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Discovery of tetrahydropyrido[4,3-*d*]pyrimidine derivatives for the treatment of neuropathic pain

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ABSTRACT

A series of tetrahydropyridopyrimidine derivatives were synthesized and evaluated for neurotoxicity and peripheral analgesic activity followed by assessment of antiallodynic and antihyperalgesic potential in two peripheral neuropathic pain models, the chronic constriction injury (CCI) and partial sciatic nerve ligation (PSNL). Compounds (**4b** and **4d**) exhibiting promising efficacies in four behavioral assays of allodynia and hyperalgesia (spontaneous pain, tactile allodynia, cold allodynia and mechanical hyperalgesia) were quantified for their ED_{50} values (15.12–65.10 mg/kg). Studies carried out to assess the underlying mechanism revealed that the compounds suppressed the inflammatory component of the neuropathic pain and prevented oxidative and nitrosative stress.

1. Introduction

Neuropathic pain is defined as an unpleasant sensory and emotional experience associated with actual or potential tissue damage or described in terms of such damage [1]. Neuropathic pain syndrome includes various diseases with heterogeneous etiology, e.g. peripheral nerve trauma (trigeminal neuralgia and phantom limb pain), neurotoxicity (cancer chemotherapy), infection (postherpetic neuralgia), infarct (stroke), and metabolic disturbances (diabetic neuropathy) [2]. The complex pathophysiology of neuropathic pain involves neuronal hyperexcitability in damaged areas of the peripheral or central nervous system characterized by allodynia (pain response to non-noxious stimuli) and hyperalgesia (exaggerated pain sensation) [3,4].

Following nerve injury, a cascade of events leads to complex inflammatory and immune mechanisms both in the periphery and the central nervous system [5]. Multiple etiologies, symptoms and complex underlying mechanisms make the treatment and management of neuropathic pain challenging [6]. The existing treatment for neuropathic pain includes anticonvulsants, antidepressants, opioids and topical agents which are not specifically indicated for neuropathic pain, and exhibit inadequate efficacy and adverse events profile [7–9].

The worldwide prevalence of neuropathic pain has imposed a burden on healthcare leading to the boom in discovery and development of many new targets which include various receptors, enzymes, transport systems, and membrane ion channels for neuropathic pain [10]. Literature review reveals various sodium channel blockers, calcium channel blockers, drugs effecting GABAergic system, NMDA, AMPA, and kainate antagonists under development for the treatment of neuropathic pain [11–14].

In recent years, numerous heterocycles with tetrahydropyridine framework have gained considerable attention in the treatment of neuropathic pain. Various tetrahydropyridopyrazoles [15], tetrahydropyridopyrimidines [16], tetrahydropyridoindoles [17], tetrahydropyridoquinazolines [18] have been found to embark antinociceptive efficacies. Literature reveals several amino and alkoxy tetrahydro-pyridopyrimidines based PDE10 inhibitors [19], P_2X_7 antagonists [20] having neuropathic pain activity. Several pyridopyrimidine derivatives mediating histaminergic pathways have also been reported for the alleviation of neuropathic pain.

In our interest to develop newer heterocyclic scaffolds for neuropathic pain, we have recently reported furoic acid hydrazones, isatin derivatives, piperazinyl derivatives, 1,2,4-triazol-5-ones, and fused triazolo-thiadiazoles as antinociceptive agents [21–25]. Hence in the course of our efforts towards the identification of new leads for the treatment of neuropathic pain, and in view of the above reports on pyridopyrimidines, synthesis of several substituted tetrahydropyridopyrimidines was accomplished followed by assessment of neuropathic pain activity and underlying mechanism of action.

Abbreviations: GABA, gamma-aminobytyric acid; NMDA, N-methyl-D-aspartate; AMPA, 2-amino-3-(3-hydroxy-5-methyl-isoxazol-4-yl)propanoic acid; PDE, phosphodiesterase; P2X7, purinoreceptor subtype; DCM, dichloromethane; THF, tetrahydrofuran; DMF, dimethyl formamide; ELISA, enzyme-linked immunosorbant assay.

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2. Experimental

2.1. Chemistry

2.1.1. General

The experimental procedures followed were as reported earlier [29]. Melting points were measured in open capillary tubes on a Buchi 530 melting point apparatus and are uncorrected. Proton nuclear magnetic resonance (^{1}H NMR) spectra were recorded for the compounds on Brucker Avance (300 MHz) instrument. Chemical shifts are reported in parts per million (ppm) using tetramethyl silane (TMS) as an internal standard. Mass spectra were measured with a Shimadzu GC–MS-QP5000 spectrophotometer. 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 silicagel-G (Merck) coated aluminum plates, visualized by iodine vapor and UV light.

2.1.2. Synthesis of N-substituted piperidin-4-one

One millimolar of 4-piperidone (1.0 equiv.) was taken in DCM (15–20 mL). To it, potassium carbonate (2.0 equiv.) was added followed by the addition of various alkyl, acid and sulphonyl halides (1.0 equiv.) at 0 °C. The reaction was stirred at room temperature overnight, diluted with water (40 mL), and neutralized to pH 6 with 0.1 N acetic acid. The solid was collected by filtration, washed with water, and dried under vacuum to provide the title compound.

2.1.3. Synthesis of methyl N-substituted-4-oxo-piperidine-3-carboxylate

A 500-mL, three-necked, round-bottomed flask was equipped with a mechanical stirrer, a reflux condenser, and a pressure-equalizing dropping funnel bearing a nitrogen inlet. The flask was flushed with nitrogen and charged with dimethyl carbonate (8.0 equiv.), 50 mL of anhydrous THF, and sodium hydride (2.2 equiv.). The suspension was stirred and heated to reflux temperature, when the slow, dropwise addition of N-substituted piperidin-4-ones (0.5 mmol) in 20 mL of dry THF was begun. The addition of N-substituted piperidin-4-ones was continued over a 1 h period. The mixture was stirred and heated at reflux for 6 h, cooled in an ice bath for 15–20 min, and hydrolyzed by slowly adding 75 mL of 3.0 M aqueous acetic acid. The contents of the flask were poured into 100 mL of saturated sodium chloride, and the aqueous mixture was extracted with (4 × 150) mL portions of DCM. The organic layers were combined, dried with anhydrous sodium sulfate, and concentrated at room temperature with a rotary evaporator. Distillation of the residual liquid under reduced pressure resulted in N-substituted-4-oxo-piperidine-3-carboxylate (79–87%) as a yellow liquid.

2.1.4. Synthesis of 2-amino-6-substituted-5,6,7,8-tetrahydropyridopyrimidin-4-ol

A solution of 0.4 mmol N-substituted-4-oxo-piperidine-3-carboxylate (1.0 equiv.) in DMF (5 mL) was treated with guanidine hydrochloride (2.0 equiv.) followed by the addition of potassium carbonate (2.0 equiv.), and the mixture was stirred at 110 °C for 16 h. The mixture was cooled to ambient temperature, diluted with water (40 mL), and neutralized to pH 6 with 0.1 N acetic acid. The solid was collected by filtration, washed with water, and dried under vacuum to provide the title compound.

2.1.5. Synthesis of 2-amino-6-substituted-5,6,7,8-tetrahydropyridopyrimidines***(1a–4d)***

0.2 mmol of substituted acids (1 equiv.) and 2-amino-6-substituted-5,6,7,8-tetrahydropyridopyrimidin-4-ols (1.0 equiv.) were taken in DCM (5 mL) followed by addition of triethylamine (1.1 equiv.), 1-ethyl-3-[3-(dimethylamino)propyl]carbodiimide hydrochloride (EDC) (1.1 equiv.) and 1-hydroxybenzotriazole (HOBT) (1.1 equiv.) at 0 °C. The reaction mixture was stirred at room temperature overnight. Following completion, the reaction mixture was washed with saturated aqueous sodium bicarbonate and brine. The organic layer was collected, dried over anhydrous sodium sulfate, filtered, and evaporated in vacuo to give the product in 78–85% yield.

*2.1.5.1. N-(6-Ethyl-4-hydroxy-5,6,7,8-tetrahydropyrido[4,3-d]pyrimidin-2-yl)-2-propyl pentanamide **(1a)**.* Yield = 78%; mp = 192–193 °C; ^{1}H NMR ($CDCl_3$) δ: 0.96–1.11 (t, *J* = 7.2 Hz, 6H), 1.23 (t, *J* = 7.6 Hz, 3H), 1.37–1.41 (m, 4H), 1.54–1.59 (q, 4H), 2.65–2.67 (t, *J* = 7.6 Hz, 1H), 2.92–3.23 (m, 4H), 3.73 (t, *J* = 7.2 Hz, 2H), 4.41 (s, 2H), 9.32 (br s, NH, D_2O exchangeable). MS (ESI) 320.4 $(M+H)^+$. Anal. ($C_{17}H_{28}N_4O_2$)C,H,N.

*2.1.5.2. N-(6-(2,6-Dimethylphenyl)-4-hydroxy-5,6,7,8-tetrahydropyrido[4,3-d]pyrimidin-2-yl)-4-nitrobenzamide **(1b)**.* Yield = 67%; mp = 201–202 °C; ^{1}H NMR ($CDCl_3$) δ: 1.23 (t, *J* = 7.2 Hz, 3H), 2.92–3.23 (m, 4H), 3.73 (t, *J* = 7.4 Hz, 2H), 4.41 (s, 2H), 8.11 (d, *J* = 7.2 Hz, 2H), 8.44 (d, *J* = 7.4 Hz, 2H), 9.11 (br s, NH, D_2O exchangeable). MS (ESI) 343.3 $(M+H)^+$. Anal. ($C_{16}H_{17}N_5O_4$)C,H,N.

*2.1.5.3. N-(6-Ethyl-4-hydroxy-5,6,7,8-tetrahydropyrido[4,3-d]pyrimidin-2-yl)-4-phenoxy-benzamide **(1c)**.* Yield = 71%; mp = 169–170 °C; ^{1}H NMR ($CDCl_3$) δ: 1.24 (t, *J* = 7.2 Hz, 3H), 2.91–3.21 (m, 4H), 3.71 (t, *J* = 7.6 Hz, 2H), 4.42 (s, 2H), 7.14–7.17 (m, 3H), 7.32–7.41 (m, 4H), 8.01 (d, *J* = 7.6 Hz, 2H), 9.41 (br s, NH, D_2O exchangeable). MS (ESI) 390.4 $(M+H)^+$. Anal. ($C_{22}H_{22}N_4O_3$)C,H,N.

*2.1.5.4. N-(6-Ethyl-4-hydroxy-5,6,7,8-tetrahydropyrido[4,3-d]pyrimidin-2-yl)isonicotinamide **(1d)**.* Yield = 72%; mp = 193–194 °C; ^{1}H NMR ($CDCl_3$) δ: 1.23 (t, *J* = 7.4 Hz, 3H), 2.92–3.23 (m, 4H), 3.73 (t, *J* = 7.6 Hz, 2H), 4.41 (s, 2H), 7.81 (d, *J* = 7.2 Hz, 2H), 8.89 (d, *J* = 7.4 Hz, 2H), 9.51 (br s, NH, D_2O exchangeable). MS (ESI) 390.4 $(M+H)^+$. Anal. ($C_{22}H_{22}N_4O_3$)C,H,N.

*2.1.5.5. N-(6-(2,6-Dimethylphenyl)-4-hydroxy-5,6,7,8-tetrahydropyrido[4,3-d]pyrimidin-2-yl)-2-propylpentanamide **(2a)**.* Yield = 63%; mp = 231–232 °C; ^{1}H NMR ($CDCl_3$) δ: 0.96–1.11 (t, *J* = 7.8 Hz, 6H), 1.23 (t, *J* = 7.4 Hz, 3H), 1.37–1.41 (m, 4H), 1.54–1.59 (q, 4H), 2.14 (s, 6H), 2.65–2.67 (t, *J* = 7.2 Hz, 1H), 2.92–3.23 (m, 4H), 3.73 (t, *J* = 7.4 Hz, 2H), 4.41 (s, 2H), 6.76 (t, *J* = 7.4 Hz, 1H), 7.03 (d, *J* = 7.4 Hz, 2H), 9.11 (br s, NH, D_2O exchangeable). MS (ESI) 396.5 $(M+H)^+$. Anal. ($C_{23}H_{32}N_4O_2$)C,H,N.

*2.1.5.6. N-(6-(2,6-Dimethylphenyl)-4-hydroxy-5,6,7,8-tetrahydropyrido[4,3-d]pyrimidin-2-yl)-4-nitrobenzamide **(2b)**.* Yield = 65%; mp = 201–202 °C; ^{1}H NMR ($CDCl_3$) δ: 2.13 (s, 6H), 2.91 (t, *J* = 7.4 Hz, 2H), 3.71 (t, *J* = 7.6 Hz, 2H), 4.52 (s, 2H), 6.76 (t, *J* = 7.4 Hz, 1H), 7.03 (d, *J* = 7.2 Hz, 2H), 7.14–7.17 (m, 3H), 7.32–7.41 (m, 4H), 8.01 (d, *J* = 7.2 Hz, 2H), 9.41 (br s, NH, D_2O exchangeable). MS (ESI) 415.4 $(M+H)^+$. Anal. ($C_{22}H_{21}N_5O_4$)C,H,N.

*2.1.5.7. N-(6-(2,6-Dimethylphenyl)-4-hydroxy-5,6,7,8-tetrahydropyrido[4,3-d]pyrimidin-2-yl)-4-phenoxybenzamide **(2c)**.* Yield = 61%; mp = 190–191 °C; ^{1}H NMR ($CDCl_3$) δ: 2.13 (s, 6H), 2.91 (t, *J* = 7.2 Hz, 2H), 3.71 (t, *J* = 7.4 Hz, 2H), 4.52 (s, 2H), 6.76 (t, *J* = 7.6 Hz, 1H), 7.03 (d, *J* = 7.2 Hz, 2H), 8.11 (d, *J* = 7.4 Hz, 2H),

8.44 (d, J = 7.6 Hz, 2H), 9.11 (br s, NH, D_2O exchangeable. MS (ESI) 466.5 $(M+H)^+$. Anal. ($C_{28}H_{26}N_4O_3$)C,H,N.

2.1.5.8. N-(6-(2,6-Dimethylphenyl)-4-hydroxy-5,6,7,8-tetrahydropyrido[4,3-d]pyrimidin-2-yl) isonicotinamide ***(2d)***. Yield = 77%; mp = 198–199 °C; ^1H NMR ($CDCl_3$) δ: 2.13 (s, 6H), 2.91 (t, J = 7.4 Hz, 2H), 3.71 (t, J = 7.2 Hz, 2H), 4.52 (s, 2H), 6.76 (t, J = 7.8 Hz, 1H), 7.03 (d, J = 7.2 Hz, 2H), 7.81 (d, J = 7.2 Hz, 2H), 8.89 (d, J = 7.2 Hz, 2H), 9.11 (br s, NH, D_2O exchangeable). MS (ESI) 374.4 $(M+H)^+$. Anal. ($C_{21}H_{21}N_5O_2$)C,H,N.

2.1.5.9. N-(4-Hydroxy-6-(4-methylbenzoyl)-5,6,7,8-tetrahydropyrido[4,3-d]pyrimidin-2-yl)-2-propylpentanamide ***(3a)***. Yield = 78%; mp = 189–190 °C; ^1H NMR ($CDCl_3$) δ: 0.96–1.11 (t, J = 7.8 Hz, 6H), 1.37–1.41 (m, 4H), 1.54–1.59 (q, 4H), 2.34 (s, 3H), 2.53 (q, 1H), 2.98 (t, J = 7.2 Hz, 2H), 3.79 (t, J = 7.4 Hz, 2H), 4.62 (s, 2H), 7.41 (d, J = 7.6 Hz, 2H), 7.93 (d, J = 7.8 Hz, 2H), 9.61 (br s, NH, D_2O exchangeable). MS (ESI) 410.5 $(M+H)^+$. Anal. ($C_{23}H_{30}N_4O_3$)C,H,N.

2.1.5.10. N-(4-Hydroxy-6-(4-methylbenzoyl)-5,6,7,8-tetrahydropyrido[4,3-d]pyrimidin-2-yl)-4-nitrobenzamide ***(3b)***. Yield = 71%; mp = 209–210 °C; ^1H NMR ($CDCl_3$) δ: 2.35 (s, 3H), 2.98 (t, J = 7.4 Hz, 2H), 3.79 (t, J = 7.4 Hz, 2H), 4.62 (s, 2H), 7.41 (d, J = 7.8 Hz, 2H), 7.91 (d, J = 7.2 Hz, 2H), 8.11 (d, J = 7.4 Hz, 2H), 8.44 (d, J = 7.8 Hz, 4H), 9.54 (br s, NH, D_2O exchangeable). MS (ESI) 433.4 $(M+H)^+$. Anal. ($C_{22}H_{19}N_5O_5$)C,H,N.

2.1.5.11. N-(4-Hydroxy-6-(4-methylbenzoyl)-5,6,7,8-tetrahydropyrido[4,3-d]pyrimidin-2-yl)-4-phenoxybenzamide ***(3c)***. Yield = 60%; mp = 231–232 °C; ^1H NMR ($CDCl_3$) δ: 2.35 (s, 3H), 2.98 (t, J = 7.2 Hz, 2H), 3.79 (t, J = 7.6 Hz, 2H), 4.62 (s, 2H), 7.14–7.17 (m, 3H), 7.32–741 (m, 6H), 7.98 (d, J = 7.6 Hz, 2H), 8.01 (d, J = 7.6 Hz, 2H), 9.44 (br s, NH, D_2O exchangeable). MS (ESI) 480.5 $(M+H)^+$. Anal. ($C_{28}H_{24}N_4O_4$)C,H,N.

2.1.5.12. N-(4-Hydroxy-6-(4-methylbenzoyl)-5,6,7,8-tetrahydropyrido [4,3-d]pyrimidin-2-yl) isonicotinamide ***(3d)***. Yield = 70%; mp = 163–164 °C; ^1H NMR ($CDCl_3$) δ: 2.35 (s, 3H), 2.98 (t, J = 7.4 Hz, 2H), 3.79 (t, J = 7.6 Hz, 2H), 4.62 (s, 2H), 7.41 (d, J = 7.4 Hz, 2H), 7.91 (d, J = 7.2 Hz, 2H), 7.81 (d, J = 7.8 Hz, 2H), 8.89 (d, J = 7.2 Hz, 4H), 9.54 (br s, NH, D_2O exchangeable). MS (ESI) 389.4 $(M+H)^+$. Anal. ($C_{21}H_{19}N_5O_3$)C,H,N.

2.1.5.13. N-(4-Hydroxy-6-tosyl-5,6,7,8-tetrahydropyrido[4,3-d]pyrimidin-2-yl)-2-propyl-pentanamide ***(4a)***. Yield = 61%; mp = 180–181 °C; ^1H NMR ($CDCl_3$) δ: 0.96–1.11 (t, J = 7.4 Hz, 6H), 1.37–1.41 (m, 4H), 1.54–1.59 (q, 4H), 2.44 (s, 6H), 2.53–2.57 (t, J = 7.6 Hz, 1H),), 2.78 (t, J = 7.4 Hz, 2H), 3.63 (t, J = 7.2 Hz, 2H), 4.42 (s, 2H), 7.40 (d, J = 7.2 Hz, 2H), 7.74 (d, J = 7.2 Hz, 2H), 9.11 (br s, NH, D_2O exchangeable). MS (ESI) 446.5 $(M+H)^+$. Anal. ($C_{22}H_{30}N_4O_4S$)C,H,N.

2.1.5.14. N-(4-Hydroxy-6-tosyl-5,6,7,8-tetrahydropyrido[4,3-d]pyrimidin-2-yl)-4-nitrobenzamide ***(4b)***. Yield = 59%; mp = 195–196 °C; ^1H NMR ($CDCl_3$) δ: 2.35 (s, 3H), 2.78 (t, J = 7.6 Hz, 2H), 3.63 (t, J = 7.4 Hz, 2H), 4.42 (s, 2H), 7.41 (d, J = 7.6 Hz, 2H), 7.75 (d, J = 7.2 Hz, 2H), 8.11 (d, J = 7.4 Hz, 2H), 8.44 (d, J = 7.8 Hz, 4H), 9.54 (br s, NH, D_2O exchangeable). MS (ESI) 469.47 $(M+H)^+$. Anal. ($C_{21}H_{19}N_5O_6S$)C,H,N.

2.1.5.15. N-(4-Hydroxy-6-tosyl-5,6,7,8-tetrahydropyrido[4,3-d]pyrimidin-2-yl)-4-phenoxy benzamide ***(4c)***. Yield = 81%; mp = 198–199 °C; ^1H NMR ($CDCl_3$) δ: 2.35 (s, 3H), 2.78 (t, J = 7.4 Hz, 2H), 3.69 (t, J = 7.2 Hz, 2H), 4.42 (s, 2H), 7.14–7.17 (m, 3H), 7.32–741 (m, 6H), 7.74 (d, J = 7.6 Hz, 2H), 8.01 (d, J = 7.8 Hz, 2H), 9.44 (br s, NH, D_2O, exchangeable). MS (ESI) 516.5$(M+H)^+$. Anal. ($C_{27}H_{24}N_4O_5S$)C,H,N.

2.1.5.16. N-(4-Hydroxy-6-tosyl-5,6,7,8-tetrahydropyrido[4,3-d]pyrimidin-2-yl)isonicotinamide ***(4d)***. Yield = 82%; mp = 205–206 °C; ^1H NMR ($CDCl_3$) δ: 2.35 (s, 3H), 2.78 (t, J = 7.4 Hz, 2H), 3.69 (t, J = 7.2 Hz, 2H), 4.42 (s, 2H), 7.41 (d, J = 7.8 Hz, 2H), 7.81–7.85 (m, 4H), 8.89 (d, 4H), 9.54 (br s, NH, D_2O exchangeable). MS (ESI) 429.4 $(M+H)^+$. Anal. ($C_{20}H_{19}N_5O_4S$)C,H,N.

2.2. Pharmacology

The detailed methodology has also been described in earlier report [29]. Swiss albino mice (either sex) with weights ranging from 20–25 g were used for the assessment of neurotoxicity, acetic acid induced writhing and formalin induced flinching model. Wistar rats of either sex (200–250 g) were used for the inflammatory and neuropathic pain models. All experiments were approved by the Institutional Animal Ethics Committee of BITS-Pilani, Hyderabad campus with a protocol number IAEC/RES/2/1. 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*.

2.2.1. Motor impairment

Minimal motor impairment was measured in mice by the rotarod test. The mice were trained to stay on an accelerating rotarod that rotates at 10 rpm. The rod diameter was 3.2 cm. Neurotoxicity was indicated by the inability of the animal to maintain equilibrium on the rod for at least 1 min in each of the three trials [26].

2.2.2. Acetic acid induced writhing

Writhing was induced in a group of mice by an intraperitoneal injection of 0.1 mL of 2% v/v acetic acid. Test group mice received acetic acid half an hour after the administration of test compounds. The number of writhings occurring for a period of 30 min 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 calculated [27].

2.2.3. Formalin induced flinching

The test involved intra-plantar injection of 25 μL of 1% formalin into the hind paw of mice, which resulted in flinches in the paw in the early phase (0–5 min) and the late phase (10–30 min) [28]. Time spent in paw licking and biting was monitored in each 5 min and calculated for both the phases. Test compounds were administered 30 min before the experiment.

2.2.4. Unilateral mononeuropathy–Chronic constriction nerve injury (CCI) model

Unilateral mononeuropathy was produced in rats using the CCI model performed essentially as described by Bennett and Xie [29]. The rats were anesthetized with an intraperitoneal dose of ketamine (55 mg/kg) and xylazine (5 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 at the mid-thigh level. 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 made with a 4–0 braided silk suture around the sciatic nerve with about 1-mm spacing. 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.

2.2.5. Induction of peripheral mononeuropathy–Partial sciatic nerve ligation model

As described by Seltzer et al. [30], in anaesthetized rats, left sciatic nerve was exposed at mid-thigh level through small incision, cleared of adhering muscle tissue, and one-half of the nerve thickness was tightly ligated using 7.0 silk suture. The wound was closed and dusted with neomycin powder. The animals were then transferred to their home-cages and left for recovery.

2.2.6. Sensory testing using nociceptive assays

Compounds (100 mg/kg, i.p.) were administered at $t = 0$, in 30% v/v PEG 400. The control group of rats received only the vehicle. Gabapentin (100 mg/kg, i.p.) was used as positive control. Paw withdrawal duration (PWD) was observed in spontaneous pain and cold allodynia and paw withdrawal threshold (PWT) was assessed in tactile allodynia and mechanical hyperalgesia. Percentage reversal in spontaneous pain, allodynia or hyperalgesia was calculated for each animal as defined below [31]

$$\%\text{Reversal} = \frac{(\text{post dose value} - \text{pre dose value})}{(\text{contralateral paw value} - \text{pre dose value})} \times 100$$

2.2.6.1. Spontaneous pain. Spontaneous pain was assessed for a total time period of 5 min as described previously by Choi et al. 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. The test consisted of noting the cumulative duration for which the rat holds its ipsilateral paw off the floor. The paw lifts associated with locomotion or body repositioning was not counted [32].

2.2.6.2. Tactile allodynia. Paw withdrawal in response to mechanical stimuli was measured using Von Frey filaments (UGO Basile, Italy). Each rat was placed on a metallic mesh floor covered with a plastic box. A set of von frey monofilaments (0.4–15 g), with intensities of mechanical stimulation increasing in graded manner with successively greater diameter filaments, were applied to the plantar surface of the hind paw five times at intervals of 1–2 s [33]. The weakest force (g) inducing withdrawal of the stimulated paw at least three times was taken as the paw withdrawal threshold with cut off value at 15 g.

2.2.6.3. Cold allodynia. 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. 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 [34].

2.2.6.4. Mechanical hyperalgesia. Mechanical paw withdrawal thresholds were assessed with a slightly modified version of the Randall–Selitto method [35] using analgesymeter (UGO Basile, Italy). The instrument exerts a force that increases at a constant rate. This force was applied to the hind paw of the rat, which was placed on a small plinth under a cone-shaped pusher with a rounded tip (1.5 mm in diameter) until the animal withdrew its paw. A cut off of 250 g was used to avoid injury. Mechanical paw withdrawal thresholds were calculated as the average of two consecutive measurements.

2.2.7. Carrageenan-induced paw edema

Paw edema was induced in wistar rats by intra-plantar injection of 50 μL of 2% carrageenan (λ-carrageenan, type IV, Sigma) diluted in saline. The volume of the paw edema (mL) was determined at 0, 60, 120 and 180 min using a water plethysmometer (Ugo Basile, Italy) Indomethacin (10 mg/kg, i.p.) was used as positive control [36]. The percentage protection against inflammation was calculated as: $V_c - V_d/V_c \times 100$, where V_c is the increase in paw volume in the absence of the test compound (control) and V_d is the increase of paw volume after administration of the test compound.

2.2.8. Estimation of total nitrite/nitrate

On 9th day post chronic constriction injury, after 2 h of administration of test compounds, the total nitrate/nitrite in brain and sciatic nerve was estimated according to the reported procedure [37]. The method involved reduction of nitrate to nitrite followed by calorimetric estimation using Griess reagent. The concentration of nitrite in the supernatant was calculated using standard curve and expressed as percentage of control.

2.2.9. DPPH (1,1-Diphenyl-2-picrylhydrazyl) assay

A solution of DPPH was prepared by dissolving 5 mg of DPPH in 2 mL of methanol, and the solution was kept in the dark at 4 °C. Varying concentrations of test compounds (200 μL) were taken in 96-well microplate. Then, 5 μL of methanolic DPPH solution (final concentration 300 μM) was added to each well. After 20 min of incubation, absorbance of the solution was read using an ELISA Reader (EL340 Biokinetic reader, Bio-Tek Instrumentation, USA) at a wavelength of 517 nm. A methanolic solution of DPPH served as a control. A dose response curve was plotted to determine the IC_{50} values. All tests and analyses were run in triplicate and averaged [38].

Percentage scavenging was calculated according to the following equation:

$$\%\text{scavenging} = \frac{\{\text{Absorbance(DPPH)} - \text{Absorbance(DPPH} + \text{compound)}\}}{\text{Absorbance(DPPH)}} \times 100$$

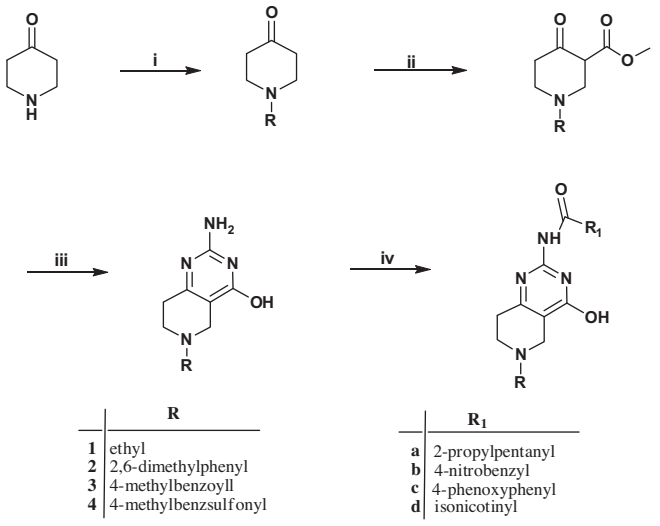
3. Results and discussion

3.1. Chemistry

Various alkyl, acid and sulphonyl halides were reacted with 4-piperidone to form N-substituted piperidin-4-one. Methoxycarbonylation of the same in the presence of sodium hydride afforded the ketoester which underwent base catalyzed reaction with guanidine to form substituted tetrahydropyridopyrimidine [16,18]. Utilizing various alkyl, aryl and hetroaryl acids, the acid amine coupling was then carried out with with the aid of 1-ethyl-3-[3-(dimethylamino)propyl]carbodiimide hydrochloride (EDC) and 1-hydroxy benzotriazole (HOBT) to form the titled compounds (Scheme 1).

3.2. Pharmacology

Compounds were assessed for neurotoxicity in the rotarod, followed by their evaluation in acute models of pain. Among all the compounds administered at the doses of 30, 100 and 300 mg/kg, compound **4e** exhibited motor impairment at 300 mg/kg dose. In acetic acid test, compounds **1b**, **1c**, **3a**, **3b**, **3d**, **4b** and **4d** suppressed the acetic acid induced writhing response significantly ($p < 0.05$) in comparison to the control (Table 1). The standard drug indomethacin exhibited the highest percentage inhibition (96.12%). In formalin induced flinching model, significant reversal



Scheme 1. Synthetic route to substituted tetrahydropyridopyrimidines. Reagents and conditions: (i) CH_2Cl_2, RX/RCOCl/RSO_2Cl, 0 °C-rt; (ii) dimethyl carbonate, NaH, THF reflux, 6 h; (iii) guanidine, K_2CO_3, DMF, reflux, 18 h; (iv) R_1COOH, Et_3N, EDC-HCl, HOBT, CH_2Cl_2, 0 °C-rt, 24 h.

of phase-I was exhibited by compounds **2b** with 53.0% inhibition. In Phase-II, all the tested compounds except **1a**, **2b**, **2d**, **3b**, **3c**, **3d**, **4b** and **4c** showed significant reversal. Compound **4d** exhibited highest reversal of 80.2%, being more efficacious than indomethacin (67.14%) (Table 1).

Compounds were further evaluated in two well-established peripheral neuropathic pain models–the chronic constriction nerve injury (CCI) and the partial sciatic nerve ligation (PSNL) model, where pain was assessed by four nociceptive tests namely spontaneous pain, tactile 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 spontaneous pain, tactile 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. Animals displaying allodynic and hyperalgesic responses in both the models, were then administered the test compound (single dose of 100 mg/kg) according to a pre-determined randomization table and testing was re-performed at 30, 60 and 120 min post drug administration. All of the behavioral responses were timed with a stopwatch.

In the CCI model, compounds **1c**, **1d**, **4a**, **4b** and **4d** completely reversed the spontaneous pain response throughout the time period of testing (30–120 min) similar to gabapentin. Compounds **1a**, **1b**, **2a**, **2b** and **3d** exhibited activity up to 60 min. The onset of action of compound **2d** was at 120 min. Other compounds were ineffective in this test (Fig. 1). Two compounds **4b** and **4d** were active in attenuating the tactile allodynia throughout the 120 min experiment. The onset of action of compounds **1d**, **2b** and **3d** was at 60 min similar to gabapentin. The duration of action of compounds **1c** and **4c** was up to 30 min, whereas for **4a** duration of action was up to 60 min. All other compounds were ineffective in this test. In the cold allodynia produced in CCI rats, significant reversal of paw withdrawal durations was observed at all time points by the administration of compounds **1c**, **1d**, **2a**, **4a**, **4b** and **4d**. Gabapentin was also found to be effective at all the time points. The onset of action for compounds **2b** and **3d** was at 60 min. Mechanical hyperalgesia was significantly attenuated at all the time points by **4b** and **4d** similar to gabapentin. The onset of action of compounds **1c** and **2b** was at 60 min. The compounds **1d**, **2a** and **3a** were active in only first 30 min of the 120 min experiment (Fig. 1).

In the PSNL model, the paw withdrawal durations due to spontaneous ongoing pain were significantly reduced by compounds **1c**, **2a**, **4b** and **4d** throughout the experiment similar to gabapentin. The onset of action for compounds **1b**, **1d**, **2b**, **2d** and **4a** was at 60 min. **1a** and **3d** exhibited irregular activity pattern, being effective at only one time point (60 min). The tactile allodynia produced in PSNL model was effectively reversed by compounds **4b** and **4d** at all the time points like gabapentin. Compound **1c** was effective up to 30 min in 120 min experiment. Compound **2d** exhibited irregular activity pattern, being effective at only one time point (60 min).

Table 1
Neurotoxicity and acute antinociceptive efficacy of tetrahydropyridopyrimidines.

Compound	Neurotoxicity[a]		Acetic acid induced writhing[b]	Formalin induced flinching[b] % Inhibition	
	0.5 h	4 h	% Inhibition	Phase-I	Phase-II
Control	–	–	–	–	–
1a	–	–	40.5	25.2	50.3*
1b	–	–	59.4*	30.4	21.0
1c	–	–	61.3*	17.4	22.4
1d	–	–	46.2	20.9	43.5
2a	–	–	46.3	25.3	15.6
2b	–	–	17.9	53.0*	55.7*
2c	–	–	11.3	4.3	24.5
2d	–	–	46.2	18.3	54.7*
3a	–	–	65.0*	15.7	40.8
3b	–	–	61.3*	14.8	70.1*
3c	–	–	46.2	13.9	56.5*
3d	–	–	61.8*	19.1	64.6*
4a	300	300	21.7	43.4	29.9
4b	–	–	75.4*	15.7	70.1*
4c	–	–	46.2	37.4	43.0
4d	–	–	74.5*	28.7	80.2*
Indomethacin[c]	–	–	96.1*	4.7	72.1*

[a] Doses of 30, 100 and 300 mg/kg were administered. The figures in the table indicate the minimum dose whereby bioactivity was demonstrated in half or more of the mice (three in each group). The animals were examined at 0.5 and 4 h. The line (–) indicates an absence of neurotoxicity at the maximum dose tested.
[b] Vehicle treated animals received 30% v/v PEG 400 in water.
[c] Indomethacin was taken as positive control at 5 mg/kg.
* Represents significance (> than 50%) at $p < 0.05$ compared to vehicle (One way ANOVA followed by Dunnett's Test, $n = 4$) at a dose of 100 mg/kg.

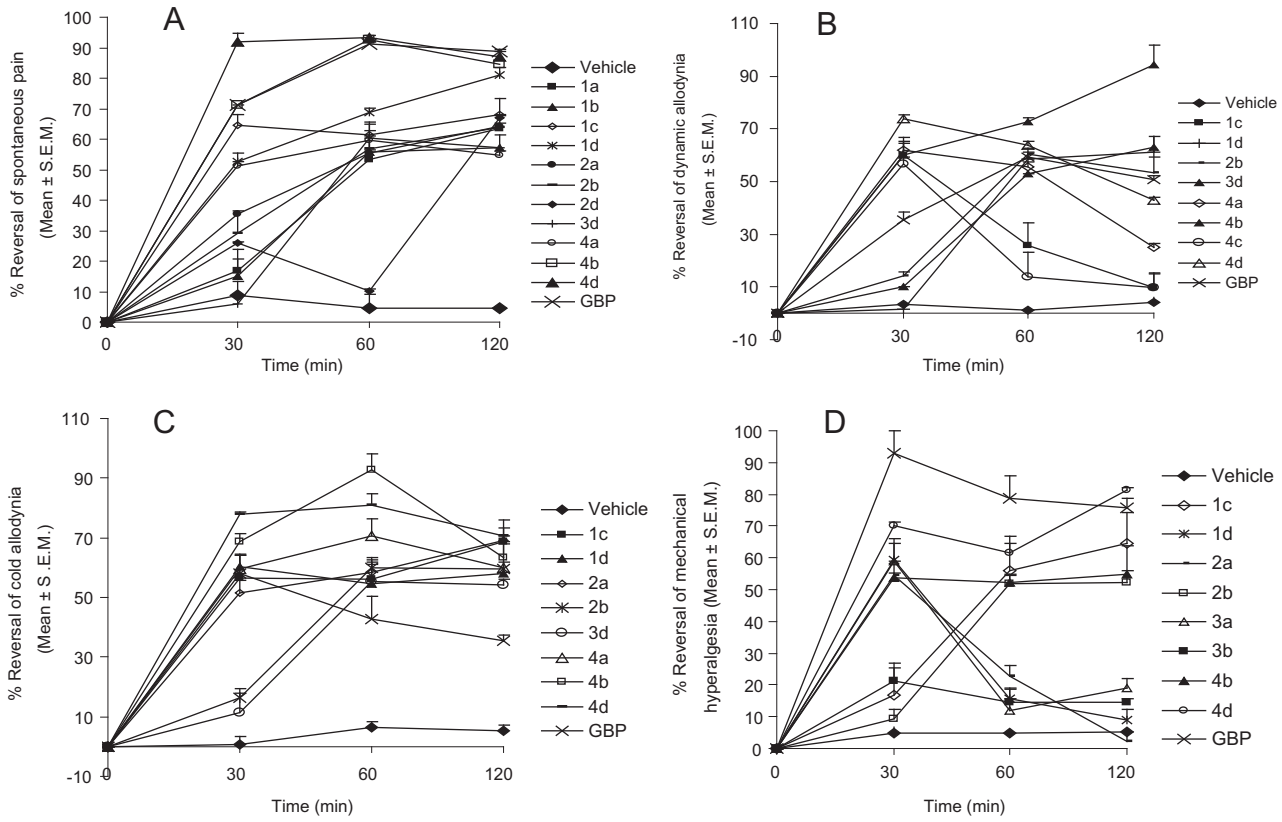


Fig. 1. Efficacy of compounds in spontaneous pain (A), tactile allodynia (B), cold allodynia (C) and mechanical hyperalgesia (D) in CCI rats. Each value represents the% reversal (mean ± SEM) in spontaneous pain, tactile allodynia, cold allodynia and mechanical hyperalgesia of four rats; ∗ denotes significant value, in comparison to their respective vehicle control at $p < 0.05$ (one-way ANOVA, followed by post-hoc Dunnett's test).

The onset of action for compounds **1d**, **2a**, **2b**, **2c** and **4a** was at 60 min. Cold allodynia produced in the PSNL model was completely reversed by the compounds **1d**, **2b**, **4b** and **4d**. Compounds **3b** and **3d** were effective up to 30 min of the experiment. The onset of action for compounds **1c**, **2a** and **4a** was at 60 min. Compounds **2b**, **4b** and **4d** significantly reversed mechanical hyperalgesia at all the time points like gabapentin. Compounds **3a** and **3d** were effective in first 30 min of the experiment whereas compounds **1c**, **1d** and **2a** were effective till 60 min of the 120 min experiment (Fig. 2).

A closer look of the structure activity relationship studies revealed that isonicotinyl substitution in compounds **1d**, **2d**, **3d** and **4d** resulted in significant attenuation of one or more nociceptive parameters in both CCI and PSNL animals. 4-nitrobenzyl substitution also proved to be additive to the antinociceptive efficacies in case of compounds **1b**, **2b**, **3b** and **4b** in both CCI and PSNL animals. Aliphatic substituent (2-propylpentanyl) in compounds **1a**, **2a**, **3a** and **4a** resulted in comparatively moderate attenuation of nociceptive parameters in neuropathic animals. However, 4-phenoxyphenyl substitution in compounds **1c**, **2c**, **3c** and **4c** proved to be detrimental for the efficacy. The general trend in antinociceptive efficacy with respect to R_1 substitution was: isonicotinyl > 4-nitrobenzyl > 2-propylpentanyl > 4-phenoxyphenyl substitution.

N-alkyaltion of the tetrahydropyridine moiety results in compounds with varied antinociceptive efficacies. The order of bioactivity of various N-alkylated tetrahydropyridopyrimidines (R substitution) in neuropathic pain model was 4-methylbenzsulfonyl > 4-methylbenzoyl > 2,6-dimethylphenyl > Ethyl.

Compounds exhibiting more than 90% reversal in one or more of the nociceptive assays (**4b** and **4d**) were taken further for ED_{50} studies. In the CCI model, compound **4d** reversed spontaneous pain with an ED_{50} value of 21.84 mg/kg at 60 min (Table 2). Compound **4b** reversed tactile and cold allodynia with an ED_{50} value of 18.24 mg/kg and 42.73 mg/kg at 120 min and 60 min respectively. Compound **4b** reversed mechanical hyperalgesia with an ED_{50} value of 26.29 mg/kg at 60 min. In PSNL model, compound **4b** reversed spontaneous pain and tactile allodynia with an ED_{50} value of 15.12 mg/kg and 41.13 mg/kg both at 60 min respectively. In cold allodynia and mechanical hyperalgesia, compound **4d** came out to be the most effective compound with an ED_{50} value of 38.21 mg/kg and 55.53 mg/kg at 60 min and 120 min respectively.

The anti-inflammatory profile of the selected compounds (**4b** and **4d**) as evidenced in formalin induced flinching test was investigated in the carrageenan induced paw edema model. **4d** showed a significant reduction in paw edema throughout the period of 180 min (Table 3). Following nerve injury, subsequent generation of free radicals leads to oxidative and nitrosative stress which exaggerates pain states. The putative role of nitric oxide (NO) in the pathophysiology of chronic nerve ligation as evident by significant increase in nitrite and nitrate levels in both brain and sciatic nerve led us to estimate the levels of nitrite, metabolite of NO in brain and sciatic nerve of CCI rats. Compound **4d** exhibited significant reduction ($p < 0.05$) of nitric oxide in sciatic nerve (Table 3). None of the compounds significantly attenuated nitrosative stress in the brain suggesting the inhibition of peripheral nitric oxide. The free radical scavenging abilities of the compounds (**4b** and **4d**) were assessed using DPPH method. Compound **4b** exhibited IC_{50}

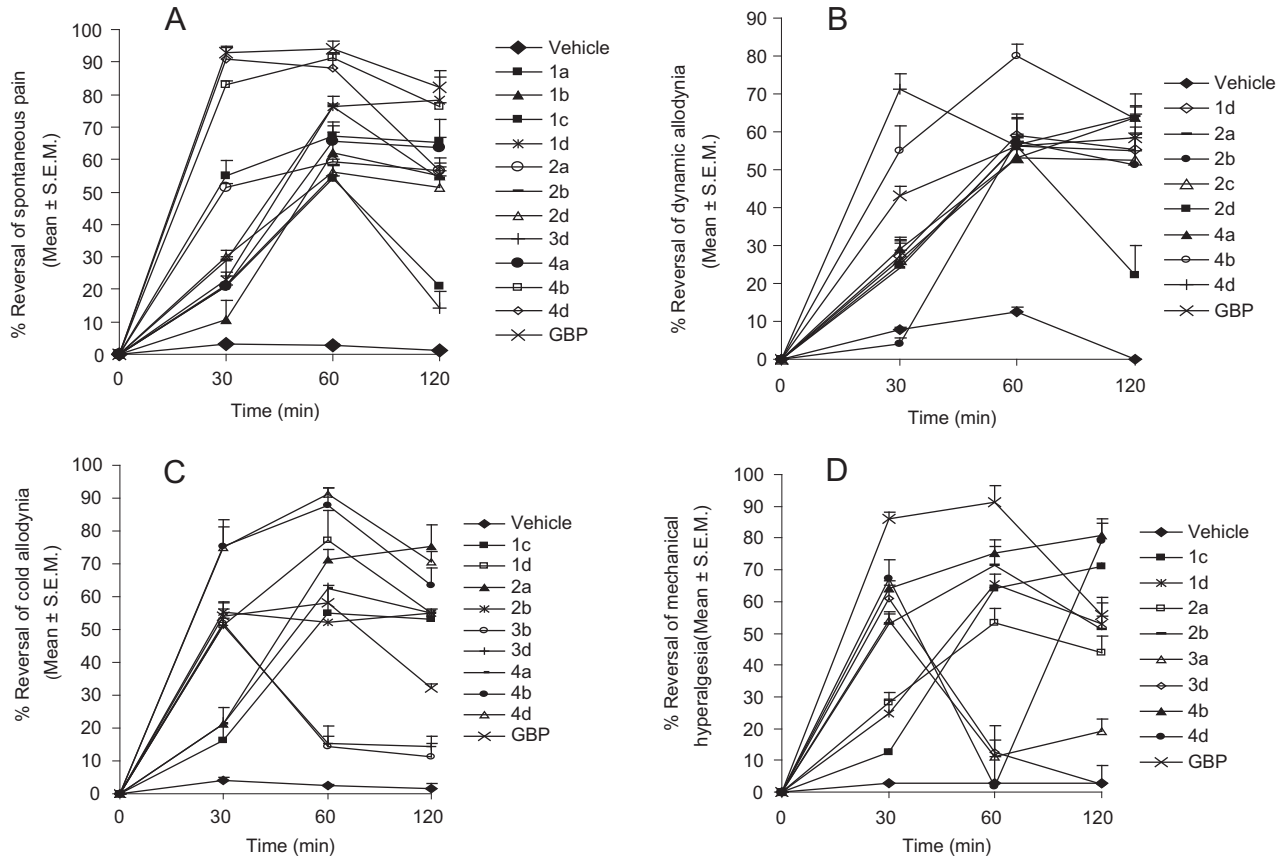


Fig. 2. Efficacy of compounds in spontaneous pain (A), tactile allodynia (B), cold allodynia (C) and mechanical hyperalgesia (D) in PSNL rats. Each value represents the% reversal (mean ± SEM) in spontaneous pain, tactile allodynia, cold allodynia and mechanical hyperalgesia of four rats; ∗ denotes significant value, in comparison to their respective vehicle control at $p < 0.05$ (one-way ANOVA, followed by post-hoc Dunnett's test.

Table 2
Quantification studies of compounds **4b** and **4d**.

Treatment	ED_{50} values (mg/kg) in CCI model (TPE in min)[a]				ED_{50} values (mg/kg) in PSNL model (TPE in min)[a]			
	SP	TA	CA	MH	SP	TA	CA	MH
4b	34.14 (60)	18.24 (120)	42.73 (60)	26.29 (60)	15.12 (60)	41.13 (60)	57.13 (60)	65.10 (60)
4d	21.84 (60)	24.16 (30)	54.33 (60)	41.26 (120)	21.64 (30)	43.22 (30)	38.21 (60)	55.53 (120)

Each value represents median effective dose in spontaneous pain (SP), tactile allodynia (TA), cold allodynia (CA) and mechanical hyperalgesia (MH).
[a] TPE represents time of peak effect in min.

Table 3
Percent protection in carrageenan induced paw edema, DPPH scavenging activity and effect of compounds **4b** and **4d** on nitrosative stress.

Treatment	% Protection in carrageenan induced paw edema			DPPH scavenging activity[b]	% Inhibition of Nitrosative stress (nitrite)[c]	
	60 min	120 min	180 min	IC_{50}(μM)	Brain	Sciatic nerve
Vehicle	–	–	–	–	–	–
4b	17.4 ± 2.3	11.8 ± 1.2	46.7 ± 1.3	254	15.2 ± 1.9	19.0 ± 3.4
4d	67.4 ± 2.3*	83.5 ± 4.5*	84.2 ± 6.4*	213	18.9 ± 3.1	50.1 ± 1.3*
Indomethacin[a]	50.3 ± 2.6*	61.2 ± 3.8*	66.9 ± 4.2*	–	–	–

* Represents significance at $p < 0.05$ compared to vehicle (One way ANOVA followed by Dunnett's test, $n = 4$).
[a] Indomethacin was tested at the dose of 10 mg/kg i.p.
[b] DPPH radical scavenging activity of the test compounds (values are represented as% scavenging calculated from the average of triplicate experiments).
[c] Percent inhibition of nitric oxide in brain and sciatic nerve of CCI rats. Compounds were tested at the respective minimal ED_{50} dose administered *i.p.*

value of 254 μM whereas IC_{50} value of 231 μM was observed with **4d** (Table 3).

4. Conclusion

In the present study, sixteen novel tetrahydropyridopyrimidines were synthesized and assessed for acute antinociceptive, antiallodynic and antihyperalgesic potential. In acute models of pain, significant inhibition of acetic acid induced writhings by seven compounds established their role as peripherally acting analgesics. Also, significant suppression of phase-I of the formalin induced flinching by one compound and phase-II by eight compounds suggested the modulation of anti-inflammatory pathways. Compound **4d** significantly reduced edema in carrageenan model at all the time points. Additionally, compound **4d** was also found to significantly attenuate nitric oxide locally in the sciatic nerve. Above findings provide evidences to the multifunctional profile of the tetrahydropyridopyrimidine scaffold and support their development as leads for treatment of neuropathic pain.

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