Neurological Sciences (2019) 40:1799–1811
https://doi.org/10.1007/s10072-019-03884-6

ORIGINAL ARTICLE

Attenuation of vincristine-induced neuropathy by synthetic cyclohexenone-functionalized derivative in mice model

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Received: 12 January 2019 / Accepted: 2 April 2019 / Published online: 30 April 2019

Abstract

Vincristine (VCR) is a well-known anticancer drug which frequently induced painful neuropathy and impairs the quality of life of patients. The present study was designed to investigate the alleviative potential of a novel cyclohexenone derivative (CHD), i.e., ethyl 6-(4-methoxyphenyl)-2-oxo-4-phenylcyclohexe-3-enecarboxylate, against VCR-induced neuropathic pain in mice model. VCR was administered intraperitoneally for 10 days in two cycles to induce neuropathic pain. Static and dynamic mechanical allodynia was evaluated using von Frey hair filaments and cotton buds, respectively. Paw thermal hyperalgesia was determined through a hot plate analgesiometer. The tail cold immersion hyperalgesia and paw cold allodynia were determined by available standard protocols. The formalin nociception was induced via subplantar injection of formalin. The antioxidant potential was evaluated via 2,2-diphenyl-1-picrylhydrazyl free radical scavenging activity. The outcome of this study revealed that CHD (30–45 mg/kg) and gabapentin (75 mg/kg) significantly enhanced the paw withdrawal threshold (PWT) and paw withdrawal latency (PWL) in static and dynamic allodynia, respectively, and increased the PWL in thermal hyperalgesia and tail withdrawal latency (TWL) as compared to the VCR-treated group. CHD significantly augmented the paw withdrawal duration (PWD) in paw cold allodynia, while the same compound only increased the paw elevation and paw licking in the delayed phase of formalin nociception. Moreover, CHD significantly inhibited the DPPH free radical scavenging action ($IC_{50} = 56$), butylated hydroxytoluene (BHT) ($IC_{50} = 39$), and ascorbic acid ($IC_{50} = 2.93$). In conclusion, CHD exhibited a profile of potential attenuative effect against the VCR-induced neuropathic pain which might be attributed to its possible antinociceptive and antioxidant effect.

Keywords Vincristine · Neuropathic pain · CHD · Ethyl 6-(4-methoxyphenyl)-2-oxo-4-phenylcyclohexe-3-enecarboxylate · Antinociceptive · Antioxidant

Introduction

Chemotherapy-induced neuropathy (CIN) is a painful situation which occurred due to damage of the somatosensory nervous system [1]. In neuropathic pain, augmented and impetuous sensitivity occurs to both noxious and innoxious stimuli [2]. Neuropathic pain is largely characterized by sensory deformities such as dysesthesia, allodynia, and hyperalgesia [3]. Complex etiological factors have been linked to the development of painful neuropathy such as physical trauma of the CNS, diabetes mellitus, cancer therapy, uremia, genetic mutations, etc. [4–6]. The pathophysiology of CIN is a complex phenomenon and that is why many preclinical models have been developed. In the clinical side, the CIN is poorly diagnosed and treated due to the complex nature of CIN [7]. Vincristine (VCR) is an effective chemotherapeutic drug indicated in cancer chemotherapy such as leukemia, lymphomas, brain tumor, and breast cancer [8]. However, VCR-induced peripheral neuropathy (VIPN) is both dose and duration dependent and causes premature termination of cancer therapy [9]. It has been reported that VIPN is correlated with axonal damage and abnormal microtubule fabrications in unmyelinated and myelinated fibers, and boosted receptiveness of C-fiber nociceptors [10–12] and is characterized by allodynia, hyperalgesia, and dysesthesia [3]. A number of useful rodent models have been reported to study VIPN [13–15] which rationally imitates chemotherapy-induced peripheral neuropathy (CIPN) in human [16, 17].

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Presently, medications of diverse chemical nature are used in the clinical management of CIN such as anticonvulsant agents (gabapentin, topiramate, carbamazepine, pregabalin, phenytoin, lamotrigine), tricyclic antidepressants (imipramine, desipramine, amitriptyline, nortriptyline), and opioids [18, 19], though such medications have been tested with an extensive continuum of undesirable effects which limit their satisfactory clinical utility in the amelioration of peripheral neuropathy [20, 21]. Consequently, there is a definite need for alternative agents to manage peripheral neuropathy with a desired safety profile and therapeutic efficacy. Hence, the current study was conducted with a novel cyclohexenone functionality and is a step forward in this direction.

The key role of cyclohexanone/hexanone ring is well established in the field of medicinal research. Literature reports have revealed that this functionality is an integral part of many interesting compounds and is of considerable significance in biomedical research for researchers to develop potentially valuable drugs. Chemically, the cyclohexenone nucleus serves as proficient intermediates for synthesizing various important heterocyclic compounds like fused pyrazoles, isoxazoles, quinazolines [22], and 2H-indazole [23]. Cyclohexenones are the derivatives of cyclohexane and chemically representing a carbonyl group at position-1 and carbon–carbon double bond at position-2. The enone functional group and substitution at carbon atom in a six-membered ring have shown high activity of cyclohexenones [24]. Considering the prominent pharmacological properties of cyclohexenone derivatives including anti-inflammatory and antinociceptive potential [25–32], the present study evaluates the therapeutic efficacy of a novel cyclohexenone functionality containing the derivative, i.e., ethyl 6-(4-methoxyphenyl)-2-oxo-4-phenylcyclohexe-3-enecarboxylate (Fig. 1), against VCR-induced neuropathy in mouse paradigms such as static and dynamic allodynia, thermal hyperalgesia, tail cold immersion hyperalgesia, paw cold allodynia, and formalin-induced biphasic nociception.

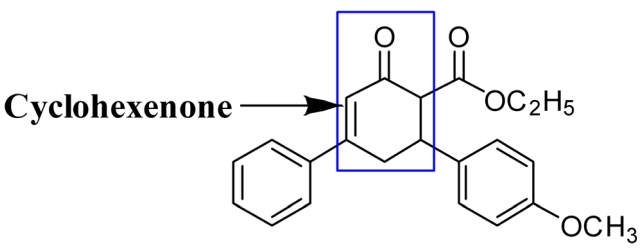

Fig. 1 Chemical structure of CHD viz ethyl 6-(4-methoxyphenyl)-2-oxo-4-phenylcyclohex-3-enecarboxylate

Materials and methods

Drugs and chemicals

The following drugs/chemicals were used: vincristine (Pharmedic Laboratories (Pvt) Ltd., Lahore, Pakistan), DPPH and BHT (Sigma-Aldrich, Germany), formaldehyde (Merck, Germany), gabapentin (Lowitt Pharmaceuticals (Pvt) Ltd., Peshawar, Pakistan), normal saline (0.9% NaCl) (Otsuka Pakistan Ltd., Lasbella, Pakistan), acetone (BDH, UK), and dimethyl sulfoxide (DMSO) (Scharlau Chemicals, Spain).

Instruments

The instruments used in the study are as follows: von Frey filaments kit (Stoelting, USA), hot plate analgesiometer (Harvard Apparatus, USA), thermostatically controlled water bath (BS-11, Lab Companion, China), and UV–visible spectrophotometer (Lambda 25, PerkinElmer, USA).

Laboratory animals

Mice (Balb/c) (18–30 g) were used during the current studies which were bred in the Animal House and Bioassay Laboratories at the Department of Pharmacy, University of Peshawar. Animals were nourished with established laboratory food and water ad libitum and housed under ambient temperature of 22 ± 2 °C through an air conditioning system and exhaust fan facility, with 12/12 h light and dark cycle.

Research ethical approval of the study

This research work was conducted under a project entitled "Studies on the nociceptive, inflammatory and neuropathic pain relieving potential of cyclohexenone derivative" by the Ethical Committee of the Department of Pharmacy, University of Peshawar, Khyber Pakhtunkhwa (KPK), Pakistan, who delivered an endorsement testament number 01/EC-18/Pharm in their meeting. Furthermore, experiments on laboratory animals were executed firmly in compliance with the Animals Scientific Procedure Act UK (1986).

Induction of vincristine-induced neuropathic pain in mice

Albino mice (Balb/c) of weight ranging 25–35 g were used in this study. The neuropathic pain was induced by i.p. injection of VCR (50 μg/kg) for 10 days in a duplet 5-day phases with an interval of 2 days. Hence, overall, 10 injections were used which were administered on different experimental days as previously reported [33, 34]. Figure 2 depicts the time line of the experimental protocol for vincristine-induced neuropathy in mice.

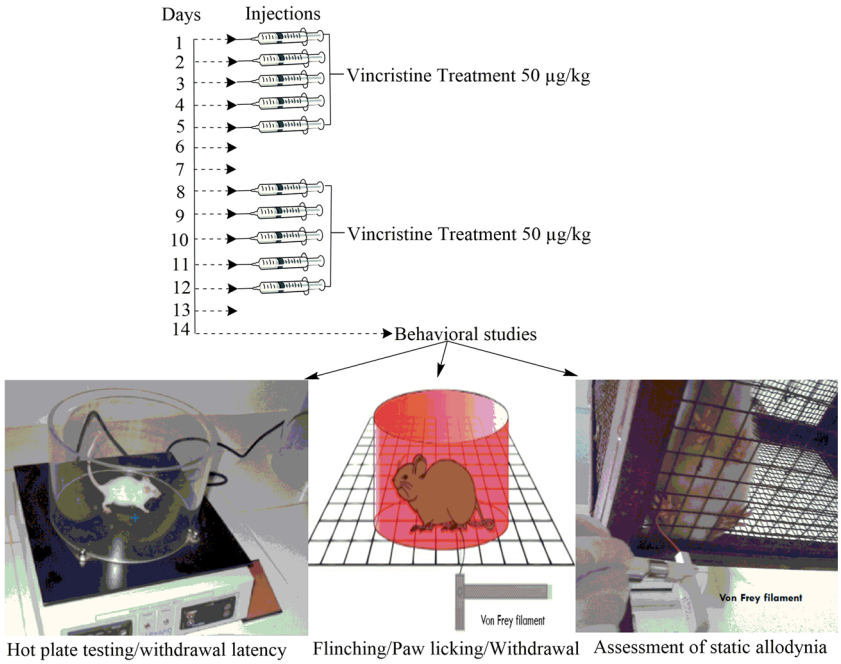

Fig. 2 Time line of experimental protocol for vincristine-induced neuropathy

General toxicity and body weight measurement in mice

The mice were observed to identify for any signs of VCR toxicity such as posterior limb debility, gastrointestinal ailments, and horripilation. Moreover, the body weights of animals were determined during the course of the experiments specified at various experimental days such as 0, 3, 6, 10, and 14 days.

Drug treatment protocol for pharmacological experiments in mice

The mice were shifted to specifically designed plexiglass cages (12 × 12 cm) with a wire mesh bottom in the experimental laboratory where they were acclimatized for 30–45 min interval in order to avoid any discrepancy during the experimentation. The Balb/c mice were randomly classified into different groups (n = 6) for investigations as follows:

Group I: saline control group. The mice in this group were administered with an equal quantity of normal saline i.p. for 10 days.
Group II: VCR disease control group. The mice in this group received VCR in a dose of 50 µg/kg, i.p. for 10 days.
Group III: standard positive control group. The mice were injected with VCR in 50 µg/kg, i.p. for 10 days. Then, on the 14th day, standard gabapentin (75 mg/kg) was administered i.p.
Group IV: test drug CHD group. The mice were served with VCR in 50 µg/kg, i.p. for 10 days. Then, CHD (30 mg/kg) was administered i.p. on the 14th day.
Group V: test drug CHD group. The mice were served with VCR in 50 µg/kg, i.p. for 10 days. Then, CHD (45 mg/kg) was administered i.p. on the 14th day.

The behavioral experiments such as dynamic and static allodynia, paw thermal hyperalgesia, tail thermal hyperalgesia, and paw cold allodynia tests were executed on days 0, 6, 10, and 14, and mice received the standard and test drug treatment on the 14th day.

Protocol of paw static mechanical allodynia in mice

The static mechanical allodynia of mice was measured with the application of von Frey hair filaments (15,10, 8, 6, 4, 2, 1.4, 1, 0.6, 0.4 g) (Stoelting, USA) which were enforced vertically at the midplantar area of the mice left posterior paw to such a degree which give rise for the hair filament to twist [35]. Initiating with a 2-g force, each von Frey hair filament was employed for 6 s interval as a cutoff time or till an affirmative response happened. Immediate flinching, licking, biting, or lifting of paw upon filament removal was documented as a positive response, and the subsequent inferior force von Frey filaments were operated for the succeeding reading, whereas in case of deprivation of response, successive higher force von Frey hair filaments were utilized. This procedure was sustained for four measurements in case of after initial positive reaction or five sequential negative responses (1, 1.4, 2, 4, and 6 g force) or four repeated positive responses (2, 1.4, 1 and 0.6 g force). The 15-g force was reserved as a cutoff force after which additional implementation of von Frey filaments was terminated. In order to evade the impact of the former stimuli on the behavioral reaction of mice, the von

Frey filaments were employed at numerous seconds interval. In case of any vague response, the stimulus was repeated. With an intermission of 5 min, the paw withdrawal threshold (PWT in grams) (the force at which the paw withdrawal happened) was averaged for three readings of hind paws.

Protocol of paw dynamic mechanical allodynia in mice

The dynamic mechanical allodynia was evaluated by lightly brushing the midplantar region of the rearmost paws with a cotton brush. Paw lifting, flinching, or licking was recognized as paw reaction and the time engaged to illustrate the paw response was contemplated as paw withdrawal latency (PWL in seconds) [36]. As a minimum, three assessments each isolated by 30 s were noted. Mice retaliating to the brushing within 8 s were incorporated in the trial; 15 s of cutoff time was executed, after which the evaluation was abolished [37].

Protocol of paw thermal hyperalgesia in mice

A hot plate analgesiometer (Harvard Apparatus, USA), comprising of a limpid cylinder utilized for confining the mice to the surface of the hot plate, conserved at a preset temperature of 54 ± 0.1 °C, was used for evaluating the paw latency reaction time (in seconds) in terms of paw licking, flinching, or jumping from the hot plate. In order to avoid any paw or body harm, a cutoff time of 30 s was executed. The paw latency reaction time was assessed at 30, 60, and 90 min after the administration of the drugs [38].

Protocol of tail cold hyperalgesia in mice

The tail cold hyperalgesia in mice was evaluated via the tail immersion method by submerging their tail terminal part up to 3 cm, in a thermostatically controlled water bath set at a temperature of 0–4 °C. The flinching, flicking, or withdrawal of mice tail was considered as escape response calculated in seconds and was designated as tail withdrawal latency (TWL). A cutoff time of 15 s was implemented to prohibit any potential tissue damage [39].

Protocol of acetone drop-induced paw cold allodynia in mice

This test was performed to determine the paw sensitivity of the animal to innocuous chemical incitement. Mice were positioned on top of the wire mesh bottom plexiglass cages (12 × 12 cm) and were allowed to acclimatize. Then, a drop (0.05 mL) of acetone was poured on the focal point of the frontal side of the mice posterior paw deprived of touching the skin utilizing a blunted needle linked to a syringe. The paw withdrawal response (stamping, licking, withdrawal, repeated and prolonged flinching of paw) duration (PWD) of mice to the cooling effect of acetone drop was recorded with a capricious least possible value of 0.5 s and a peak value of 15 s [40]. Acetone was alternatively operated three times to each hind paw, and the mean of three measurements for each paw was taken as the cumulative value of each mice.

Conduction of inverted wire mesh screen test in mice (motor activity testing)

The inverted wire mesh screen test was performed to evaluate the motor function of mice. The mice were positioned on a vertical wire mesh screen, which was then upturned such that the mice became suspended upside down. The test was ended after 3 min or when the mice drop off the screen or any came primarily. Pausing at a minimum of 30 min between each trial, each mouse was tried thrice. The times taken by each mouse in three measurements were then averaged [41].

Protocol of formalin-induced biphasic nociceptive threshold in mice

The mice were acclimatized for 20 min in a wire mesh base plexiglass container (12 × 12 cm), preceding to the formalin-induced nociceptive test. Then, pain was induced by administering 20 μL of formulated 5% formalin subcutaneously (s.c.) in the subplantar region of the right hindmost paw of mice. The nociceptive response time (calculated in seconds) was assessed by enumerating the paw elevation, licking, and biting and determined in two phases, i.e., acute phase (0–5 min) and delayed or chronic phase (20–40 min) [42].

Protocol of antioxidant activity for the test compound CHD

The in vitro antioxidant potential of the test compound was assessed via DPPH free radical scavenging assay [43]. Concisely, 1 mM solution of DPPH free radical was prepared in methanol. Then, a 10-mg/mL stock solution of CHD was formulated in DMSO and various concentrations such as 1, 10, 30, 50, 100, 200, 400, 600, 800, and 1000 μg/mL were prepared. One milliliter of DPPH free radical solution was then mixed with 1 mL of various concentrations of CHD

in test tubes. The solutions were vortexed vigorously and incubated at room temperature in the dark for 30 min. Then, the absorbance of CHD and standard compounds was evaluated at 517 nm with a UV–visible spectrophotometer. Ascorbic acid and BHT were utilized as standards, while control was prepared by dissolving 1 mL of 1 mM DPPH free radical solution in 1 mL methanol. The percent DPPH free radical hunting potential of CHD was measured using the following equation:

$$\text{Percent DPPH scavenging effect} = [(A_0 - A_1/A_0) \times 100]$$

Where absorbance of the control was A_0, while that of the standard or test sample was A_1. The concentration of the compound which reduces 50% of DPPH radicals scavenging termed as IC_{50} was calculated using nonlinear regression analysis of concentrations and absorbance.

Statistical analysis

GraphPad Prism version 5 was used for data analysis as mean ± standard error (SEM) and one-way ANOVA followed by post hoc Dunnett's test or Bonferroni's multiple comparison tests, where the pertinent Mann–Whitney *U* test was also applied for analysis. Values were considered statistically significant at $P < 0.05$.

Results

Effect of CHD on static mechanical allodynia in VCR-induced neuropathic pain model in mice

Repeated injections of vincristine have shown pain threshold on different treatment days in the form of decreased PWT (g) (Fig. 3a). Then, co-administration of the test compound (30–45 mg/kg) and standard gabapentin (75 mg/kg) has shown statistically significant antiallodynic effect (static) in the form of a significant increase in PWT (g) (Fig. 3b). One-way ANOVA followed by Bonferroni's multiple comparisons test demonstrated a considerable decline ($\$\$\$P < 0.001$) in the PWT of VCR-treated animals in relation to the saline-treated group, whereas a significant upsurge in PWT (g) has been observed for the standard-treated group ($\#\#\#P < 0.001$) and test compound ($^{***}P < 0.001$, $^{**}P < 0.01$), respectively, at cited doses at 0.5, 1, and 1.5 h intervals as compared to the VCR-treated group (Fig. 3b).

Effect of CHD on paw dynamic mechanical allodynia in VCR-induced neuropathy

The PWL (s) in response to cotton brushing, after repeated administration of VCR in four groups ($n = 6$), was significantly reduced on the 6th and 10th days and a maximum decrease on the 14th day as compared to the saline control group (Fig. 4a). On the 14th day, co-administration (VCR+gabapentin 75 mg/kg; VCR+CHD 30 and 45 mg/kg) indicated a significant antiallodynic effect (dynamic) (Fig. 4b). The PWL of mice was significantly increased ($\#\#\#P < 0.001$) in the gabapentin+VCR-treated group as compared to the VCR-treated group only. Similarly, CHD at 45 mg/kg enhanced the PWL ($^{***}P < 0.001$, $^{**}P < 0.01$) and at 30 mg/kg ($^{**}P < 0.01$, $^{*}P < 0.05$) compared to the VCR-treated group at 0.5, 1, and 1.5 h interval (Fig. 4b).

Effect of CHD and standard in paw thermal hyperalgesia in VCR-induced neuropathic pain model in mice

A comparative study of the VCR-treated groups versus the normal saline-treated group was conducted on a hot plate analgesiometer to predict the pain threshold at different days of VCR treatment as shown in Fig. 5a. However, the PWL (s) of the VCR+gabapentin 75 mg/kg and VCR+CHD 30 and 45 mg/kg treated groups was significantly increased as compared to the VCR as an indicator of protective effect against VCR-induced neuropathy by test and standard treatment. As displayed in Fig. 5a, the test compound effect was significant in augmenting the PWL of mice paw during 0.5 and 1 h interval, at 45 mg/kg ($^{***}P < 0.001$, $^{**}P < 0.01$, and $^{*}P < 0.05$) and 30 mg/kg, while it became inefficient after 1.5 h as compared to the VCR-treated group.

Effect of CHD on tail cold hyperalgesia in VCR-induced neuropathic pain model in mice

As depicted in Fig. 6a, cold hyperalgesia response in the form of decreased TWL (s) was noted in all VCR-treated groups at different days of treatment protocol as compared to the normal saline-treated group. However, when the VCR-treated groups were treated on the 14th day with the test compound (30–45 mg/kg) and standard (75 mg/kg), a significant protective effect was observed in the form of increased TWL (s) as compared to the VCR-treated group, i.e., ($^{***}P < 0.001$ and $^{*}P < 0.05$) CHD at 45 mg/kg and ($^{**}P < 0.01$) CHD at 30 mg/kg after 0.5 and 1 h

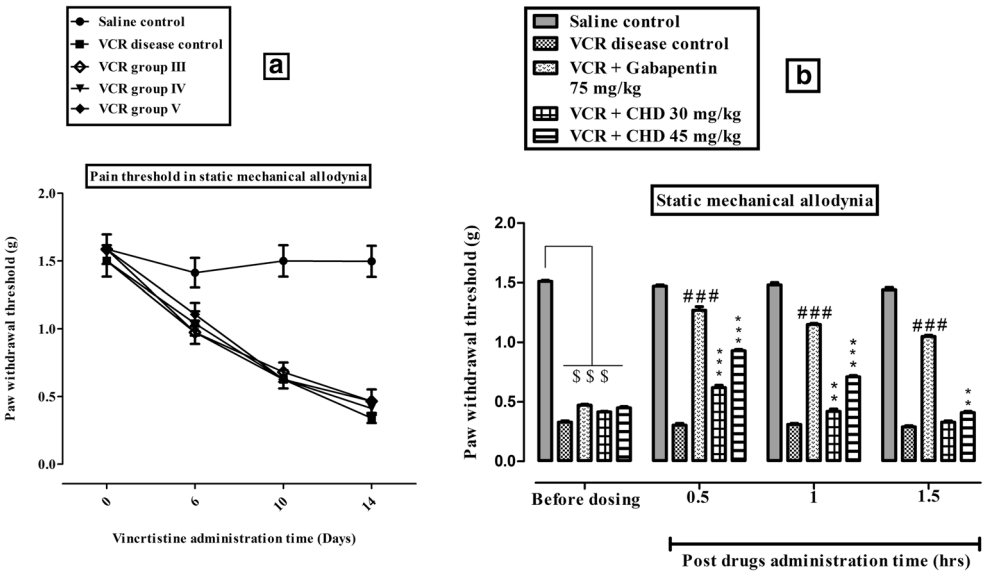

Fig. 3 The static antiallodynic effect of CHD in VCR-induced neuropathy in mice. Results are expressed as mean ± SEM ($n = 6$). One-way analysis of variance (ANOVA) followed by multiple comparison Bonferroni's test displayed a notable decrease ($\$\$\$P < 0.001$) in PWT of the VCR-treated group as compared to the saline-treated group (**a**). As shown in **b**, ($^{***}P < 0.001$, $^{**}P < 0.01$) the test compound CHD compared with the VCR group; ($\#\#\#P < 0.001$) gabapentin-treated group compared with the VCR-induced neuropathy group

interval individually, ineffective after 1.5 h of drug administration, while gabapentin showed a significant activity ($\#\#\#P < 0.001$) at 0.5–1.5 h (Fig. 6b).

Effect of CHD on chemical (acetone drop)-induced paw cold allodynia in VCR-induced neuropathic pain model in mice

Figure 7 depicts prereading at 0 day of all group animals with no significant changes in PWD (s). Then, groups 2–4 were treated with VCR, and at 14 days, predose reading was recorded, indicating a significant increase in PWD (s) as an indicator of neuropathic pain ($\$\$\$P < 0.001$) as compared to normal saline. Then, cold allodynia was induced in VCR-treated animals with application of acetone drops (0.05 mL). Post-treatment (at 1 h) results at the 14th day of CHD and standard gabapentin exhibited a statistically significant effect in the form of decreased PWD (s) as compared to the VCR-treated group, i.e., ($^{***}P < 0.001$, 6.29 s) at 45 mg/kg and ($^{***}P < 0.001$, 8.013 s) at 30 mg/kg doses and standard gabapentin ($\#\#\#P < 0.001$, 4.963 s).

Effect of CHD in formalin-induced biphasic nociception model in VCR-induced neuropathic pain model in mice

The effect of CHD in formalin-induced nociception test was determined in dual segments: acute and delayed phases.

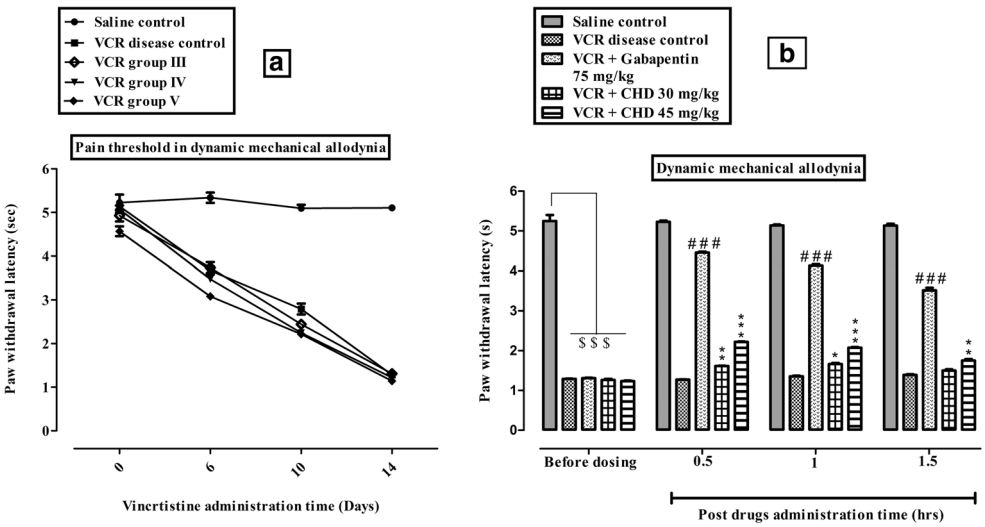

Fig. 4 The dynamic antiallodynic effect of CHD in VCR-induced neuropathy in mice. **a** Decrease in PWT of VCR-treated groups on the 14th day ($\$\$\$P < 0.001$) as compared to the saline-treated group ($n = 6$). **b** ($^{*}P < 0.05$, $^{***}P < 0.001$, $^{**}P < 0.01$) test compound compared with the VCR group; ($\#\#\#P < 0.001$) gabapentin-treated group compared with the VCR group only. The values were presented as mean ± SEM ($n = 6$) and data was analyzed by one-way ANOVA with Bonferroni's post hoc multiple comparison test

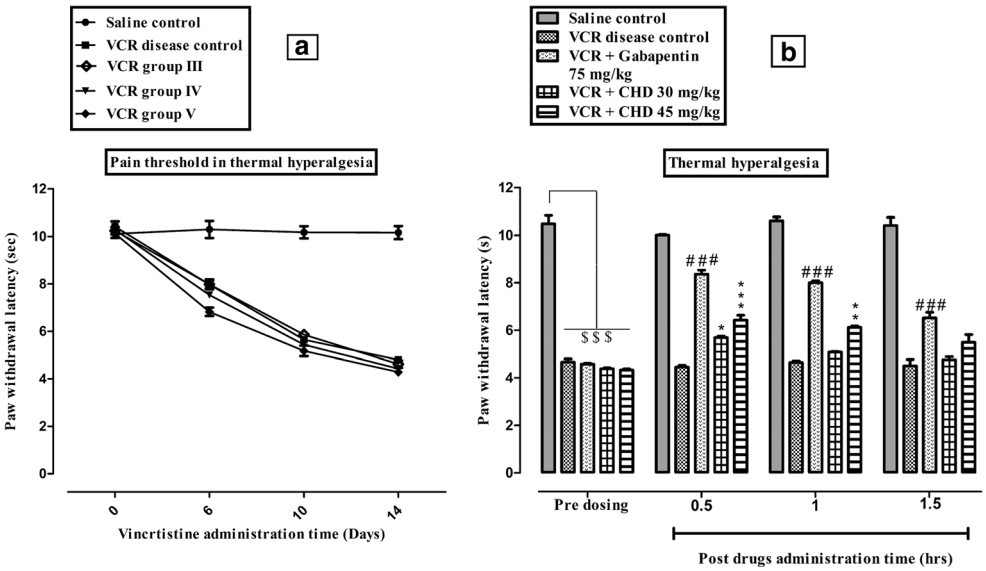

Fig. 5 Protective effect of CHD in thermal hyperalgesia in VCR-induced neuropathy in mice. The data was expressed as mean ± SEM and was analyzed by one-way ANOVA followed by Bonferroni's post hoc multiple comparison test. VCR-treated groups indicated decrease in PWL ($\$\$\$P < 0.001$) as compared to the saline-treated category at different treatment days (**a**, **b**). However, a significant increase in PWL (s) ($^{***}P < 0.001$, $^{**}P < 0.01$, and $^{*}P < 0.05$) was observed with CHD at respected doses, and likewise the gabapentin-treated group ($\#\#\#P < 0.001$) upsurge in PWL as compared to the VCR-treated group

Acute phase

Formalin s.c. injection in VCR-treated animals produced a significant increase ($\$\$\$P < 0.001$) in both paw elevation and paw licking (indicators of pain) as compared to the saline-treated group as shown in Fig. 8a. Treatment of CHD (30–45 mg/kg) did not execute any significant change in paw elevation and licking, whereas gabapentin provoked a significant decrease in paw elevation time (s) ($\#P < 0.05$) in comparison to the VCR-treated group (Fig. 8a).

Delayed phase

Like in Fig. 8a, formalin s.c. injection in VCR-treated animals produced a significant increase ($\$\$\$P < 0.001$) in both paw elevation and paw licking as compared to the saline-treated group (Fig. 8b). However, in the delayed phase, CHD at a dose of 30 mg/kg significantly reduced ($^{***}P < 0.001$) the paw elevation, whereas at 45 mg/kg dose, it also significantly reduced ($^{***}P < 0.001$, $^{**}P < 0.01$) both the paw elevation and paw licking as compared to the VCR-treated

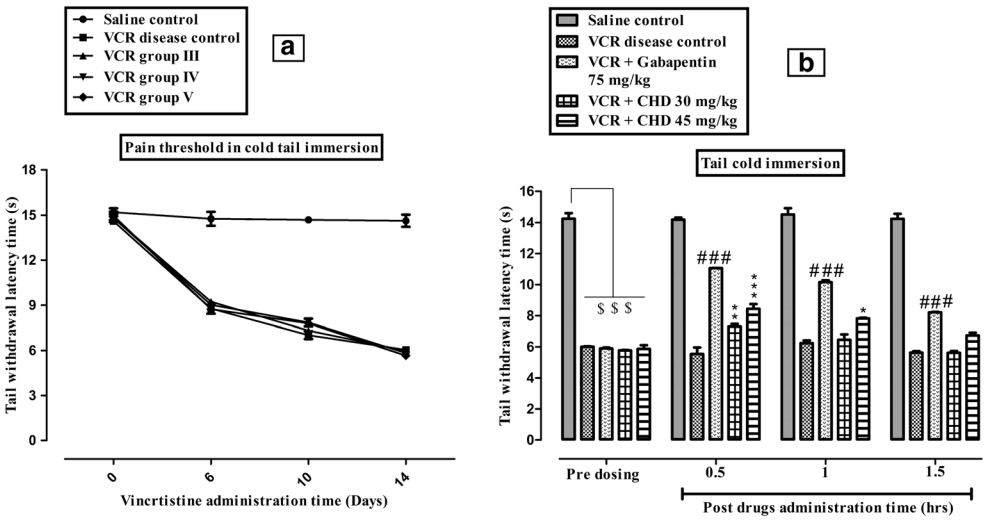

Fig. 6 Protective effect of CHD in cold hyperalgesia test in VCR-induced neuropathy in mice. The data was expressed as mean ± SEM ($n = 6$) and was analyzed by one-way ANOVA followed by post hoc multiple comparison Bonferroni's test. ($\$\$\$P < 0.001$) decrease in TWL (s) of the VCR-treated group as compared to saline-treated mice (**a**, **b**). **b** ($^{**}P < 0.01$) at 30 mg/kg and ($^{***}P < 0.001$, $^{*}P < 0.05$) at 45 mg/kg amplification with CHD after 0.5 and 1 h interval, and the gabapentin-treated group ($\#\#\#P < 0.001$) upsurge in TWL (s) of mice as compared to the VCR-treated group

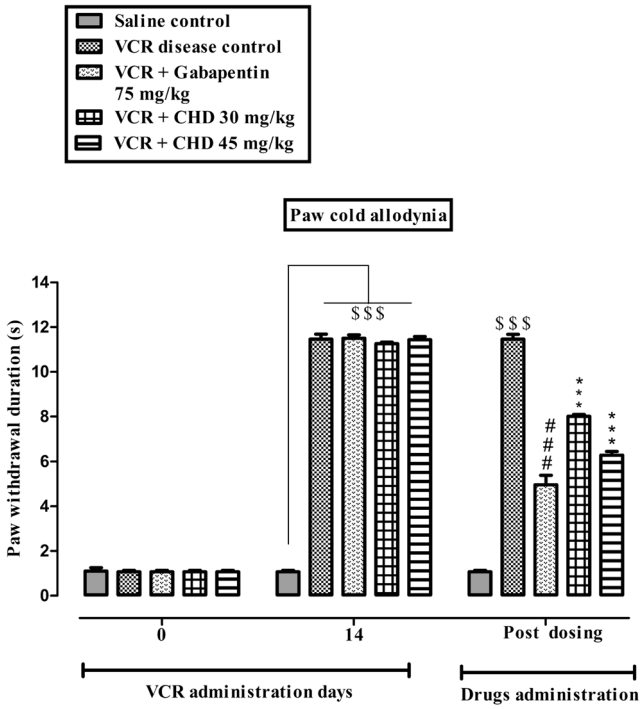

Fig. 7 Protective effect of CHD on chemical (acetone drop)-induced paw cold allodynia in VCR-induced neuropathy. The data was presented as mean ± SEM ($n = 6$) and analyzed by one-way ANOVA followed by post hoc multiple comparison Bonferroni's test. A decrease in PWD (s) of the VCR-treated groups on the 14th day ($\$\$\$P < 0.001$) as compared to the saline group. ($^{***}P < 0.001$) test compound CHD compared with the VCR group and standard gabapentin ($\#\#\#P < 0.001$) as compared to the VCR group

group (Fig. 8b). Gabapentin (75 mg/kg) also significantly diminished ($\#\#\#P < 0.001$) both paw elevation and paw licking as compared to the VCR-treated group (Fig. 8b).

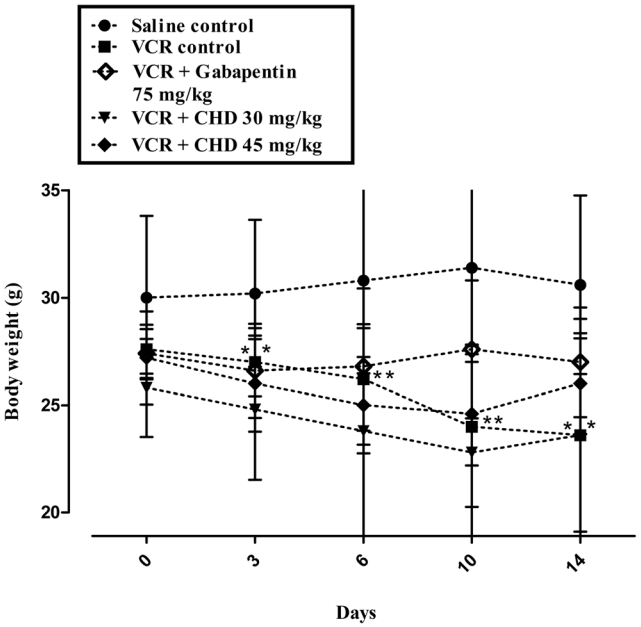

Fig. 9 The effect of VCR (50 μg/kg i.p.) on body weights of mice determined on days 0, 3, 6, 10, and 14 of experimental procedure. Values for VCR-treated mice were significantly changed as compared to saline-treated control animals ($n = 6$ in each category), (expressed as mean ± SEM, $^{**}P < 0.01$, analyzed via Mann–Whitney *U* test)

Determination of body weight variation in VCR-induced neuropathic pain model in mice

The general body toxicity was evaluated on various days; no animals died; however, only mild horripilation was observed at day 14 of VCR dose used for neuropathy. The weight of the animals was measured at various intervals (0, 3, 6, 10, and 14 days) during the experiments and showed that there was a noteworthy decline ($P < 0.01$) in the weight of the animals

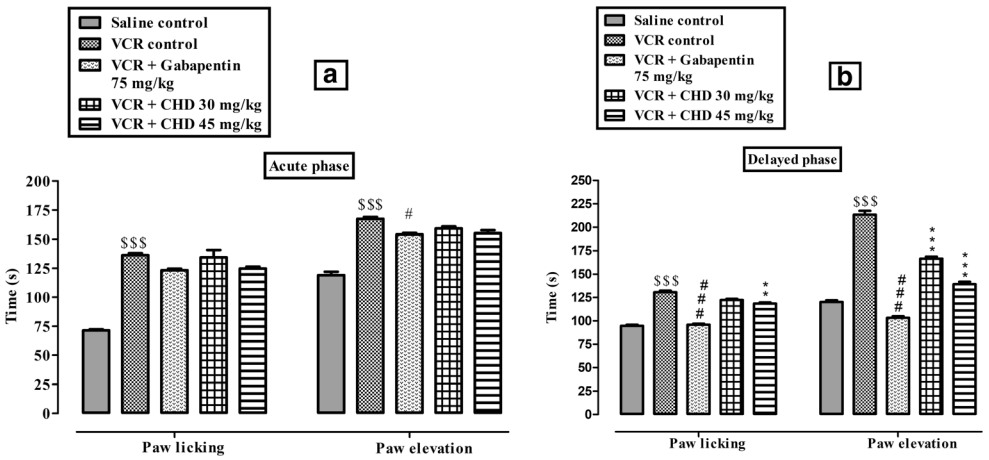

Fig. 8 Protective effect of CHD **a** in acute phase and **b** delayed phase of formalin-induced nociception in VCR-induced neuropathy. **a** Revealed no significant effect of CHD on paw licking and elevation ($P > 0.05$) in the acute phase. However, standard exhibited a significant decrease in paw elevation (s) in acute phase ($P < 0.05$). In the delayed phase (**b**), both the test compound and standard exhibited a statistically significant effect by decreasing both paw licking and elevation as compared to the VCR-treated group, i.e., ($^{***}P < 0.001$, $^{**}P < 0.01$) of CHD and ($\#\#\#P < 0.001$) for standard gabapentin in their respective doses as compared to the VCR-treated group. The data was expressed as mean ± SEM ($n = 6$) and was analyzed by one-way ANOVA followed by post hoc multiple comparison test

treated with vincristine as compared to the saline control category (Fig. 9).

Effect of CHD on balance and motor activity of mice in VCR-induced neuropathic pain model

There was no noteworthy variance in the capability of mice to grip the overturned mesh screen as an indication of general body weakness measured in time taken to grasp the screen pre- and post-vincristine administration in the vincristine control group in contrast to the saline control group.

Antioxidant activity of CHD in DPPH free radical scavenging

The maximum free radical scavenging effect in the form of suppression of DPPH observed with CHD was 71.61%, while that of ascorbic acid was 96.15 and 93.15% with BHT at specified concentrations in a dose-dependent manner. The antioxidant activity of CHD was related to standard ascorbic acid and BHT and was noted in ascending order of ascorbic acid > BHT > CHD as shown in Table 1. The IC_{50} values for CHD, ascorbic acid, and BHT determined were 56, 2.93, and 39 as displayed in Table 1 which follow the descending order of CHD < BHT < ascorbic acid.

Discussion

Chemotherapy including vincristine-induced neuropathy is a well-documented adverse effect in humans and rodent models. However, the onset of sign and symptoms of neuropathy depends on the cumulative dose, duration, and frequency of the chemotherapeutic agent [44, 45]. Chemotherapy-induced neuropathy is one of the most common etiological factors and major adverse effects which limits the effectiveness of current cancer chemotherapy [46, 47]. VCR is an effective antineoplastic agent indicated in cancer chemotherapy such as lymphomas, leukemia, primary brain tumor, breast cancer, and Hodgkin's disease [8, 48, 49]. However, the limiting factor for the clinical use of VCR in cancer patients is its neurotoxicity and sensorimotor or peripheral neuropathy [50, 51]. VCR induced its anticancer and neurotoxicity effect by binding to β tubulin and interrupt the polymerization of microtubules [10]. It is previously reported that VCR triggers free radical production and discharge of inflammatory mediators which ultimately results in axonal degeneration and decreasing conduction velocity of motor neuron [17, 52].

In the field of antineuropathic pain drug development, preclinical rodent models are commonly used to assess the attenuative effect of investigational drugs on pain behavior [45, 47, 53]. The currently available regimens for painful neuropathy are either partially effective (anticonvulsants/opioids/antidepressants) or not fully responsive (SAIDs/NSAIDs) [54]. Hence, neuropathic pain model development and validation is an active area of research for development of new investigational analgesics. Experimental nerve damages to model clinical neuropathic pain can be accomplished by treatment with different chemotherapeutic drugs such as vincristine, taxanes, etc., which develop painful neuropathy in humans [44, 55]. The same cancer chemotherapy induces painful neuropathy in rodent models including VCR-induced neuropathic pain model [45]. The findings of various preclinical chemotherapy-induced neuropathic pain models including the VCR model are considered mimetic for extrapolation to neuropathic pain (allodynia) in clinical cases of cancer patients undergoing chemotherapy [56].

In the current investigation, the protective effect of a novel cyclohexenone derivative (CHD), i.e., ethyl 6-(4-methoxyphenyl)-2-oxo-4-phenylcyclohexe-3-enecarboxylate (Fig. 1), was evaluated in VCR-induced neuropathic pain mice model. The VCR administration (50 μg/kg, i.p.) for

Table 1 DPPH scavenging activity of CHD, ascorbic acid, and BHT

Parameter	Concentration (μg/mL)	Percent inhibition		
		CHD	BHT	Ascorbic acid
	1	19.03 ± 0.537	13.03 ± 0.7372	43.03 ± 0.837
	10	32.62 ± 0.517	21.62 ± 0.5171	71.62 ± 0.417
	30	42.45 ± 0.389	45.45 ± 0.5804	76.45 ± 0.228
	50	49.11 ± 0.324	66.11 ± 0.0693	81.11 ± 0.044
	100	59.218 ± 0.734	86.18 ± 0.0358	87.18 ± 0.161
	200	63.97 ± 1.837	91.97 ± 0.4648	91.97 ± 0.035
	400	69.77 ± 1.252	92.77 ± 0.1347	95.77 ± 0.066
	600	70.468 ± 2.381	92.68 ± 0.2432	95.68 ± 0.072
	800	71.672 ± 0.842	92.72 ± 0.2492	95.72 ± 0.054
	1000	71.615 ± 1.683	93.15 ± 0.1491	96.15 ± 0.067
IC_{50}		56	39	2.93

10 days in dual 5-day phases with a pause of 2 days resulted in the development of painful neuropathy (Fig. 2). Painful behavior was noted in the form of static and dynamic mechanical allodynia, tail cold hyperalgesia, paw thermal hyperalgesia, acetone-induced paw allodynia, and formalin-induced nociception, assessed on various experimental days such as 0, 6, 10, and 14 (Figs. 3a, 4a, 5a, 6a, 7, and 8). The perceived modifications and highly reproducible neuropathic conditions developed in the current study were according to previous reports [33, 34, 57–59]. In our study, CHD (30–45 mg/kg) possibly changed the nociceptive threshold in the form of various pain parameters such as PWT (g) (Fig. 3b), brushing PWL (s) (Fig. 4b), paw thermal hyperalgesia as PWL (s) (Fig. 5b), increased TWL (s) (Fig. 6b), PWD (s) (Fig. 7), and paw licking and elevation behaviors (Fig. 8b). There was a significant antiallodynic effect (static and dynamic) in the form of increase in PWT (g) and PWL (s) as compared to the VCR-treated group (Figs. 3b and 4b). Moreover, promising protective effects were observed when compared with the VCR-induced neuropathy group in paw thermal hyperalgesia such as increased PWL (s), increased TWL (s) in tail cold hyperalgesia, decreased paw withdrawal duration (PWD) in chemical (acetone drop)-induced paw cold allodynia, and reduced paw elevation and licking time (s). Chronic VCR dosing indicated a decline in the weight of VCR-treated mice as compared to saline-treated control animals (Fig. 9). VCR displays oversensitivity in C and A-δ fibers of nociceptive nerves, which causes the excitement of dorsal horn neurons resulting in central sensitization which ultimately leads to allodynia and hyperalgesia [60]. A significant static antiallodynic effect was observed inthe form of increase in PWT (g) (Fig. 2b). The impulsive discharge of C and A-δ fibers is primarily connected with pain behavior as a result of VCR intoxication [61]. Various research studies have featured the significance of central glial activation in hyperalgesia and allodynia development in VCR-induced neuropathy as well as in peripheral inflammatory model [62]. The stimulated glial cells provoke and discharge the pronociceptive mediators such as prostaglandins, nitric oxide (NO), TNF-α, and proinflammatory interleukins. Consequently, any agent which can inhibit the proinflammatory and pronociceptive mediators has been reported as efficacious in diminishing the neuropathic pain [63, 64]. Therefore, the antinociceptive effect revealed with CHD treatment in VCR-induced neuropathic pain can be vindicated. Previous reports have shown that cyclohexenone derivatives have been tested for anti-inflammatory and antinociceptive activity both in standard streptozocin-induced neuropathic and normal rodent models which support the current findings [30, 32, 65–71]. The biphasic nociception induced by formalin is mainly linked with an injured tissue which integrates the clinical pain more closely as compared to other tests [72]. The acute phase is short existed (0–10 min), mostly governed by C fiber stimulation because of peripheral stimuli, whereas delayed phase is longer (20–40 min), caused by dorsal horn functional alterations and local tissue inflammation. Peripherally acting agent block only delayed phase while both phases would be inhibited by centrally acting analgesic [73]. CHD demonstrated antinociceptive effect only in delayed phase which proposes that the cyclohexenone derivative would obstruct the inflammatory proteins persuaded pain discernment and hence it's peripheral action. Though, interestingly CHD also showed activity in thermal hyperalgesia test describing its possible central activity for antinociception.

Oxidative stress has been reported as an etiological factor in many diseases including chemotherapy-induced neuropathy (CTIN) [45, 74, 75]. Oxidative stress arises due to imbalance between oxidants such as reactive oxygen species (ROS), reactive nitrogen species (RNS), and antioxidants (AOX) within the cell. Different antioxidant assays are used to measure antioxidant activities of substances. In our study, we have used a standard DPPH assay to test the antioxidant potential of our compound CHD by comparing its activity with standard ascorbic acid and BHT. The DPPH assay revealed that the maximum suppression of DPPH as a free radical scavenging effect with CHD was 71.61%, while that of ascorbic acid was 96.15 and 93.15% with BHT at 1000 (μg/mL) concentration (Table 1). Oxidative stress and enhanced free radicals are the major contributors in the development of neuropathy in VCR-administered mice [17, 34]. Therefore, any drug with an antioxidant or free radical scavenger activity can attenuate the mechanical allodynia [76]. CHD may possibly attenuate the mechanical allodynia due to its antioxidant activity as shown in Table 1, which is in accordance with previous reports for various cyclohexenone derivatives to exhibit a significant antioxidant effect [43, 77, 78]. Gabapentin is presently recommended as a principal line therapy for the management of neuropathic pain and is efficient in alleviating hyperalgesia and allodynia in diabetic neuropathy because of its unique action on post-synaptic voltage-dependent calcium ion channels located in the spinal cord [79–81]. In our study, we have used gabapentin as a standard and we have compared the results of CHD (30–45 mg/kg) and gabapentin (75 mg/kg) simultaneously with the VCR-induced treated group and exhibited comparable activity in terms of protection.

In conclusion, the present study revealed the attenuative potential of our selected cyclohexenone derivative (CHD), i.e., ethyl 6-(4-methoxyphenyl)-2-oxo-4-phenylcyclohexe-3-enecarboxylate, against VCR-induced neuropathic pain in mice model. The study is supported by highly reproducible standard in vivo models commonly used in chemotherapy-induced neuropathy and in vitro DPPH assay. The alleviative potential against painful vincristine-induced neuropathy might be possibly attributed to the antinociceptive and antioxidant profiles, though further molecular studies are warranted to confirm the underlying neuropathic protective mechanism of CHD. The outcomes of the current preclinical study of CHD in VCR-induced neuropathic

pain model further validate this model as predictive in clinical cases of chemotherapy-induced neuropathy.

Acknowledgments The selected cyclohexenone compound has been synthesized as part of a series of compounds and their structures were confirmed by Dr. Rasool Khan, Associate Professor, Institute of Chemical Science, University of Peshawar. We are grateful to him for providing a series of compounds and after preliminary study; we have selected the cited CHD compound for neuropharmacological study.

Authors' contributions GA initiated the research project and directed the research scholars as supervisor in conducting pharmacological experiments. GA critically evaluated the contents of the final version of the manuscript. JK accomplished the pharmacological experiments and performed calculations and statistical analysis. He developed the preliminary draft of the manuscript. RU helped JK in the conduction of the experiments. SU conducted experiment-related synthesis and structure confirmation under the supervision of RK. RK helped in planning and supervising the experiments related to chemistry of our selected compounds (chemistry structural data not included in this manuscript). All authors read and approved the final manuscript.

Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

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