morph tolerance, both PGB and TOLP restore the developed Morph tolerance in isolated MVD**.**

4.6. The Effect of PGB, TOLP, and PGB/TOLP Combination on Motor Coordination and Balance in Naïve Rats

Acute oral treatment with either 100 mg/kg or 150 mg/kg of TOLP and 25 mg/kg of PGB failed to affect motor coordination and balance. On the other hand, rats treated with 50 mg/kg and 100 mg/kg doses of PGB exhibited motor dysfunction and coordination imbalance. Furthermore, treatment with the oral TOLP/PGB combination (both at 25 mg/kg) did not alter the rats' motor coordination and balance compared to the vehicle-treated rats.

4.7. The Impact of PGB, TOLP, and PGB/TOLP Combination on Gastrointestinal Transit in Naïve Rats

Treatment with 25 and 50 mg/kg of TOLP, 25 mg/kg of PGB, and the TOLP/PGB combination, both at 25 mg/kg, didn’t elicit any delay in GI transit after oral charcoal suspension in naïve rats. On the other hand, acute treatment with 50 mg/kg of PGB induced a moderate delay in the GI transit in rats compared to the vehicle group, albeit such delay was statistically significant.

5. Conclusions

1. Like PGB, the onset of the antiallodynic effect of TOLP requires chronic treatment to be achieved.
2. The current results indicate, for the first time, that the combination of PGB/TOLP elicits an acute anti-tactile allodynic effect. This finding supports the use of medication combinations targeting both VGSC and VGCC for the treatment of NP.
3. The fast onset of the TOLP/PGB combination may be attributed to the ability of the combination to inhibit glutamate release, the key transmitter in the neurochemical changes that occur in NP.
4. The fast onset of action of the TOLP/PGB combination may also be attributed to the ability of the single drugs to influence the glutamatergic system, a key player in NP development, through different mechanisms.
5. In the therapy of diabetic polyneuropathic pain, inhibition of VGCCs is more relevant than inhibition of VGSCs.
6. PGB's capacity to restore and even promote spinal opioid system function is one of its main advantageous effects in diabetic polyneuropathic pain.
7. The effect of PGB on delaying the development of Morph antinociceptive tolerance can be mediated by lowering the spinal level of D-serine, a co-agonist of NMDARs, thus achieving normal activity for NMDARs, key receptors in the development of opioid tolerance..
8. The augmented effect between drugs targeting VGSCs and VGCCs on MVD muscle contractions further supports the cooperation between the two channels in the context of transmitters' release.
9. The in vitro data related to Morph antinociceptive tolerance suggest that both VGSC and VGCC inhibitors can inhibit the development of opioid tolerance and point to the possibility of interference of pharmacokinetic factors when TOLP is investigated in the whole animal.

6. Bibliography of the Candidate's Publications

6.1. The Publications of the Candidate Involved in the Current Thesis

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