Combined carbamazepine and pregabalin therapy in a rat model of neuropathic pain


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Editor’s key points

- Optimal therapy for neuropathic pain has not been established.
- There is a need for exploration of combination therapies with different modes of action.
- A variety of dose combinations of pregabalin and carbamazepine were assessed using behavioural and functional measures.
- Synergy was detected mainly in the higher dose range.

Background. Carbamazepine and pregabalin have proven effects against neuropathic pain. Carbamazepine blocks voltage-dependent Na⁺ channels, whereas pregabalin blocks voltage-dependent Ca²⁺ channels. The authors hypothesized that the co-administration of these drugs would synergistically reduce neuropathic pain.

Methods. Neuropathic pain was induced by L5 nerve ligation in Sprague–Dawley rats. To determine their ED₅₀ values, carbamazepine and pregabalin were orally administered at 0.3, 3, 10, or 30 mg kg⁻¹. The drugs were then co-administered at 0, 1/4 × ED₅₀, 1/2 × ED₅₀, 1.5 × ED₅₀, and 2 × ED₅₀ to determine the ED₅₀ and ED₇₅ values of the drugs in combination. Allodynia was determined using the von Frey hair test and dose–effect curves and isobolograms were used to investigate drug interactions. Levels of the acute reactive protein c-Fos in the dorsal horn were evaluated as an indicator of pathological nerve excitation.

Results. At ED₅₀ levels, carbamazepine and pregabalin did not exhibit synergy, but doses higher than ED₇₅ were found to be synergistic. The combination index was 0.18 (strong synergy) and dose reductions were 35.7-fold for carbamazepine and 6.8-fold for pregabalin when co-administered when compared with a single administration at ED₇₅. The percentage allodynia relief was only 60% for carbamazepine and 80% for pregabalin by single administration, whereas their co-administration relieved allodynia by 100%. Furthermore, treatment decreased c-Fos expression in the dorsal horn, but expression differences between animals treated with carbamazepine plus pregabalin were not significantly different from those treated with single drug.

Conclusions. Carbamazepine and pregabalin ameliorate neuropathic pain synergistically at higher doses.

Keywords: carbamazepine; ion channels; neuralgia; pregabalin

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Neuropathic pain is a chronic condition arising from injury or disease of the peripheral or central nervous system. It is a common problem and may affect around 8% of the population. Furthermore, neuropathic pain is difficult to treat and the therapeutic efficacies of drugs are only about 50%, which makes the management of neuropathic pain challenging for physicians.

The mechanisms responsible for neuropathic pain are not well established, and therefore, treatment largely depends on empirical measures, previous drug efficacies, and trial-and-error. In clinical practice, physicians choose a drug and increase its dosage until side-effects are intolerable, and often, these side-effects prevent further administration.

Recently, changes in ion channels have attracted attention as a possible aetiology of neuropathic pain. Treatments modulating targeted ion channels are based on the known reorganizations of ion channels in neuromas, dorsal root ganglia, the spinal cord, and the brain after nerve injury. Na⁺ and Ca²⁺ channels are known to be abnormally activated, up-regulated, or both, and thus, blocking these channels offers the possibility of reducing neuropathic pain.

Anti-epileptic drugs that target neuronal excitability by modulating ion channels, receptors, and intracellular signalling pathways have been shown to reduce neuropathic pain. Carbamazepine, the first anti-convulsant studied in clinical trials, probably alleviates pain by decreasing Na⁺ channel conductance, whereas pregabalin acts on Ca²⁺ channels to inhibit pre-synaptic glutamate release. In general, when drugs with different mechanisms of action are combined, analgesic efficacy is achieved using smaller doses and side-effects are reduced. In patients with refractory partial seizures, the co-administration of carbamazepine and pregabalin was found to be highly effective. However,
few studies have been conducted on neuropathic pain, although in one study on co-administered carbamazepine and gabapentin, it was found that they had a synergistic effect on trigeminal neuralgia.\(^{13}\) Therefore, we hypothesized that the co-administration of carbamazepine and pregabalin would have a synergistic effect on neuropathic pain. In this study using a nerve ligation neuropathic pain model, behavioural measures were used to assess the effects of drug interaction on mechanical allodynia. In addition, c-Fos levels (a well-known indicator of rapid and transient neuronal activity in the central nervous system) were measured in the dorsal horn neurones,\(^{14,15}\) to determine the extent to which different treatments suppress neuronal activity.\(^{16}\)

**Methods**

Experiments were approved by our Institutional Animal Care Committee and were performed in accordance with the ARRIVE (Animals in Research: Reporting In Vivo Experiments) guidelines.

**Animal preparation**

Male Sprague–Dawley rats, weighing 130–180 g, were housed in separate cages under a 12/12 h day/night cycle and provided food and water ad libitum. Rats were acclimatized for 5–7 days before the experiments.

**Model of neuropathic pain**

Neuropathic pain was induced using the procedure described by Kim and Chung.\(^{17}\) Briefly, rats were anaesthetized with 2.5% enflurane in O\(_2\). A dorsal midline incision was then made from L3 to S2, and under microscopic guidance, the left L6 transverse process was partly resected to visualize the L4 and L5 spinal nerves. The left L5 spinal nerve immediately distal to the dorsal root ganglion was then isolated and ligated tightly with 6-0 black silk.

One week later, rats showing any sign of motor dysfunction, including abnormal ambulation or placing/stepping reflex, were excluded. Animals with a paw withdrawal threshold (PWT) of >5 g (no allodynia) for the operated hindpaw were also excluded.\(^{18}\)

**Test for tactile alldynia**

To avoid circadian cycle effects, all studies were performed at 8 a.m. An observer unaware of the drugs/doses used performed the tactile allodynia testing, which was performed using the von Frey hair test (vFT). Tactile allodynia peaks at 1–5 weeks after surgery and recovers 10–18 weeks later.\(^{19}\) In a previous study, the maximum anti-allodynic effect of oral doses of carbamazepine and pregabalin was observed at 2 h post-dosing.\(^{10}\) Therefore, the vFT was performed before and 2 h after drug administration at 1 week after surgery.

For vFT, a rat was placed in a clear plastic cage (24×13×13 cm) with a 4×4 mm wire-mesh grid floor and allowed to acclimatize for at least 15 min. A series of von Frey hairs (numbers: 4.17, 4.31, 4.56, 4.74, 4.93, 5.07, and 5.18; Stoelting, Wood Dale, IL, USA) starting with number 4.31 were applied in sequence through the grid floor to the ventral surface of the operated hindpaw with sufficient pressure to cause the filament to buckle.

Brisk paw lifting within 5 s was defined as a positive response and this was followed by the application of the next weakest filament. The absence of a paw withdrawal response after five trials prompted the use of the next strongest filament. This was continued until five additional measurements had been made after recording the initial change in the paw withdrawal response. PWT results were calculated using the following formula:

\[
50\%\text{g PWT} = \frac{10^{X_f + \kappa \times 0.22}}{10^{000}}
\]

where \(X_f\) is the number of the final von Frey filament used, \(\kappa\) the statistic from the tabular value for the pattern of positive/negative responses.\(^{20}\)

We also recorded side-effects such as sedation and motor dysfunction at the time of allodynia testing.

**Carbamazepine and pregabalin administration**

Carbamazepine was purchased from Sigma Chemical Company (St Louis, MO, USA), and pregabalin from Pfizer (Ann Arbor, MI, USA). Drugs were randomly administered 1 week after nerve ligation, that is, 0.3, 3, 10, or 30 mg kg\(^{-1}\) of carbamazepine (\(n=15\) in each dose) or 0.3, 3, 10, or 30 mg kg\(^{-1}\) of pregabalin (\(n=15\) in each dose) via an oral gavage tube. Carbamazepine was prepared in 100% DMSO and pregabalin in 0.9% saline. Stock drugs were prepared at 20 mg ml\(^{-1}\) and all doses were delivered as 1 ml solutions, which was achieved by adding normal saline. vFT was performed before drug administration and 2 h afterwards.

The PWT at each concentration was converted to degrees of anti-allodynic effect using the following formula

\[
\text{Anti - allodynic effect} = \frac{a}{8.81}
\]

where \(8.81=\frac{\text{[post-treatment PWT} - \text{pre-treatment PWT}]}{\text{at maximal anti-allodynia and } a=\frac{\text{[post-treatment PWT} - \text{pre-treatment PWT}]}}{\text{at each dose.}}\)

**Determining ED\(_{50}\) values**

ED\(_{50}\) values and dose–effect curves were obtained using the Calcsusyn program (BIOSOFT, Cambridge, UK). The ED\(_{50}\) of carbamazepine was found to be 10.3 mg (95% range: 2.6–40.2 mg) and that of pregabalin was 3.3 mg (95% range: 1.56–7.6 mg).

These ED\(_{50}\) values were used to determine doses for co-administration. Doses of 0×ED\(_{50}\), 1/4×ED\(_{50}\), 1/2×ED\(_{50}\), 1.5×ED\(_{50}\), and 2×ED\(_{50}\) of each drug were co-administered, that is, carbamazepine 0 mg, pregabalin 0 mg; carbamazepine 2.6 mg, pregabalin 0.8 mg; carbamazepine 5.2 mg, pregabalin 1.7 mg; carbamazepine 15.5 mg, pregabalin 5.0 mg; and carbamazepine 20.6 mg, pregabalin 6.6 mg, respectively (\(n=15\) per dose combination). Methods of drug
administration and vFT were the same as used for single drug administrations.

Combination index (CI) and dose-reduction index (DRI) were calculated using the multiple drug-effect equation of Chou.21 in the Calcusyn program. CIs reflect the type of interaction between co-administered drugs. CI values in the range 0.9 and 1.1 indicate an additive effect, whereas CI values of <0.9 indicate synergism and CI values of >1.1 indicate antagonism. DRI is a measure of the fold-reduction in dosage in a synergistic combination required to achieve the same effects as the individual drugs. DRI is important clinically, because toxicity is reduced but therapeutic efficacy is retained.

An isobologram provides a convenient graphical display in which equipotent pairs of doses of two drugs are connected. The line produced represents the additive effect exhibited by the two drugs. However, if the dose of the combined drugs is lower than this line, it indicates a synergistic effect between the two drugs.22

### c-Fos measurement by immunohistochemistry

vFT was performed 1 week after L5 ligation. Baseline PWT before drug administration was similar for the control, carbamazepine, pregabalin, and combination groups (n=5 in each group). The carbamazepine group received 51 mg kg\(^{-1}\) and the pregabalin group received 17 mg kg\(^{-1}\). These doses represented the start of the plateau in drug dose–effect curves, that is, the minimum dose required to induce a maximal anti-allodynic effect. The combined group was administered 50% of these drug levels, that is, carbamazepine 25.5 mg kg\(^{-1}\)+pregabalin 8.5 mg kg\(^{-1}\).

Spinal cord samples were obtained 2 h after drug administration by an overdose of sodium pentobarbital and perfusing the ascending aorta with 200 ml of saline followed by 250 ml 4% paraformaldehyde in 0.1 M phosphate buffer (pH 7.4). Briefly, the previous incision was reopened and widened. The L5 nerve was identified with a black silk tie and dissected carefully to the point of the spinal cord. The spinal cord at L5 was then resected, soaked in the same fixative for 24 h, and preserved at −70°C in a refrigerator.

Cross-sections were cut using a Microslicer (25 μm thickness, Cryocut Micromot, CM3050S, Leica, Germany) and staining was performed by free-floating immunohistochemistry (IHC). Sections were washed three times for 10 min in phosphate-buffered saline (PBS), permeabilized with 0.25% Triton-X for 10 min, incubated in 3% normal goat serum (NGS, diluted in PBS, pH 7.4) for 1 h, then with primary antibody in 3% NGS (1:200) overnight at 4°C, and washed three times. The primary antibody used was rabbit polyclonal antibody against N-terminal amino acids 1–14 of human c-Fos (ab7963; Abcam, USA). Sections were then washed three times in PBS, incubated with Alexa Fluor 488-labelled secondary antibody (1:200, Molecular Probes, USA) in 3% NGS for 2 h, and washed three times. Finally, sections were mounted on chrome-alum–gelatin-coated slides, allowed to dry, counterstained with Vectashield medium containing 4,6-diamidino-2-phenylindole (DAPI) (Vector Laboratories, USA), and coverslipped. Slides were observed under a confocal microscope LSM700 (Zeiss, Germany). c-Fos-positive nuclei in laminae I and II of the left dorsal horn of L5 within a 319×319 μm\(^2\) grid were counted in three sequential sections at ×20 (five rats per group). c-Fos-positive nuclei counting was performed in a double-blind fashion.

### Statistical analysis

Fifteen rats in each group were required to detect the minimum difference in means (post-treatment PWT−pre-treatment PWT) of 1 g with the expected standard deviation of residuals of 0.8 g, a power of 0.8, and a- error of 0.05. Statistical differences between means were determined by the t-test or one-way analysis of variance followed by Tukey's honestly significant difference test. The difference between theoretical and experimental ED\(_{50}\) and ED\(_{75}\) values was examined using Student's t-test. The Kruskal–Wallis test and subsequent post hoc comparisons (Mann–Whitney test of ranks with Bonferroni's correction) were performed on c-Fos levels in the four groups. Statistical significance was accepted for P-values of <0.05.

### Results

Tactile alldynia (PWT < 5 g), as measured by the vFT, developed in 134 of 145 rats by 4 days after surgery (87%). The 134 rats with evidence of tactile alldynia were used for drug experiments.

The ED\(_{50}\) of carbamazepine was 10.3 mg (95% range: 2.6–40.2 mg) and that of pregabalin 3.3 mg (95% range: 1.56–7.6 mg), whereas the ED\(_{75}\) of carbamazepine was 333.7 mg (95% range: 12.0–9256.3 mg) and that of pregabalin 20.2 mg (95% range: 6.6–62.2 mg) (Table 1). Carbamazepine or pregabalin alone did not achieve a 100% anti-allodynic effect. The percentage alldynia relief was only 60% for carbamazepine and 80% for pregabalin by single administration (Fig. 1).

The ED\(_{50}\) of the combined drugs for carbamazepine was 6.5 mg (95% range: 3.9–10.9 mg) and pregabalin 2.1 mg

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<th>ED(_{50})</th>
<th>ED(_{75})</th>
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<td>Carbamazepine (mg)</td>
<td>10.3 (2.6–40.2)</td>
<td>333.7 (12.0–9256.3)</td>
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<tr>
<td>Pregabalin (mg)</td>
<td>3.3 (1.56–7.6)</td>
<td>20.2 (6.6–62.2)</td>
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<tr>
<td>Combined (mg)</td>
<td>6.5 (3.93–10.9)</td>
<td>9.3 (5.7–15.4)*</td>
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<tr>
<td>Carbamazepine</td>
<td>6.5 (3.93–10.9)</td>
<td>9.3 (5.7–15.4)*</td>
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<tr>
<td>Pregabalin</td>
<td>2.1 (1.3–3.5)</td>
<td>3.0 (1.8–4.9)*</td>
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(95% range: 1.3–3.5 mg), and the ED_{75} of the combined drugs for carbamazepine was 9.3 mg (95% range: 5.7–15.4 mg) and pregabalin 3.0 mg (95% range: 1.8–4.9 mg) (Table 1). Combination treatment relieved allodynia by 100% (Fig. 1).

At ED_{50}, no synergistic effect was observed, but doses higher than the ED_{75} showed a synergistic effect according to the isobologram, CI, and DRI. The CI value was 1.3 at the ED_{50} and 0.18 at the ED_{75}. The DRI is a measure of how many fold the dose of each drug in a synergistic combination may be reduced at a given effect level when compared with drugs alone. Doses of carbamazepine and pregabalin were found to be reduced by 35.7- and 6.8-folds, respectively, by co-administration at ED_{75} (Table 1, Fig. 2).

Expressions of nucleus c-Fos in laminae I and II of the dorsal horn were higher in the control group than in the treatment groups. No differences were observed between the treatment groups in terms of c-Fos expression (Table 2, Fig. 3).

We did not observe any difference between the control and treatment groups in degree of sedation, interaction with other rats, or motor functions, such as, standing, walking, or grooming.

**Discussion**

Injured sensory nerves exhibit spontaneous or ectopic firing, particularly in larger A-fibres. These processes are dependent on voltage-dependent Na\(^+\) channel activity and contribute to the development of hyperalgesia and allodynia, characteristic of neuropathic pain.\(^2\) Therapeutic concentrations of carbamazepine suppress sustained, repetitive, high-frequency neuronal firing by blocking voltage-dependent Na\(^+\) channels in the somata of primary sensory neurones,\(^2\) in peripheral nerve neuromas,\(^2\) and in spinal cord neurones.\(^2\) This leads to the stabilization of the pre-synaptic neuronal membrane and a subsequent reduction in neurotransmitter release, particularly, of the excitatory amino acids glutamate and aspartate.\(^2\) For this reason, carbamazepine continues to be important in the management of trigeminal neuralgia.\(^1\)

An increase in intracellular Ca\(^{2+}\) level in the spinal cord also causes allodynia and hyperalgesia.\(^2\) A longer depolarization and concomitant Ca\(^{2+}\) entry facilitate the release of glutamate and substance P from nerve endings, which activate N-methyl-D-aspartate receptors and results in a wind-up phenomenon.\(^2\) Pregabalin is a novel central nervous system drug that does not interact with benzodiazepine or GABA receptors. Pregabalin is structurally related to the anti-epileptic drug gabapentin and both drugs act at the \(\alpha_2\delta\)-subunit, an auxiliary subunit of voltage-dependent Ca\(^{2+}\) channels.\(^3\) This subunit enhances Ca\(^{2+}\) channel currents and voltage-dependent Ca\(^{2+}\) channel binding-site affinity.\(^3\) Pregabalin decreases Ca\(^{2+}\) influx and subsequent abnormal neuronal activity by blocking the \(\alpha_2\delta\)-subunit,\(^3\) and is being increasingly used instead of gabapentin for...
the treatment of neuropathic pain because it potentially has higher potency and fewer side-effects. In previous studies, single administrations of carbamazepine or gabapentin were found to have low efficacies in chronic constriction injury or spinal ligation models, and accordingly, in the present study, single administration of carbamazepine or pregabalin provided only 60–80% efficacy against allodynia at maximum doses.

The combination of carbamazepine and pregabalin offers a potential means of synergistically treating neuropathic pain. However, no clinical or experimental studies have been undertaken on this topic.

Anti-neuropathic efficacies of new drug combinations are often difficult to determine in clinical practice due to methodological variations and ethical issues. Therefore, in this study, we used an L5 nerve ligation model to characterize the interaction between carbamazepine and pregabalin. Allodynia is a prominent manifestation of central sensitization, and is a state in which normally innocuous input is perceived as pain. Allodynia is considered both a hallmark and one of the most troublesome components of neuropathic pain symptoms. Furthermore, measurements of allodynia using the vFT are well standardized and reproducible. Therefore, we measured anti-allodynic effects of these drugs and quantified it by using dose–effect curves, isobolograms, CI, and DRI analysis.

By measuring anti-allodynic effect quantitatively, we found that the Na$^+$-channel blocker carbamazepine and the Ca$^{2+}$-channel blocker pregabalin act synergistically to treat allodynia.

Previously, carbamazepine was found to be highly effective in combination with pregabalin in cases of refractory partial seizure, and no pharmacokinetic drug–drug interactions were observed. Furthermore, concentrations of carbamazepine were unaffected by concomitant pregabalin administration and carbamazepine–pregabalin combinations were generally well tolerated.

In another study, gabapentin was added to carbamazepine and found to have a synergistic effect in trigeminal neuralgia. In particular, multiple sclerosis patients with refractory trigeminal neuralgia receiving therapeutic level of carbamazepine showed that the addition of gabapentin rendered a significant reduction in the dose of carbamazepine to a level below that considered effective for relieving pain.

Carbamazepine and pregabalin act synergistically to treat allodynia in our study. However, the combination did not show a synergistic effect at ED$50$. The reason for this is not clear, although lower doses of the drugs might have failed to cover all the abnormal electrical firings in damaged nerves. In clinical practice, doses of carbamazepine or pregabalin often do not reach levels that provide effective neuropathic pain relief.

High-frequency spontaneous discharges from ectopic sites in peripheral nerves after injury increase the responsiveness of dorsal horn neurones, which constitute central sensitization. c-fos is an immediate early gene, and the IHC detection of c-Fos protein in dorsal horn is widely used to identify neural response after extracellular stimuli. In the present study, we measured c-Fos in laminae I and II, because little c-Fos expression was observed in other laminae which was in line with a previous report.
and colleagues showed that peripheral injury to C-fibres may have induced sprouting of Aδ-fibre terminals from deeper laminae (III and IV) to laminae I and II, an area in which they do not normally terminate, by identifying the manifestation of myelinated fibres in laminae I and II. Changes in central A-fibre connectivity is considered one of the major mechanisms underlying the development of allodynia, although it is unclear whether this is actually related to Aδ afferents sprouting into superficial laminae.

Carbamazepine and pregabalin alone and in combination reduced c-Fos expression vs controls in the present study. In previous studies, a single intraperitoneal injection of carbamazepine before noxious stimulation reduced the number of c-Fos immunoreactive neurones to 30–80% of control levels in the dorsal horn. Chronic treatment with carbamazepine also significantly attenuated the amount of c-Fos. Kaneko and colleagues reported that pre-treatment with gabapentin suppresses c-Fos expression in the mouse spinal cord induced by noxious stimulation. In another study, pregabalin reduced colorectal distension-induced increases in c-Fos expression in the spinal cord. No report has yet described the effect of pregabalin on c-Fos in the context of neuropathic pain except ours.

However, in contrast to the results shown by vFT, no synergistic effect was observed for carbamazepine and pregabalin in c-Fos expression. c-Fos reflects a rapid and transient index of neuronal activity in the central nervous system, and thus, its expression might have been partially dissipated during spinal cord preparation. Another possibility is that c-Fos expression might not be linearly correlated with degree of allodynia, especially when allodynia becomes less intense after treatment. It is also possible that the statistical power was too weak to detect small differences between c-Fos expressions in the treatment groups (n=5 in each group for the measurement of c-Fos) as the power calculation was not based on c-Fos detection. Thus, we could not show any c-Fos differences between three treatment groups, although a false-negative finding is also a possibility.

For limitation, we chose allodynia evaluation as a physiological test and c-Fos detection as a biochemical test. They are the most commonly performed tests for neuropathic pain measurement. However, the measurements of Na+ and Ca2+ channels directly or other aspects of neuropathic pain (hyperalgesia and hyperaesthesia) might have yielded more information concerning neuropathic pain. Secondly, we could not observe any difference between the control and treatment groups in terms of side-effects. The co-administration of carbamazepine and pregabalin is known to be safe and free of most side-effects, including dizziness, somnolence, and nystagmus, and ~95% of reported adverse events were considered mild to moderate and transient in nature. However, we did not measure side-effects quantitatively using objective tests, which might have revealed subtle difference in those side-effects. Finally, rats with no operation or sham operation may have served as a true control group instead of rats with nerve ligation only.

In conclusion, carbamazepine and pregabalin were found to act synergistically on neuropathic pain. Based on these results, we recommend that further clinical trials be conducted on carbamazepine and pregabalin co-treatment in the context of neuropathic pain.

Declarations of interest
None declared.

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