ANTIALLODYNIC AND ANTIHYPERALGESIC ACTIVITIES OF ANTICONVULSANT GABA DERIVATIVES IN BOTH SCIATIC NERVE AND SPINAL NERVE LIGATION MODELS OF NEUROPATHIC PAIN

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Summary

There is considerable research evidence supporting a palliative role for γ-aminobutyric acid (GABA)ergic neurotransmission and voltage-gated sodium channel blockade in neuropathic pain conditions. Hence, the present study was undertaken to assess the peripheral analgesic, antiallodynic and antihyperalgesic activities of the synthesized structural analogues of GABA. The screening study included an acute tissue-injury, chronic constriction injury (CCI), and spinal nerve ligation (SNL) models of neuropathic pain. All of the tested compounds suppressed the acetic acid induced writhing response significantly in comparison to the control. In particular, compound **12** was observed to be the most active compound with percent inhibition greater than that of the standard drug aspirin (97.8% inhibition of writhing response as against 97.0% shown by aspirin). In neuropathic pain studies, compounds 7 and **12** (100 mg/kg, i.p.) emerged as the most active compound affording maximum protection in the CCI model, and compounds **4** and **5** (100 mg/kg, i.p.) in SNL rats.

Keywords: γ-aminobutyric acid; Thiosemicarbazones; GABA; neuropathic pain; analgesic activity; antiallodynic; antihyperalgesic.

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Introduction

Conventional antiepileptic drugs, such as phenytoin and carbamazepine, have been used to treat neuropathic pain since the 1940s. [1] Other important approaches to the control of chronic neuropathic pain have involved γ-aminobutyric acid (GABA) mechanisms. Activation of GABA-mediated inhibition can result in analgesia and a number of agents have been examined in this context. Sodium valproate enhances GABA function by an unknown mechanism, but it is thought to be either through binding to the $GABA_A$ complex or enhancing GABA synthesis or release. [2] More recently, some of the newer anticonvulsants, in particular gabapentin and to a lesser extent topiramate and lamotrigine, received increased attention as analgesics for treating neuropathic pain. [3] It is worth noting, however, that despite the progress made with these compounds, neuropathic pain remains under treated and in many patients gabapentin does not provide adequate pain relief. In behavioral studies, it has been shown that various chemical analogues of GABA attenuate allodynic and hyperalgesic responses in chronic constriction injury (CCI) in rats. [4-7] Hence there is a current focus on screening the potential of anticonvulsants as therapeutic agents and also in development of newer lead molecules for neuropathic pain treatment. Recently, we reported the anticonvulsant and antinociceptive activities of variously substituted GABA hydrazones. [8] GABA semicarbazones, designed and synthesized as pharmacophoric hybrids.[9] Various aryl thiosemicarbazides and thiosemicarbazones have also been reported by our group to exhibit anticonvulsant activity in MES and scPTZ tests. These agents were also found to block the expression of fully kindled seizures.[10-12] Given the promising biological profile of GABA derivatives and aryl thiosemicarbazones, we initiated a drug discovery program focusing on the design and synthesis of newer GABA derivatives. These N-terminal derivatives of GABA have exhibited anticonvulsant activity in the subcutaneous picrotoxin-induced seizure and febrile seizure models.[13] In the present paper, we undertook to test the effect of these new anticonvulsant GABA derivatives in rodent models of neuropathic pain.

Materials and methods

Animals used for the experiments: The experimental protocols used were approved by the Institutional Animal Ethics Committee of Birla Institute of Technology & Science Pilani (Protocol Nos: IAEC/RES/6/3 and IAEC/RES/6/2). Swiss albino mice (either sex) with weights ranging from 20-25 g were used for the acetic acid induced writhing model. Wistar rats of either sex (200-320 g) were used for both the neuropathic pain models. All experiments were approved by the Institutional Animal Ethics Committee. Animals were housed six (mice) and four (rats) per cage at constant temperature under a 12 h light/dark cycle (lights on at 7:00 AM), with food and water *ad libitum*.

Acetic acid Induced Writhing: Mice were divided into groups of six each. Using the method of Siegmund et al.[14] writhing was induced by an intraperitoneal injection of 0.1 mL of 3% v/v acetic acid. Test group mice received acetic acid one hour after drug-treatment. The number of writhings occurring for a 30 min time period was recorded. For scoring purposes, a writhe

was indicated by stretching of the abdomen with simultaneous stretching of at least one hind limb. The percentage inhibition of the writhing response was then calculated.

Induction of Peripheral Mononeuropathy (CCI Model): Unilateral mononeuropathy was produced in rats using the CCI model performed essentially as described by Bennett and Xie.[15] The rats were anesthetized with an intraperitoneal dose of pentobarbital sodium (65 mg/kg) with additional doses of the anesthetic given as needed. Under aseptic conditions, a 3-cm incision was made on the lateral aspect of the left hindlimb (ipsilateral) at the mid-thigh level with the right hindlimb serving as the control (contralateral). The left paraspinal muscles were then separated from the spinous processes and the common left sciatic nerve was exposed just above the trifurcation point. Four loose ligatures were then made with a 4-0 braided silk suture around the sciatic nerve with about 1-mm spacing as reported elsewhere. [16] The wound was then closed by suturing the muscle using chromic catgut with a continuous suture pattern. Finally, the skin was closed using silk thread with horizontal-mattress suture pattern. A sham surgery (n=4) was performed by exposing the sciatic nerve as described above, but not damaging it. Povidone iodine ointment was applied topically on the wound and gentamicin antibiotic (4 mg/kg) was given intramuscularly for five days after surgery. The animals were then transferred to their home-cages and left for recovery.

Selective Segmental L5 SNL Model: A left L5 spinal nerve ligation, as described by Kim and Chung,[17] was performed. The rats were anesthetized with an intraperitoneal dose of pentobarbital sodium (65 mg/kg) with additional doses of the anesthetic given as needed. Under aseptic conditions, using the transverse processes of L6 as a guide, the left paraspinal muscles were exposed and separated from the spinous processes of L4 to S2 by blunt dissection. The L5 spinal nerve was then exposed at the level of the dorsal root ganglion, and ligated tightly with a 4-0 braided silk suture. Only one tight ligature was made in this model. After confirmation of hemostasis, the wound was then closed by suturing at both muscle and skin levels. A sham surgery (n=4) was performed by exposing the L5 spinal nerve as described above, but not damaging it. Povidone iodine ointment was applied topically on the wound and gentamicin antibiotic (4 mg/kg) was given intramuscularly for five days after surgery. The animals were then transferred to their home-cages and left for recovery.

Sensory Testing (Nociceptive Assays): Four nociceptive assays aimed at determining the severity of behavioral neuropathic responses namely allodynia and hyperalgesia were performed. The assays involved measurement of the degree of spontaneous (ongoing) pain and tests of hind limb withdrawal to cold and mechanical stimuli (dynamic mechanical allodynia, cold allodynia and mechanical hyperalgesia). A minimum of 10 min separated the testing procedures to reduce the influence of prior nociceptive testing. The order of testing was as follows: spontaneous pain, dynamic allodynia, cold allodynia and lastly mechanical hyperalgesia. All of the behavioral responses were timed with a stopwatch.

Spontaneous Pain

Spontaneous pain was assessed for a total time period of 5 min as described previously by Choi et al. [18] The operated rat was placed inside an observation cage that was kept 5 cm from the ground level. An initial acclimatization period of 10 min was given to each of the rats. A total number of four rats (n=4) were assigned to this group. The test consisted of noting the cumulative duration that the rat holds its ipsilateral paw off the floor. The paw lifts associated with locomotion or body repositioning were not counted. It's been suggested that those paw lifts in the absence of any overt external stimuli are associated with spontaneous pain, and are correlative of ongoing pain.[18]

Dynamic Component of Mechanical Allodynia

All of the operated rats were assessed for dynamic allodynic response according to the procedure described by Field et al. [19-20] The operated rat was placed inside an observation cage that was kept 5 cm from the ground level. An initial acclimatization period of 10 min was given to each of the rats. A total number of four rats (n=4) were assigned to this group. A positive dynamic allodynic response consisted of lifting the affected paw for a finite period of time in response to mild stroking on the plantar surface using a cotton-bud. This stimulus is non-noxious to a normal-behaving rat. The latency to paw-withdrawal was then noted down. If no paw withdrawal was shown within 15 s, the test was terminated and animals were assigned this withdrawal time. Hence, 15 s effectively represented no withdrawal.

Cold Allodynia

The rats demonstrating unilateral mononeuropathy were assessed for acute cold allodynia sensitivity using the acetone drop application technique as described by Caudle et al. [21] The operated rat was placed inside an observation cage that was kept 5 cm from the ground level and was allowed to acclimatize for 10 min or until exploratory behaviour ceased. A total number of four rats (n=4) were assigned to this group. Few drops (100-200 μL) of freshly dispensed acetone were squirted as a fine mist onto the midplantar region of the affected paw. A cold allodynic response was assessed by noting down the duration of paw-withdrawal response. For each measurement, the paw was sampled three times and a mean calculated. At least 3 min elapsed between each test.

Mechanical Hyperalgesia

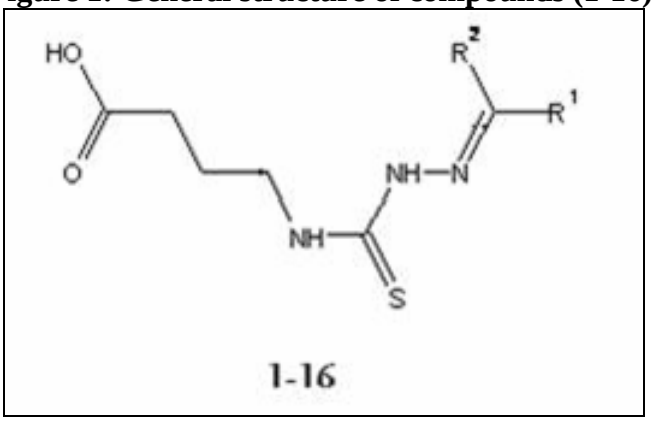
Mononeuropathic rats were assessed for mechanical hyperalgesia sensitivity according to the procedure described by Gonzalez et al. [22] The operated rat was placed inside an observation cage that was kept 5 cm from the ground level. An initial acclimatization period of 10 min was given to each of the rats. A total number of four rats (n=4) were assigned to this group. Hindpaw withdrawal duration was measured after a mild pin-prick stimulus to the midplantar surface of the ipsilateral (left) hindpaw. A withdrawal was defined as being abnormally prolonged if it lasted at least 2 s. The mean withdrawal duration was taken from a set of three responses.

Pharmacological Interventions

Drugs : Compounds **(1-16)** { 1: 4-(Hydrazine carbothioamido)butanoic acid; **2:** 4-(2-(2-Hydroxybenzylidene)hydrazine carbothioamido)butanoic acid; **3:** 4-(2-(4-

Nitrobenzylidene)hydrazine carbothioamido)butanoic acid; **4:** 4-(2-(4-Chlorobenzylidene)hydrazine carbothioamido)butanoic acid; **5:** 4-(2-(3-Nitrobenzylidene)hydrazine carbothioamido)butanoic acid; **6:** 4-(2-(4-(Dimethylamino)benzylidene)hydrazine carbothioamido)butanoic acid; 7**:** 4-(2-(1-Phenylethylidene)hydrazine carbothioamido)butanoic acid; **8:** 4-(1,3-dioxo-1,3-dihydro-2*H*-isoindol-2-yl)-*N*-(4-methylphenyl)butanamide; **9:** 4-(2-(1-(3-Aminophenyl)ethylidene)hydrazinecarbothioamido)butanoic acid; **10:** 4-(2-(1-(4-Nitrophenyl)ethylidene)hydrazinecarbothioamido)butanoic acid; **11:** 4-(2-(Diphenylmethylene)hydrazinecarbothioamido)butanoic acid; **12:** 4-[2-(4-Bromophenyl) (phenyl)methylene)hydrazine carbothioamido)butanoic acid; **13:** 4-(2-(1,3-Diphenylpropan-2-ylidene)hydrazine carbothioamido)butanoic acid; **14:** 4-(2-Cyclohexylidene hydrazine carbothioamido)butanoic acid; **15:** 4-(2-Cyclopentylidene hydrazine carbothioamido)butanoic acid; **16:** 4-(2-(2-Oxoindolin-3-ylidene)hydrazine carbothioamido)butanoic acid} were synthesized according to previously reported procedures.[13] General structure of these compounds is given in **figure 1**. Lamotrigine and carbamazepine were obtained as gift samples from M/s IPCA Laboratories, India. Gabapentin was obtained as a generous gift sample from M/s Wockhardt Laboratories, India. Aspirin used for the study was commercially available from Central Drug House, India.

Figure 1: General structure of compounds (1-16).



Acetic acid Induced Writhing

Compounds (**1-16)**; 100 mg/kg, i.p.) were administered in 30% v/v PEG 400, one hour prior to acetic acid administration. The control group mice received 30% v/v PEG 400 (10 mL/kg). Aspirin at a dose of 100 mg/kg, i.p. suspended in 30% v/v PEG 400 was used as the standard drug.

Peripheral Nerve Injury

Baseline sensory response values were measured for each group of animals (n=4) pre-operatively and 9 days post-operatively. Animals displaying allodynic and hyperalgesic responses in both the models, were then administered the relevant drug according to a pre-determined randomization table and testing was re-performed at 30, 60, 90, 120 and 150 min post drug administration. Each group of animals was used for only one drug administration protocol to ensure no 'carry-over' effects. Compounds **(1-16)** (100 mg/kg, i.p.) were administered at t=0, in 30% v/v PEG 400. The vehicle control group of rats received only the solvent (30% v/v PEG 400). Three positive control groups were run alongside drug

treatment groups using lamotrigine, carbamazepine and gabapentin (100 mg/kg, i.p.). The treatment protocol remained the same for these three drugs. No drug testing was performed for sham-operated rats.

Statistical Analysis : All data are expressed as means ± standard error of mean (SEM). The data were analyzed using Student's *t* test only when two means were compared (acute pain assay). In the case of neuropathic pain studies, statistical significance was determined for drug effects by one-way ANOVA, and bonferroni's post hoc test was used for individual comparisons with the control values. In the first assay, significance was assigned to a P value of less than 0.01 and in the chronic pain assay; comparison results with a P value of less than 0.05 were considered statistically significant. The statistical software package PRISM (Graphpad Software Inc, San Diego, CA) was used for the analyses.

Results

Acetic acid Induced Writhing: The aim of this drug discovery program was to prepare newer GABA derivatives effective in the treatment of pain conditions. To examine the potential therapeutic value of the synthesized compounds (**1-16**) in the treatment of neuropathic pain, the first part of this study examined the ability of the synthesized derivatives of GABA to inhibit writhing responses in the acetic acid induced writhing test, a chemical pain test used to evaluate acute antinociceptive function (**Table 1**).

Table 1. Effects of compounds on writhing induced by acetic acid in mice

#	Acetic acid induced writhing [a]	
	Number of writhes (per 30 min)	% Inhibition
1	17.45 ± 2.26	91
2	38.04 ± 2.13	80
3	18.00 ± 1.15	91
4	26.00 ± 1.15	86
5	14.33 ± 2.03	93
6	11.67 ± 1.20	94
7	38.67 ± 0.67	80
8	16.67 ± 1.45	91
9	26.34 ± 2.98	86
10	18.67 ± 1.45	90
11	28.67 ± 0.88	86
12	8.00 ± 1.73	96
13	27.33 ± 1.45	85
14	23.04 ± 3.16	88
15	24.33 ± 0.88	87
16	27.67 ± 1.45	86
Control	191.00 ± 4.04	-
Aspirin	5.67 ± 3.48	97

[a] Control animals were administered 30% v/v PEG 400 in water. Each value represents the mean ± SEM of six mice significantly different from the control at $P < 0.01$ (Student's t-test).

All of the tested compounds suppressed the acetic acid induced writhing response significantly (P<0.01) in comparison to the control. The standard drug, aspirin exhibited the highest percentage inhibition (97.0%). Compounds **1**, **3**, **5**, **6**, **8**, **10** and **12** were the most active compounds with percentage inhibition values more than 90%. Of these compounds, **12** was observed to be the most active with 96% inhibition

Peripheral Nerve Injury: In the next phase of screening two well known peripheral neuropathic pain models were used that included the chronic constriction injury (CCI) and L5 spinal nerve ligation (SNL) models in rats. In the CCI model, the left sciatic nerve proximal to the trifurcation point was constricted with four loose ligatures using 3-0 braided silk thread, while in the SNL model, a tight ligation was tied around the L5 spinal nerve using 3-0 braided silk thread. Four nociceptive assays aimed at determining the severity of behavioral neuropathic responses namely allodynia and hyperalgesia were performed. The assays involved measurement of the degree of spontaneous (ongoing) pain and tests of hind limb withdrawal to cold and mechanical stimuli (dynamic mechanical allodynia, cold allodynia and mechanical hyperalgesia). A minimum of 10 min separated the testing procedures to reduce the influence of prior nociceptive testing. The order of testing was as follows: spontaneous pain, dynamic allodynia, cold allodynia and lastly mechanical hyperalgesia. Baseline sensory response values were measured for each group of animals (n=4) pre-operatively and 9 days post-operatively. All animals included in the study showed altered sensory responses in all the four behavioral nociceptive tests, 9 days following CCI and SNL. The sham-operated animals showed no significant difference from the pre-operative baseline sensory response values. In the animals, where only the solvent was administered as a control, there was no significant difference between the pre-drug and post-treatment values, at all time points of observation.

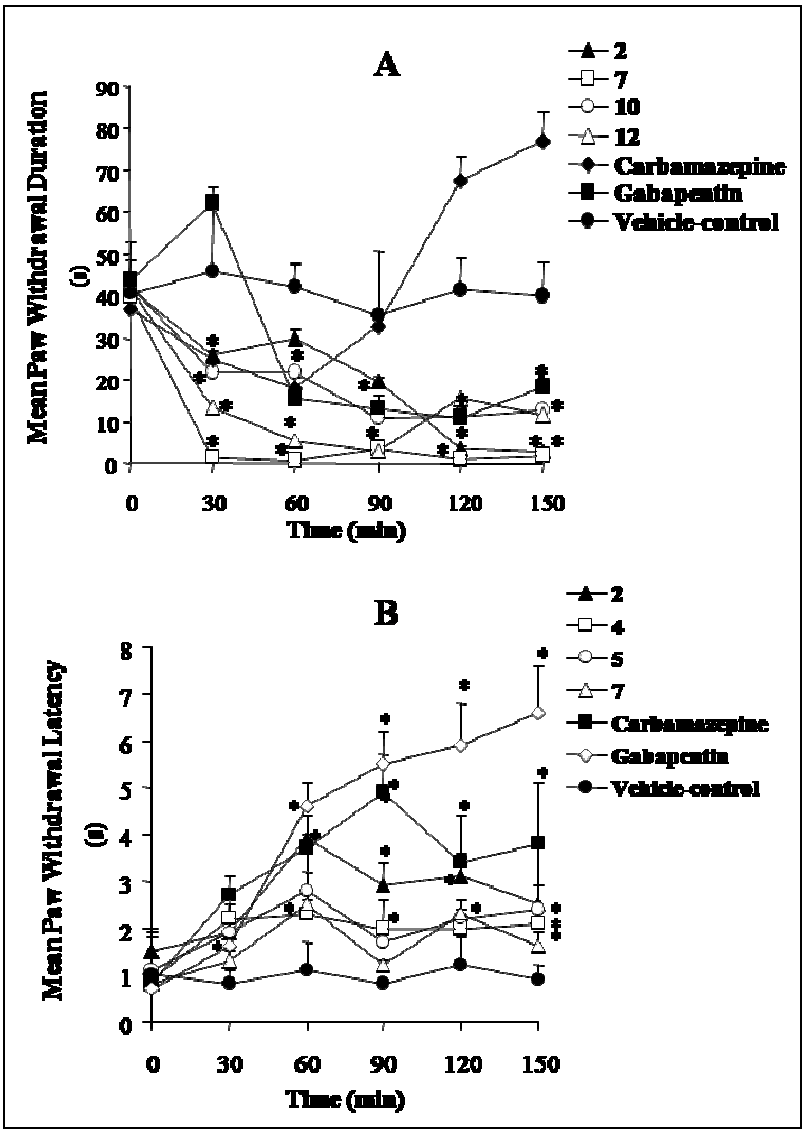
Spontaneous Pain in the CCI model

In the CCI model, compounds **7**, **10**, and **12** completely reversed the spontaneous pain response, at the dose tested over 2.5 h, more effective when compared to carbamazepine (**Figure 2A**). Of these compounds, **7** was observed to be 10 times more active than gabapentin and **10** was equipotent with gabapentin. The time to peak-effect of these compounds were 1 h, 2 h, and 1.5 h respectively. Gabapentin showed activity from 1 h till 2.5 h of testing. The onset of action of compound **2** was 1.5 h and all other compounds were ineffective in this test. In this test, the standard drug carbamazepine reversed the spontaneous pain response only till 1 h significantly, and lamotrigine was devoid of any activity.

Dynamic Allodynia in the CCI model

Three compounds (**2**, **4**, and **5**) were active in attenuating the dynamic allodynic response throughout the entire 2.5 h experiment as the standard drugs carbamazepine and gabapentin (**Figure 2B**). Compound **7** showed response between 1-2 h period and all other compounds were inactive. In this assay, the standard drug carbamazepine showed complete protection till 2.5 h, while gabapentin exhibited antiallodynic activity from 60-2.5 h of sensory testing.

Figure 2. Effects of compounds on spontaneous pain and dynamic allodynia in CCI rats.



*P<0.05, in comparison with the pre-drug values

Cold Allodynia in the CCI model

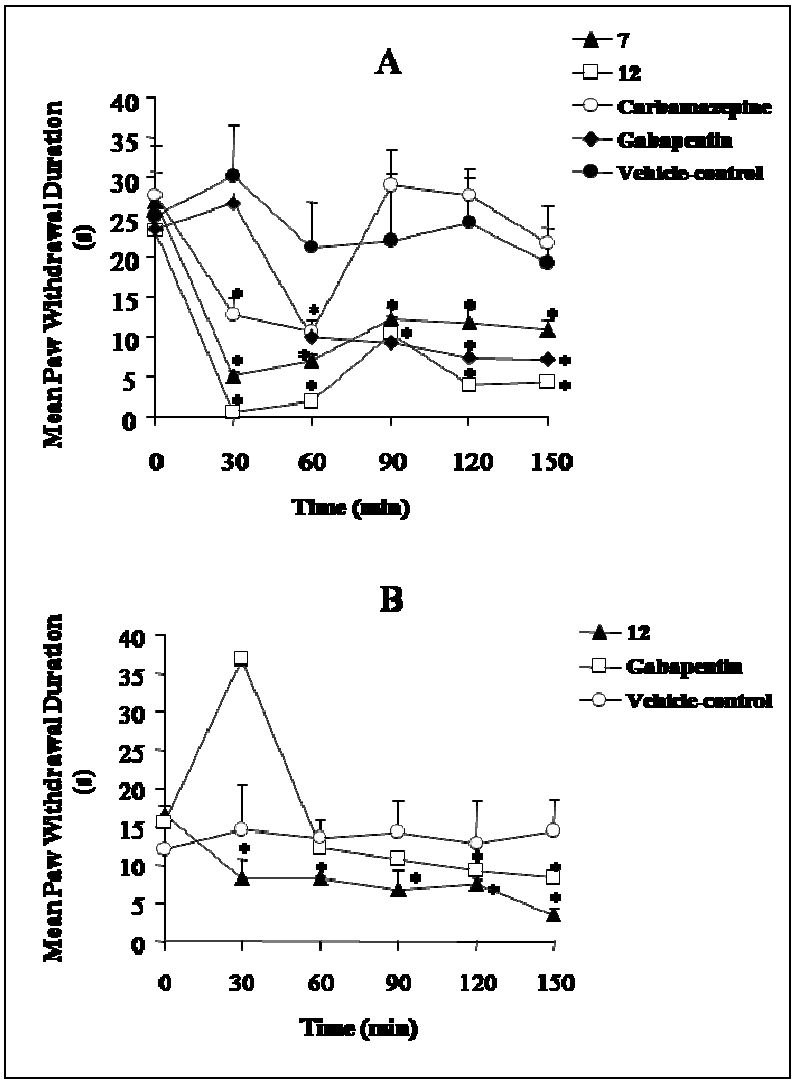
In the cold allodynia produced in CCI rats, the paw withdrawal durations (PWDs) were significantly reduced by the administration of compounds **7** and **12**, throughout the entire 2.5 h **(Figure 3A)**. Of these, **7** showed 1.5 times more activity than carbamazepine and compound **12** was found to be 5.6 and 1.7 times more active than carbamazepine and gabapentin respectively. Carbamazepine was effective only till 1 h and gabapentin showed activity from 1 h till 2.5 h of testing. All other compounds were found to be ineffective in this test. So was the standard drug lamotrigine.

Mechanical Hyperalgesia in the CCI model

Hyperalgesia evoked by a mechanical pin-prick stimulus was effectively attenuated at all time-points of study by compound **12** (**Figure 3B**). This compound showed 2.4 times more activity than the standard drug gabapentin.

All other compounds including the standard drugs lamotrigine and carbamazepine were found to be completely inactive in this assay. Gabapentin was effective in this assay from 2-2.5 h of testing.

Figure 3. Effects of compounds on cold allodynia and mechanical heperalgesia in CCI rats.

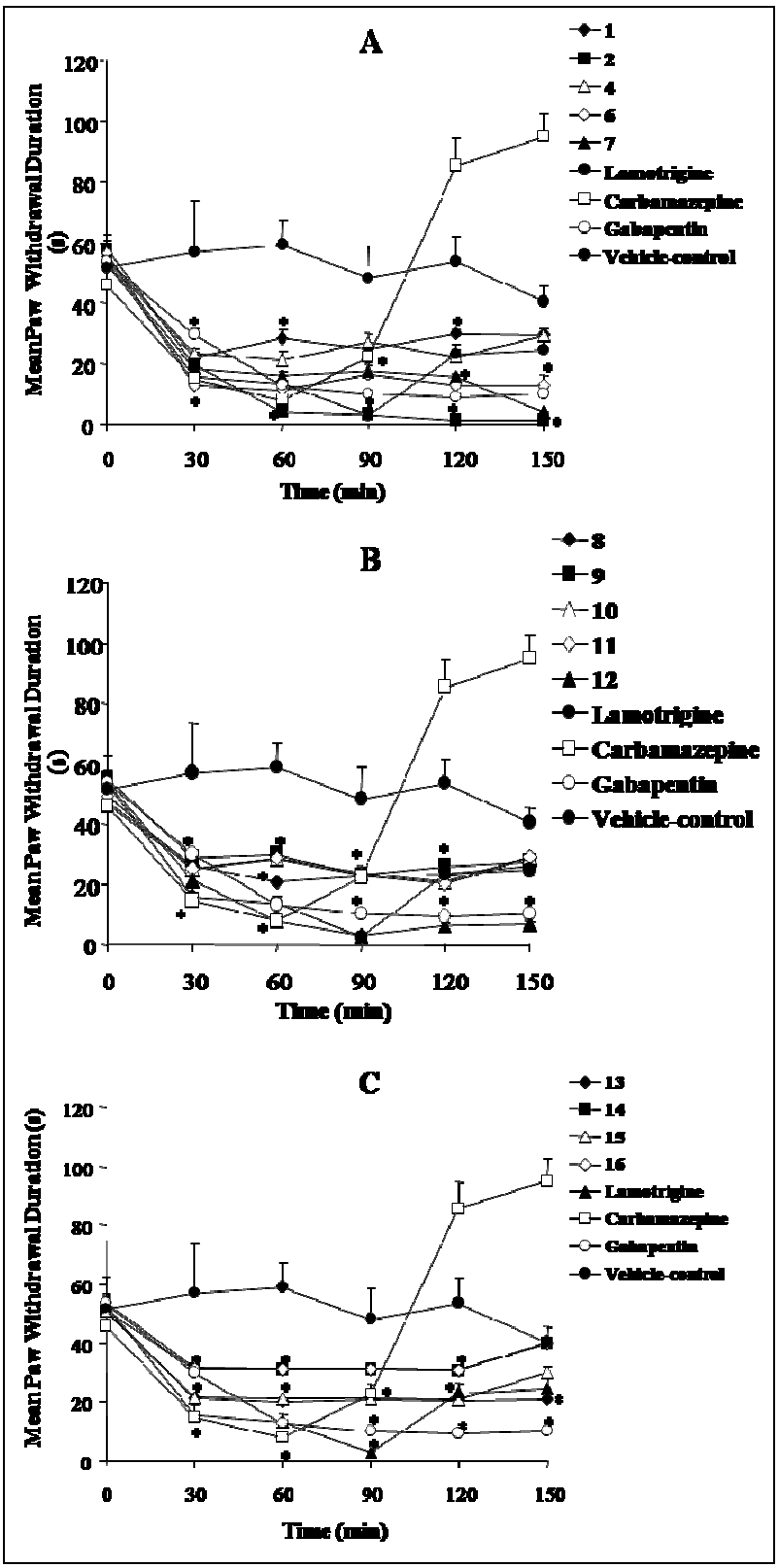


*P<0.05, in comparison with the pre-drug values

Spontaneous Pain in the SNL model

In the SNL model, the paw withdrawal durations due to spontaneous ongoing pain were significantly reduced by all compounds except **3** and **5**. All the active compounds showed a significant reduction in paw withdrawal duration till 2.5 h similar to gabapentin, while compounds **14** and **16** showed activity till 2 h (**Figure 4A-C**). Compounds **10** and **11** showed onset of action at 2 h, while compound **13** at 1.5 h. Carbamazepine showed significant activity till 1.5 h only, while lamotrigine was active throughout the 2.5h period of sensory testing.

Figure 4. Effects of compounds on spontaneous pain in SNL rats.



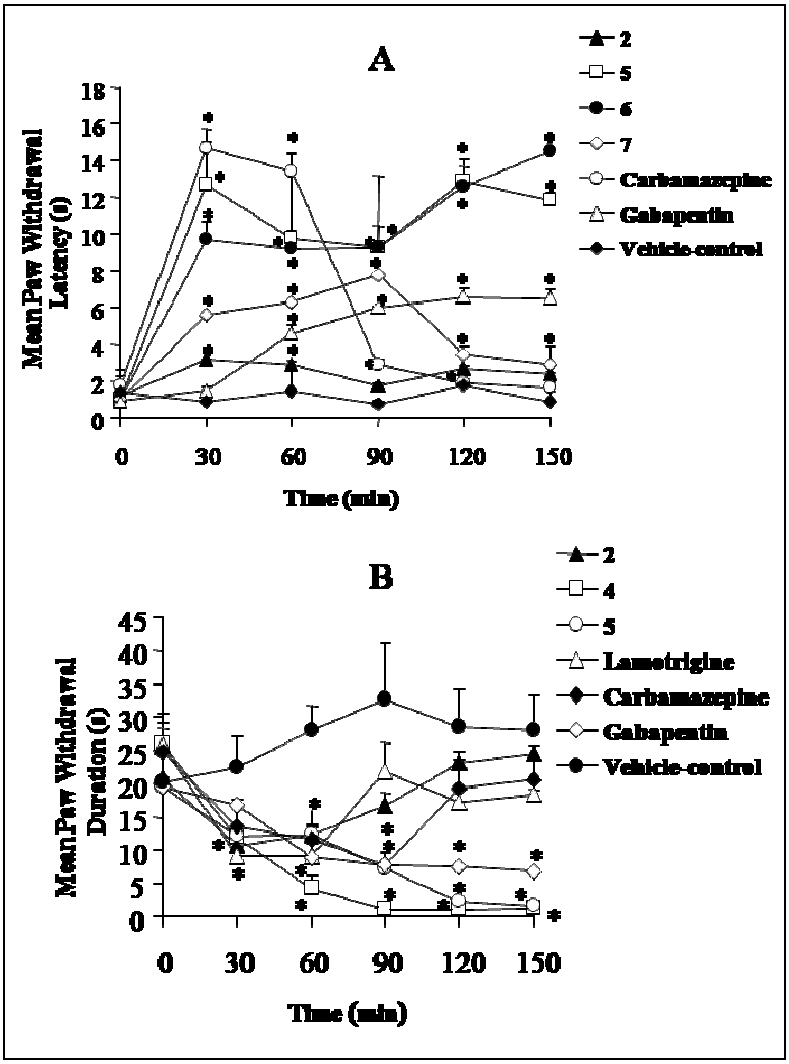
*P<0.05, in comparison with the pre-drug values

Dynamic Allodynia in the SNL model

The dynamic mechanical allodynia produced by SNL was effectively reversed by compounds **2**, **5**, **6**, and **7** (**Figure 5A)** completely effective till 2.5 h, hence more

effective than carbamazepine which showed activity till 1 h. Of these compounds, **5** and **6** exhibited 1.95 and 1.9 times more activity than the standard drug gabapentin, which reversed the dynamic allodynia from 1 to 2.5 h. Compound **4** which was active in completely reversing the dynamic allodynia response in the CCI model, was inactive in the SNL model. Carbamazepine was active from 0.5-1 h, while lamotrigine showed no antiallodynic effect.

Figure 5. Effects of compounds on dynamic and cold allodynia in SNL rats.



*P<0.05, in comparison with the pre-drug values

Cold Allodynia in the SNL model

Cold allodynia produced by SNL model was completely reversed by compounds **2**, **4**, and **5** (**Figure 5B**). Of these compounds, **4** was observed to be 6.6 and 5.3 times more active than carbamazepine and gabapentin respectively. Compound **5** was found to be equipotent with carbamazepine and 4.3 times more active than the standard drug gabapentin. Administration of all other compounds to animals with an allodynic response to cold stimuli, showed no antiallodynic activity. Lamotrigine was active from 0.5 h-1 h min, while carbamazepine showed significant activity between 1-1.5 h only. Gabapentin reversed the cold allodynia from 1 to 2.5 h in this assay. Compounds **7** and **12** which were active in CCI rats were inactive in the SNL rats.

Mechanical Hyperalgesia in the SNL model

In the mechanical hyperalgesia assay, none of the compounds were effective in reversing hyperalgesia. Only carbamazepine showed significant reduction in paw withdrawal duration from 1 h to 2.5 h period. As in the case of CCI rats, here again gabapentin was observed to be completely inactive throughout the 2.5 h period of nociceptive testing.

Discussion

This study examined the potential therapeutic value of GABA derivatives (**1-16)** in the treatment of neuropathic pain using the CCI and SNL models of neuropathic pain. The first part of this study examined the ability of the synthesized derivatives of GABA to inhibit writhing responses in the acetic acid induced writhing test, a chemical pain test used to evaluate acute antinociceptive function. The results of this study indicate that the newer GABA derivatives, apart from exhibiting significant analgesic activity in the acetic acid induced writhing model, also possess antiallodynic and antihyperalgesic actions in both the CCI and SNL models of neuropathic pain. Overall, it appears that in the CCI model of neuropathic pain, compounds that showed promising results include **7** and **12** effective in three out of four tests, and **2** in two tests. Other compounds that showed activity in at least one test include **4**, **5**, and **10**. It is well established that GABAergic mechanisms are involved in antinociceptive processes. In 1999, Eaton et al.[23] provided evidence for the involvement of GABA in spinal antinociception, when a single intrathecal injection of GABA permanently reversed neuropathic pain after nerve injury. Moreover, a reduced spinal GABAergic tone has been suggested just after nerve injury.[24] Intrathecal administration of GABA receptor antagonists dose-dependently produced tactile allodynia, [25] suggesting that inhibiting endogenous GABA can lead to an excited sensory state. Hence, taking into consideration both of the above-mentioned findings, it is concluded that the analgesic activity of compounds (**1-16)** observed in both tissue-injury and peripheral nerve injury models could be primarily mediated by the peripheral GABAergic pathway.

In conclusion, we have shown that the synthesized derivatives of GABA produce antinociceptive actions in the acetic acid induced writhing test and peripheral nerve injury (CCI and SNL) models of neuropathic pain. The underlying mechanisms are expected to be enhancement of peripheral GABAergic neurotransmission owing to various reports on the involvement of GABAergic pathway in peripheral models of neuropathic pain. This study presents the first report on the antiallodynic and antihyperalgesic activities of GABA thiosemicarbazones. Further research is required to confirm the hypothesized molecular mechanisms of action of the reported compounds.

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