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Original article

Discovery of molecules for the treatment of neuropathic pain: Synthesis, antiallodynic and antihyperalgesic activities of 5-(4-nitrophenyl)furoic-2-acid hydrazones

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ABSTRACT

Neuropathic pain is a chronic pain condition that occurs and persists in a heterogeneous group of etiologically different diseases characterized by a primary lesion or dysfunction of the peripheral or central nervous system. Current treatment options do not provide adequate relief for many patients and a significant number of the agents used have dose limiting side effects. During the course of our work on the synthesis and screening of new drugs for the treatment of neuropathic pain, we have identified 5-(4-nitrophenyl)furoic-2-acid hydrazones which showed significant antiallodynic and antihyperalgesic activities in a chronic constriction injury (CCI) model of neuropathic pain in rat. Synthesized compounds thus represent a promising lead for new drug development for the treatment of neuropathic pain.

1. Introduction

Neuropathic pain has an important prevalence in the general population, and a severe impact on quality of life and mood of affected patients. It results from injury to the peripheral and/or central sensory pathways where the painful state exists without apparent noxious input, and is associated with hyperexcitability and spontaneous action potential firing in sensory neurons [1]. In contrast to acute nociceptive pain, the cascade of events that arise following peripheral nerve injury leads to a maintained abnormality in the sensory system, resulting in an abnormal pain phenomenon that can be grossly debilitating. Currently therapy for neuropathic pain is based on tricyclic antidepressants and antiepileptic drugs, the most frequently studied drug classes [2,3]. Opioids and analgesics are a second-line choice [4]. Current treatment options do not provide adequate relief for many patients and a significant number of the agents used have dose limiting side effects.

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. Earlier reports from our research group have identified many leads with antiallodynic and antihyperalgesic activity in both chronic constriction injury and spinal nerve ligation models of neuropathic pain [5,6]. *N*-phthaloyl GABA amides and acid hydrazones have been found to exhibit both anticonvulsant and antiallodynic and antihyperalgesic activities [7]. The furan-amide derivatives have been reported to have both affinity and selectivity for block of Na_v1 and it is widely appreciated that blockade of sodium channels contributes to the analgesic activity. Jarvis et al. had reported a furan-amide derivative A-803467 (5-(4-chlorophenyl)-*N*-(3,5-dimethoxyphenyl)furan-2-carboxamide) which showed potent and selective $Na_v1.8$ sodium channel blockade and attenuated neuropathic and inflammatory pain in rats [8]. 5-Aryl-2-furfuramide derivatives were also found to be effective in animal models of neuropathic and inflammatory pain, through blockade of the $Na_v1.8$ sodium channel, which is a tetrodotoxin-resistant (TTx-r) voltage-gated sodium channel (VGSC) expressed on small diameter sensory neurons and has been implicated in the pathophysiology of inflammatory and neuropathic pain [9]. Piperazine derivatives of furamide derivatives are also reported to have block the tetrodotoxin-resistant sodium channel $Na_v1.8$ (PN3) as well as the

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$Na_v1.2$ and $Na_v1.5$ subtypes for efficacy in neuropathic pain [10]. A United States Patent (Application 20040048889) claims the use of non-competitive and selective gluR 5 antagonists including 5-carboxyl-3,4-di-benzamido-2-furoic acid; 4-carboxyl-2,5-di-benzamido-3-furoic acid as glutamate receptor modulating compounds for the treatment of various central nervous system diseases including chronic and neuropathic pain [11]. Based in part on this literature support, various hydrazones of 5-(4-nitrophenyl) furoic-2-acid were synthesized by a three step reaction and were screened in chronic constriction injury (CCI) model of neuropathic pain in rats in the present work.

2. Synthesis

The compounds (presented in Fig. 1) were prepared by a three-step reaction. 5-(4-nitrophenyl)furoic-2-acid was first esterified using ethanol and sulphuric acid to yield 79% of ethyl 5-(4-nitrophenyl)furan-2-carboxylate (A) [12]. Then the ethyl ester was further treated with hydrazine-hydrate to form 64% of 5-(4-nitrophenyl)furan-2-carbohydrazide (B) [13]. Various aldehydes and ketones were reacted with this hydrazide in presence of glacial acetic acid under microwave irradiation to yield the titled compounds (**1**–**10**) [14]. The yields ranged from 46% to 70%. The physical properties of the synthesized compounds are presented in Table 1. The structures were characterized by both spectral and elemental analysis and the data were within ±0.4% of the theoretical values.

3. Results and discussion

The chronic constriction injury (CCI) was used as a peripheral neuropathic pain model, where the left sciatic nerve proximal to the trifurcation point was constricted with four loose ligatures using 3-0 braided silk thread [15]. All the synthesized compounds (**1**–**10**) were examined for potential therapeutic value in the treatment of neuropathic pain. 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. The sham-operated animals showed no significant difference from the pre-operative baseline sensory response values. In the animals, where only the vehicle was administered as a control, there was no significant difference between the pre-drug and post-treatment values, at all time points of observation. All of the behavioral responses were timed with a stopwatch. All of the compounds were tested at a single dose of 100 mg/kg. The results of active compounds along with data on standard drugs, lamotrigine, carbamazepine and gabapentin are represented in Figs. 2–5.

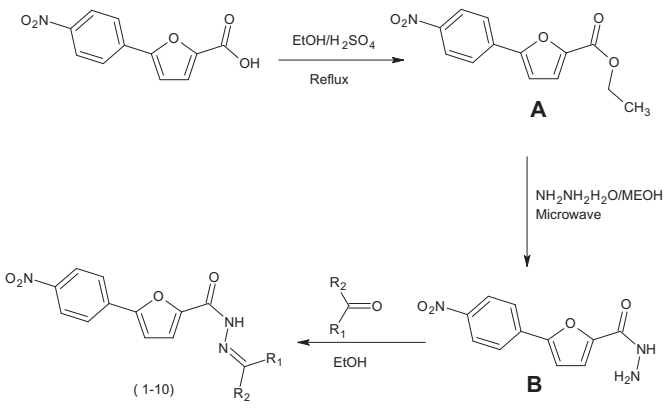


Fig. 1. Synthetic route to 5-(4-nitrophenyl)furoic-2-acid hydrazones.

In the CCI model, compounds **1**, **3**, **4**, **6**, **9** and **10** completely reversed the spontaneous pain response, at the tested dose over 2.5 h, more effective when compared to carbamazepine (Fig. 2). These compounds had onset of action from 0.5 h except compound **4**, which was effective from 1 h. Compound **3** was five times more active than gabapentin in this model at 100 mg/kg and emerged as the most effective compound in reducing spontaneous pain.

Gabapentin showed activity from 1 h through 2.5 h of testing. The onset of action of these compounds was 0.5 h, with one compound at 1 h and all other compounds (**2**, **5**, **7**, and **8**) 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 active only at 2 h.

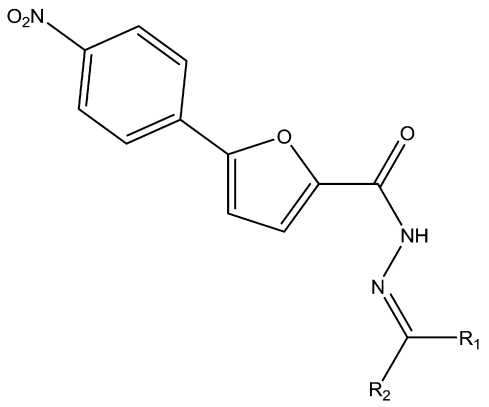
Five compounds (**6**, **7**, **8**, **9** and **10**) were active in attenuating the dynamic allodynic response throughout the entire 2.5 h experiment as the standard drugs carbamazepine and gabapentin, while compound **1** was active through 2 h (Fig. 3). Compound **9** showed onset of action at 1.5 h, while others (**6**–**8**) had onset effect at 0.5 h. All other compounds (**2**–**5**) were inactive. In this assay, the standard drug carbamazepine showed complete protection through 2.5 h, lamotrigine was active only at 0.5 h, while gabapentin exhibited antiallodynic activity from 1 to 2.5 h of sensory testing.

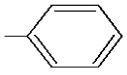
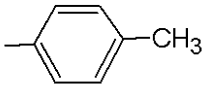
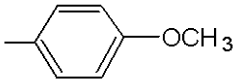
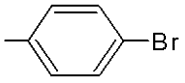
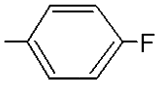
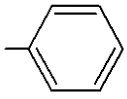
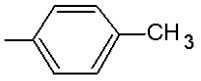
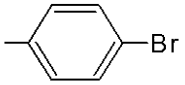
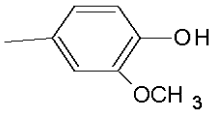
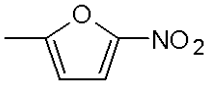
In cold allodynia produced in CCI rats, the paw withdrawal durations (PWDs) were significantly reduced by the administration of compounds **1**, **3**, **4**, **6**, **8**, and **10**, throughout the entire 2.5 h period (Fig. 4). Of these, compound **4** was the most effective, showing four times more activity than gabapentin and six times more activity than carbamazepine and lamotrigine in the model at 100 mg/kg. Carbamazepine was effective at 0.5 h and 1 h and gabapentin showed activity from 1 h through 2.5 h of testing. All other compounds (**2**, **5**, **7** and **9**) were found to be ineffective in this test. Hyperalgesia evoked by a mechanical pinprick stimulus was effectively attenuated at all time-points of study by compound **3** and **4** (Fig. 5). Compound **2** and **10** were effective from 1.5 h. Other compounds (**1**, **5**, **8** and **9**) were ineffective in this assay. Compound **6** was effective at 0.5 and 1 h, while compound **7** was effective between 0.5 and 1.5 h. All other compounds including the standard drugs gabapentin, lamotrigine and carbamazepine were found to be completely inactive in this assay.

Of these 10 compounds, **6** and **10** were effective in all the assays, while compounds **1**, **3** and **4** were effective in three nocciceptive assays, and **7**, **8** and **9** were effective in two assays. Compound **2** was effective in one assay, while compound **5** was totally inactive in all the assays.

The bioevaluation led to an understanding of the importance of the group at carbimino carbon atom. Replacement of the proton on the carbimino carbon atom by methyl function had beneficial effect with respect to **6** and **7** (active in 4 and 2 assays, respectively) compared to **1** and **2** (active in 3 and 1 assays, respectively). Replacement of carbimino hydrogen in compound **4** with a methyl group afforded analog **8** with diminished activity. The distal aryl ring at the carbimino terminal was found to be important for activity as any variation in the substitution on this ring affected bioactivity. Substitution at the C-4 position with bromo, methyl and

Table 1
General structure and physical data of the synthesized compounds (**1**–**11**).



#	R_1	R_2	Molecular formula	Molecular weight	M.P. (°C)	Yield %
1	H		$C_{18}H_{13}N_3O_4$	335.31	200–203	52
2	H		$C_{19}H_{15}N_3O_4$	349.34	173–175	68
3	H		$C_{19}H_{15}N_3O_5$	365.34	145–147	52
4	H		$C_{18}H_{12}BrN_3O_4$	414.21	138–140	68
5	CH_3		$C_{19}H_{14}FN_3O_4$	367.33	153–155	59
6	CH_3		$CH_{19}H_{15}N_3O_4$	349.34	208–210	70
7	CH_3		$CH_{20}H_{17}N_3O_4$	363.37	213–215	60
8	CH_3		$C_{19}H_{14}BrN_3O_4$	428.24	218–220	58
9	H		$C_{19}H_{15}N_3O_6$	365.34	145–147	65
10	H		$C_{19}H_{15}N_4O_7$	384.99	123–125	46

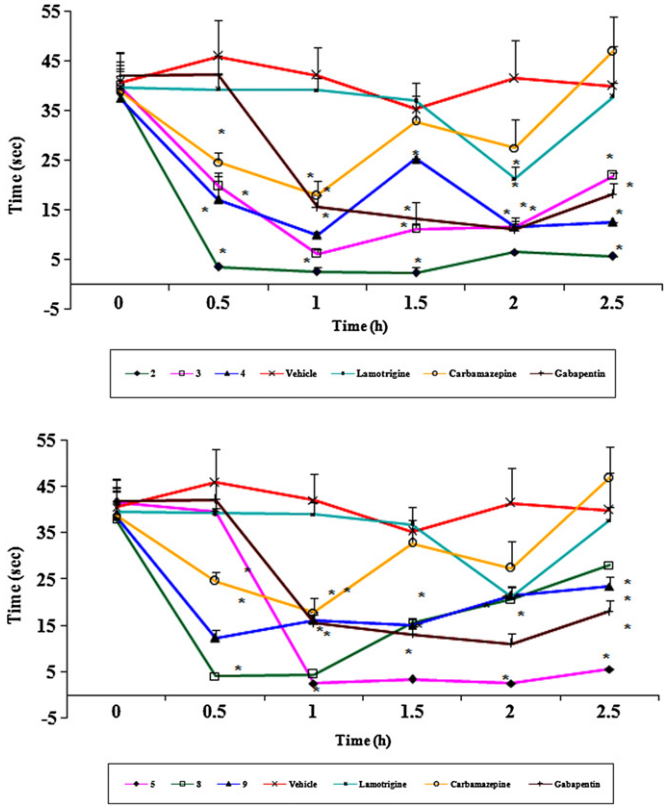


Fig. 2. Effects of compounds in the spontaneous pain assays in CCI rats, along with lamotrigine, carbamazepine and gabapentin. Graphs depict the effects of compounds (100 mg/kg, i.p.) in reversing the spontaneous pain response. The results are shown as mean paw withdrawal duration (PWD ± SEM) of 4 rats per group. $^{*}P < 0.05$, in comparison with the control values (ANOVA followed by post-hoc Bonferroni test).

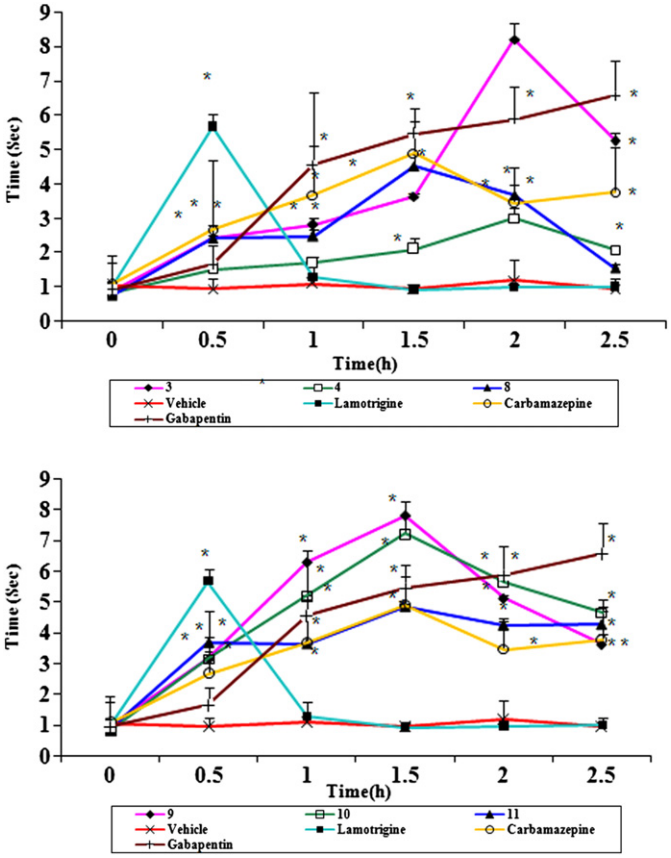


Fig. 3. Effect of compounds in reversal of the dynamic allodynia in CCI rats. The results are shown as mean paw withdrawal latency (mean PWL ± SEM) of 4 rats per group. $^{*}P < 0.05$, in comparison with the control values (ANOVA followed by post-hoc Bonferroni test).

methoxy was well tolerated while with fluoro a complete loss of bioactivity was observed. Replacement of the distal phenyl ring with a 2-nitrofuryl substituent analog 10, a compound found to be efficacious in all the four behavioral assays.

4. Conclusion

In conclusion, we have shown that the synthesized derivatives of furoic acid hydrazones produce antinociceptive actions in the peripheral nerve injury (CCI) model of neuropathic pain. The underlying mechanisms are expected to be blockade of sodium ion channel owing to various reports on the involvement of sodium ion channel in peripheral models of neuropathic pain and based on the literature evidence on the earlier report on furan amide as selective $Na_v1.8$ sodium channel blocker [4]. This study presents the report on the antiallodynic and antihyperalgesic activities of furoic acid hydrazones. Further research is required to confirm the hypothesized molecular mechanisms of action of the reported compounds.

5. Experimental protocols

5.1. Chemistry

Melting points were measured in open capillary tubes on a Buchi 530 melting point apparatus and are uncorrected. Infrared (IR) and proton nuclear magnetic resonance (^{1}H NMR) spectra were recorded for the compounds on Jasco IR Report 100 (KBr) and Brucker Avance (300 MHz) instruments, respectively. Chemical shifts are reported in parts per million (ppm) using tetramethyl silane (TMS) as an internal standard. All exchangeable protons were confirmed by addition of D_2O. Elemental analyses (C, H and N) were undertaken with a Perkin–Elmer model 240C analyzer and all analyses were consistent with theoretical values (within ±0.4%) unless indicated. The homogeneity of the compounds was monitored by ascending thin layer chromatography (TLC) on silica gel-G (Merck) coated aluminum plates, visualized by iodine vapor and UV light. Developing solvents were chloroform-methanol (9:1).

5.1.1. Synthesis of ethyl 5-(4-nitrophenyl)furan-2-carboxylate (**A**)

5-(4-Nitrophenyl) furan-2- carboxylic acid (2 gm, 8.58 mmol), ethanol (20 ml), concentrated sulphuric acid (1 ml) were taken in a round bottom flask and refluxed for 48 h. The reaction progress was monitored by TLC using a mixture of dichloromethane and methanol in the ratio of 9.2:0.8 as the mobile phase. The reaction mixture was then subjected to distillation to remove the excess ethanol and added in ice, filtered and dried to afford the titled compound with 79% yield and the compound melted at 155 °C. ^{1}H NMR (DMSO-d_6) δ (ppm): 1.27 (t, 3H, CH_3), 4.26 (q, 2H, CH_2), 7.3–7.4 (m, 2H, CH of furan), 7.4–8.4 (m, 4H, Ar–H). Calculated for $C_{13}H_{11}NO_5$: C, 59.77; H, 4.24; N, 5.36, O, 30.62; found: C, 60.01; H, 4.37; N, 5.39, O, 30.6.

5.1.2. Synthesis of 5-(4-nitrophenyl) furan-2-carbohydrazide (**B**)

Ethyl 5-(4-nitrophenyl) furan-2-carboxylate **(A)** (1 g, 3.83 mmol) was dissolved in methanol, hydrazine-hydrate (1 g, 20 mmol) was added and stirred at room temperature followed by microwave reflux for 10 min. The reaction progress was monitored by TLC using a mixture of DCM and methanol in the ratio 9.2:0.8 as the mobile phase. After the completion of reaction, the reaction mixture was

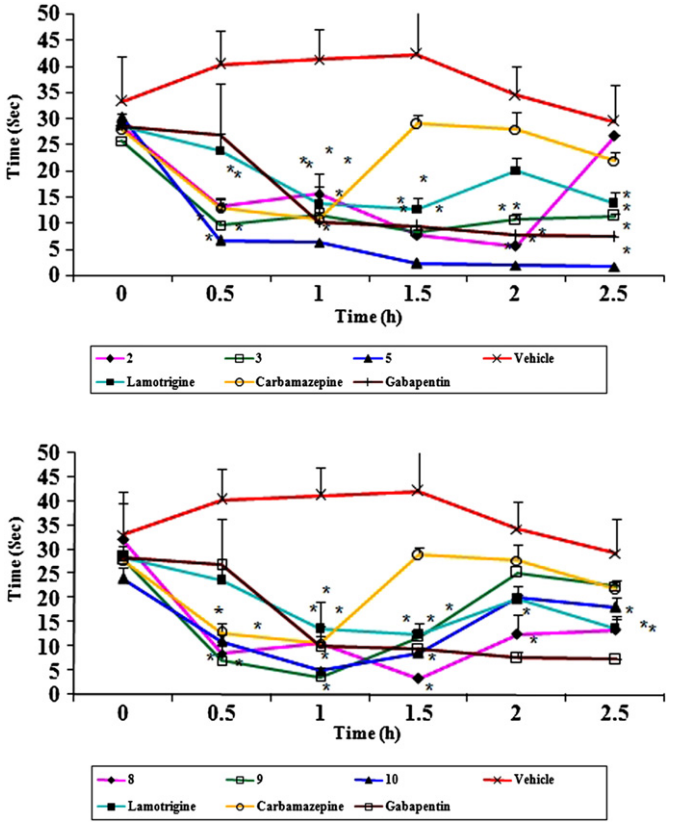


Fig. 4. Graphs show the effects of compounds (100 mg/kg, i.p.) against cold allodynia in CCI rats and Panel. The results are shown as either mean paw withdrawal duration (mean PWD ± SEM) of 4 rats per group. $^{*}P < 0.05$, in comparison with the control values (ANOVA followed by post-hoc Bonferroni test).

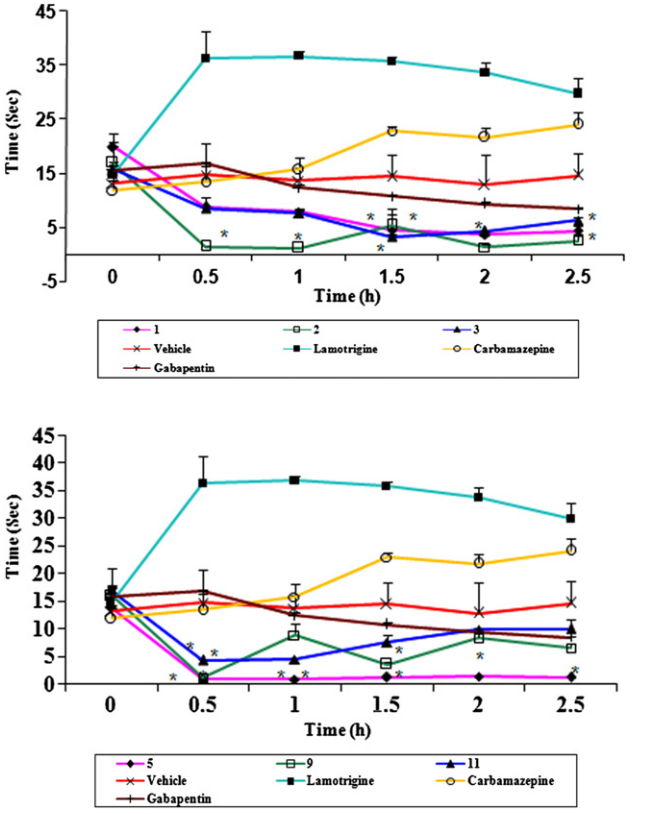


Fig. 5. Graphs depict the effects of compounds (100 mg/kg, i.p.) against mechanical hyperalgesia in CCI rats. The results are shown as mean paw withdrawal duration (mean PWD ± SEM) of 4 rats per group. $^{*}P < 0.05$, in comparison with the control values (ANOVA followed by post-hoc Bonferroni test).

then distilled to remove the excess alcohol and then the reaction mass was added to ice. It was then filtered and dried to afford the title compound with 64% yield and the compound melts at 176 °C. ^{1}H NMR (DMSO-d_6) δ (ppm): 2.2 (s, 2H, NH_2), 7.2–7.4 (m, 2H, CH of furan), 7.9–8.4 (m, 4H, Ar–H), 7.99 (s, 2H, NH). Calculated for $C_{11}H_9N_3O_4$: C, 59.77; H, 4.24; N, 5.36, O, 30.62; found: C, 60.01; H, 4.37; N, 5.39, O, 30.6.

*5.1.3. General procedure for the synthesis of acid hydrazones (**1**–**10**)*

5-(4-Nitrophenyl)furan-2-carbohydrazide (**B**) (1 g, 4.04 mmol) was dissolved in ethanol followed by the addition of equimolar quantity of the aldehyde or ketone. To this few drops of glacial acetic acid was added to lower the pH. It was then subjected to microwave radiation for 5–10 min. The reaction progress was monitored by TLC using a mixture of DCM and methanol in the ratio 9.2:0.8 as the mobile phase. After completion the reaction mass was dumped into cold water, the obtained precipitate was filtered and dried to give the final compound. The yields ranged from 46% to 70%. The physical data of the compounds are presented in Table 1. The structures were characterized by both spectral and elemental analysis and the data were within ±0.4% of the theoretical values. The IR spectra of the compounds were identical in the following aspects: 3300, 3035, 1680, 1550 cm^{-1}; ^{1}H NMR (DMSO-d_6, 400 MHz, δ ppm) spectra of some of the representative compounds are as follows.

*5.1.4. 5-(4-Nitrophenyl)-N′-(1-phenylethylidene)furan-2-carbohydrazide (**1**)*

IR (KBr): 3300, 3035, 1680, 1660, 1550, 1400, 1380, 1350 cm^{-1}; ^{1}H NMR (DMSO-d_6) δ (ppm): 7.15 (d, 1H, Ar–H), 7.25 (d, 1H, Ar–H), 7.31–7.39 (m, 4H, Ar–H), 7.76–7.83 (m, 4H, Ar–H), 8.22 (s, 1H, NH), 8.56 (s, 1H). Calculated for $C_{18}H_{13}N_3O_4$: C, 64.47; H, 3.91; N, 12.53, O, 19.09; found: C, 64.36; H, 3.97; N, 12.49, O, 19.18.

*5.1.5. N′-(4-methylbenzylidene)-5-(4-nitrophenyl)furan-2-carbohydrazide (**2**)*

IR (KBr): 3300, 3035, 1680, 1660, 1550, 1400, 1380, 1350 cm^{-1}; ^{1}H NMR (DMSO-d_6) δ (ppm): 2.53 (s, 3H), 7.15 (d, 1H, Ar–H), 7.25 (d, 1H, Ar–H), 7.31–7.39 (m, 4H, Ar–H), 7.83 (d, 2H, Ar–H), 8.1 (d, 2H, Ar–H), 8.1 (s, 1H, NH), 8.5 (s, 1H). Calculated for $C_{19}H_{15}N_3O_4$: C, 65.32; H, 4.33; N, 12.03, O, 18.32; found: C, 65.23; H, 4.39; N, 12.09, O, 18.29.

*5.1.6. N′-(4-bromobenzylidene)-5-(4-nitrophenyl)furan-2-carbohydrazide (**4**)*

IR (KBr): 3300, 3035, 1680, 1660, 1550, 1400, 1380, 1350, 780 cm^{-1}; ^{1}H NMR (DMSO-d_6) δ (ppm): 7.19 (d, 1H, Ar–H), 7.23 (d, 1H, Ar–H), 7.31–7.39 (m, 4H, Ar–H), 7.53–7.64 (m, 4H, Ar–H), 8.3 (s, 1H, NH), 8.6 (s, 1H). Calculated for $C_{18}H_{12}BrN_3O_4$: C, 52.19; H, 2.92; Br, 19.29 N, 10.14; O, 15.45; found: C, 52.23; H, 2.99; Br, 19.27; N, 10.09, O, 15.42.

*5.1.7. 5-(4-Nitrophenyl)-N′-(1-p-tolylethylidene)furan-2-carbohydrazide (**7**)*

IR (KBr): 3300, 3035, 1680, 1660, 1550, 1400, 1380, 1350 cm^{-1}; ^{1}H NMR (DMSO-d_6) δ (ppm): 2.53 (s, 3H), 2.59 (s, 3H), 7.19 (d, 1H, Ar–H), 7.23 (d, 1H, Ar–H), 7.29–7.35 (m, 4H, Ar–H), 7.83 (d, 2H, Ar–H), 8.4 (d, 2H, Ar–H), 8.1 (s, 1H, NH). Calculated for $C_{20}H_{17}N_3O_4$: C, 66.11; H, 4.72; N, 11.56, O, 17.61; found: C, 66.23; H, 4.69; N, 11.49, O, 17.59.

*5.1.8. N′-(5-nitrofuran-2-yl)methylene))-5-(4-nitrophenyl)furan-2-carbohydrazide (**10**)*

IR (KBr): 3300, 3035, 1680, 1660, 1550, 1400, 1360, 1350, 835 cm^{-1}; ^1H NMR (DMSO-d_6) δ (ppm): 7.10 (d, 1H, Ar–H), 7.22 (d, 1H, Ar–H), 7.38–7.42 (m, 2H, Ar–H), 7.79 (d, 2H, Ar–H), 7.93 (d, 2H, Ar–H), 8.13 (s, 1H, NH), 8.3 (s, 1H). Calculated for $C_{16}H_{10}N_4O_7$: C, 51.90; H, 2.72; N, 15.13, O, 30.25; found: C, 52.01; H, 2.61; N, 15.09, O, 30.29.

5.2. Pharmacology

Albino mice (Swiss strain, 20–25 g) and albino rats (Wistar, 200–320 g) of either sex were used as experimental animals. 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*. The synthesized compounds **1**–**10** were suspended in 30% v/v polyethylene glycol (PEG) 400. 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.

5.2.1. 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.

5.2.2. Pharmacological interventions

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 CCI rat models, were then administered the relevant drug according to a pre-determined randomization table and testing was re-performed at 0.5, 1, 1.5, 2 and 2.5 h post-drug administration. Each group of animals was used for only one drug administration protocol to ensure no 'carry-over' effects. Compounds **1**–**10** (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.

5.2.3. 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.

5.2.3.1. Spontaneous pain. Spontaneous pain was assessed for a total time period of 5 min as described previously by Choi et al. [17]. 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 was 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 [13].

5.2.3.2. 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. [18,19]. 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.

5.2.3.3. 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. [20]. 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 behavior 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.

5.2.3.4. Mechanical hyperalgesia. Mononeuropathic rats were assessed for mechanical hyperalgesia sensitivity according to the procedure described by Gonzalez et al. [21]. 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 pinprick 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.

5.2.4. Statistical analysis

All data are expressed as means ± standard error of mean (SEM). The data were analyzed using one-way ANOVA, and Bonferroni's

post-hoc for individual comparisons with the control values. *P* value of less than 0.05 was considered statistically significant. The statistical software package PRISM (Graphpad Software Inc, San Diego, CA) was used for the analyses.

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