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Evaluation of the Neuroprotective Activity of a New Allylmorpholine Derivative in a Rat Model of Traumatic Brain Injury

Veronika A. Prikhodko^{1,2}, Aleksandra V. Kan¹, Yuriy I. Sysoev^{1,2,3,4}, Irina A. Titovich¹, Natalia A. Anisimova¹, Sergey V. Okovityi^{1,2}

¹ Saint-Petersburg State Chemical and Pharmaceutical University, 14A, Professor Popov str., St. Petersburg, 197376, Russia

² N. P. Bechtereva Institute of the Human Brain of the Russian Academy of Sciences, 12A, Akademik Pavlov str., St. Petersburg, 197376, Russia

³ Institute of Translational Biomedicine, Saint Petersburg State University, 7-9/1050, Universitetskaya emb., St. Petersburg, 199034, Russia

⁴ Pavlov Institute of Physiology of the Russian Academy of Sciences, 6, Makarova emb., St. Petersburg, 199034, Russia

*Corresponding author: Veronika A. Prikhodko. E-mail: veronika.prikhodko@pharminnotech.com

ORCID: Veronika A. Prikhodko – <https://orcid.org/0000-0002-4690-1811>; Aleksandra V. Kan – <https://orcid.org/0000-0002-7833-9878>; Yuriy I. Sysoev – <https://orcid.org/0000-0003-4199-5318>; Irina A. Titovich – <https://orcid.org/0000-0002-1343-4663>; Natalia A. Anisimova – <https://orcid.org/0000-0002-2620-2976>; Sergey V. Okovityi – <https://orcid.org/0000-0003-4294-5531>.

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Abstract

Introduction. The search for and development of new drugs capable of reducing the severity of neurological deficit in traumatic brain injury are a critical task for investigational pharmacology. Chromone-containing allylmorpholines are a new group of neuroprotective drug candidates that have been shown to inhibit acetylcholinesterase and butyrylcholinesterase, and block N-methyl-D-aspartate receptors *in vitro*.

Aim. This study aimed to evaluate the neuroprotective activity of the allylmorpholine derivative (E)-4-[3-(8-bromo-6-methyl-4-oxo-4H-chromen-3-yl)-1-cyclohexylallyl]morpholin-4-ium chloride (33b) *in vivo* using a rat model of traumatic brain injury.

Materials and methods. Traumatic brain injury was induced using the controlled cortical impact model. The allylmorpholine derivative was administered intraperitoneally at 1, 10, or 50 mg × kg⁻¹ b.w. at 1 h after trauma induction, and then daily for the next 6 d. The neurological deficit was assessed using the Limb Placing, Open Field, Elevated Plus Maze, Beam Walking, and Cylinder tests.

Results and discussion. At all doses administered, the allylmorpholine derivative had no positive effect on the motor function or exploratory behavior following traumatic brain injury. In the Elevated Plus Maze, 10 mg × kg⁻¹ b.w. of the compound further suppressed exploratory behaviour in the injured animals, which appears to be consistent with its sedative properties observed previously in zebrafish.

Conclusion. Despite the previously described *in vitro* affinity of allylmorpholines towards several molecular targets crucial for the pathogenesis of brain trauma and posttraumatic functional recovery, an allylmorpholine derivative had no neuroprotective effect in a rat model of traumatic brain injury in this study. These results further emphasize the importance of *in vivo* evaluation of potential neuroprotective drug candidates.

Keywords: allylmorpholines; allylmorpholine derivatives; brain injuries, traumatic; neuroprotection; neuroprotective agents; neurological rehabilitation; neuropharmacology

Conflict of interest. The authors declare that they have no obvious and potential conflicts of interest related to the publication of this article.

Contribution of the authors. Veronika A. Prikhodko analyzed the literature, conducted the experiments, analyzed and interpreted the data, drafted the manuscript, and prepared the figures. Aleksandra V. Kan and Irina A. Titovich analyzed the literature, conducted the experiments, analyzed the data, and drafted the manuscript. Yuriy I. Sysoev analyzed the literature, designed the study, analyzed and interpreted the data, drafted and reviewed the manuscript. Sergey V. Okovityi and Natalia A. Anisimova designed the study, interpreted the data, and reviewed the manuscript.

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Оценка нейропротекторной активности нового производного аллилморфолина на модели черепно-мозговой травмы у крыс

В. А. Приходько^{1,2*}, А. В. Кан¹, Ю. И. Сысоев^{1,2,3,4}, И. А. Титович¹, Н. А. Анисимова¹, С. В. Оковитый^{1,2}

¹ Федеральное государственное бюджетное образовательное учреждение высшего образования «Санкт-Петербургский государственный химико-фармацевтический университет» Министерства здравоохранения Российской Федерации, 197376, Россия, г. Санкт-Петербург, ул. Проф. Попова, д. 14, лит. А

² Федеральное государственное бюджетное учреждение науки «Институт мозга человека имени Н. П. Бехтерева Российской академии наук», 197376, Россия, г. Санкт-Петербург, ул. Акад. Павлова, д. 12А

³ Институт трансляционной биомедицины, Санкт-Петербургский государственный университет, 199034, Россия, г. Санкт-Петербург, Университетская наб., д. 7-9, пом. 1050

⁴ Федеральное государственное бюджетное учреждение науки «Институт физиологии им. И. П. Павлова Российской академии наук», 199034, Россия, г. Санкт-Петербург, наб. Макарова, д. 6

*Контактное лицо: Приходько Вероника Александровна. E-mail: veronika.prikhodko@pharminnotech.com

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ORCID: В. А. Приходько – <https://orcid.org/0000-0002-4690-1811>; А. В. Кан – <https://orcid.org/0000-0002-7833-9878>; Ю. И. Сысоев – <https://orcid.org/0000-0003-4199-5318>;
И. А. Титович – <https://orcid.org/0000-0002-1343-4663>; Н. А. Анисимова – <https://orcid.org/0000-0002-2620-2976>; С. В. Оковитый – <https://orcid.org/0000-0003-4294-5531>.

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Резюме

Введение. Поиск и изучение новых лекарственных средств, способных снижать выраженность неврологического дефицита у пациентов, перенесших черепно-мозговую травму, является актуальной задачей фармакологии. Среди потенциальных нейропротекторных средств следует отметить хромонсодержащие производные аллилморфолина, для которых *in vitro* была показана блокирующая активность в отношении ацетилхолинэстеразы, бутирилхолинэстеразы и рецепторов N-метил-D-аспартата.

Цель. Оценка нейропротекторной активности производного аллилморфолина (E)-4-[3-(8-бром-6-метил-4-оксо-4H-хромен-3-ил)-1-циклогексилаллил]морфолин-4-ия хлорида (33b) *in vivo* на модели черепно-мозговой травмы у крыс.

Материалы и методы. Черепно-мозговую травму у крыс моделировали методом контролируемого кортикального удара. Производное аллилморфолина вводили в дозах 1, 10 и 50 мг/кг внутривентрикулярно спустя час после травмы и далее 1 раз в сутки в течение 6 дней. Степень неврологического дефицита у животных оценивали в тестах «Стимулирование конечностей», «Открытое поле», «Приподнятый крестообразный лабиринт», «Сужающаяся дорожка» и «Цилиндр».

Результаты и обсуждение. Производное аллилморфолина ни в одной из вводимых доз не оказывало положительного влияния на двигательную функцию или исследовательское поведение травмированных животных. Доза 10 мг/кг вызвала у травмированных животных снижение исследовательской активности в тесте «Приподнятый крестообразный лабиринт», что согласуется с ранее показанным седативным действием изучаемого соединения у рыб *Danio rerio*.

Заключение. В настоящем исследовании было показано отсутствие нейропротекторного действия производного аллилморфолина на модели черепно-мозговой травмы у крыс, несмотря на показанную ранее *in vitro* активность по отношению к биологическим мишеням, играющим ведущую роль в развитии патологических процессов и функциональном восстановлении после черепно-мозговой травмы. Полученные результаты показывают важность проведения исследований потенциальных нейропротекторных средств *in vivo*.

Ключевые слова: производные аллилморфолина, черепно-мозговая травма, нейропротекция, нейропротекторные средства, нейрореабилитация, нейрофармакология

Конфликт интересов. Авторы декларируют отсутствие явных и потенциальных конфликтов интересов, связанных с публикацией настоящей статьи.

Вклад авторов. В. А. Приходько анализировала литературные данные, провела эксперименты, анализировала и интерпретировала данные, подготовила текст рукописи и иллюстрации. А. В. Кан, И. А. Титович анализировали литературные данные, провели эксперименты, анализировали данные, подготовили текст рукописи. Ю. И. Сысоев анализировал литературные данные, разработал дизайн исследования, анализировал и интерпретировал данные, подготовил и редактировал текст рукописи. С. В. Оковитый и Н. А. Анисимова разработали дизайн исследования, интерпретировали данные, редактировали текст рукописи.

Финансирование. Результаты работы получены с использованием оборудования ЦКП «Аналитический центр ФГБОУ ВО СПХФУ Минздрава России» в рамках соглашения № 075-15-2021-685 от 26 июля 2021 года при финансовой поддержке Минобрнауки России.

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INTRODUCTION

Traumatic brain injury (TBI) is a major problem of modern health care due to the high mortality and disability rates, prevalence among different segments of the population, and the associated social and economic burden. According to the recent estimates, the global annual incidence of TBI, regardless of the etiology and severity, is 939 cases per 100,000 people, and the lifetime prevalence is up to 15 % of the population [1]. The general mortality rate in TBI is 5–10 %, while 41–85 % of severe cases are fatal. TBI can lead to motor function impairment, cognitive and memory deficits, reduced, or lost working capacity [2]. Treatment of acute TBI, complication prevention and management, and patient

rehabilitation in the post-traumatic period require rational drug therapy including neuroprotective agents of different groups. Pharmacological neuroprotection in TBI is predominantly aimed at sustaining the metabolism of neurons and glia, and minimizing cell loss due to oxygen and energy deprivation [3]. Modern complex rehabilitation of TBI patients may involve the use of nootropics, antihypoxants, and antioxidants, which prevent or reduce the formation of free radicals, increase neuronal survival under hypoxia and/or ischemia, increase brain acetylcholine levels, and suppress lipid peroxidation [4]. However, the level of evidence for the clinical efficacy and safety of currently marketed neuroprotective drugs remains insufficient [5]. In view of

the above, the search for and development of new neuroprotective agents can be considered an urgent task for modern experimental pharmacology [6, 7].

Novel chromone-containing allylmorpholines (CCAM) derived at the Department of Organic Chemistry of the St. Petersburg State Chemical and Pharmaceutical University, are of interest as potential neuroprotective agents for TBI. The CCAM have been shown to block N-methyl-D-aspartate (NMDA) receptors, and inhibit acetylcholinesterase and butyrylcholinesterase *in vitro* [8]. According to the literature data, various morpholine derivatives are able to promote neuronal survival, thus improving cognitive and motor function in traumatic, ischemic, and neurodegenerative lesions of the central nervous system [9]. According to the above, the CCAM may represent a novel potential treatment option for TBI.

The present work was aimed at evaluating the neuroprotective activity of a novel allylmorpholine derivative, (*E*)-4-[3-(8-bromo-6-methyl-4-oxo-4*H*-chromen-3-yl)-1-cyclohexylallyl]morpholin-4-ium chloride (33b), *in vivo* using a rat model of TBI.

MATERIALS AND METHODS

The animal experiments were carried out in compliance with the Order of the Ministry of Health of the Russian Federation No. 199n (2016 April 1) "On the approval of the Rules of Good Laboratory Practice", and the recommendations of the Bioethics Committee of the St. Petersburg State Chemical and Pharmaceutical University. A total of 50 white outbred male rats weighing 250–300 g were purchased from the Rappolovo laboratory animal supplier (Leningrad Region, Russia), received in a single shipment, and quarantined for 14 d prior to use. All animals were provided *ad libitum* access to normal chow ("Complete feedstuff for laboratory animals", Laboratorkorm, Russia) and drinking water meeting the requirements of GOST 2874-82 "Drinking water". Prior to the experiment, the animals were assigned randomly using a random number method into 5 groups: intact (Intact; NaCl 0.9 %, $n = 10$), control (Control; TBI + NaCl

0.9 %, $n = 10$), TBI + 1 mg · kg⁻¹ b.w. 33b ($n = 10$), TBI + 10 mg · kg⁻¹ b.w. 33b ($n = 10$) and TBI + 50 mg · kg⁻¹ b.w. 33b ($n = 10$). The experiments were carried out according to the schedule shown in Figure 1.

TBI was induced in control and treated rats using the controlled cortical impact model. Prior to the surgery, the animals were anesthetized with intraperitoneal chloral hydrate (400 mg · kg⁻¹ b.w.). A craniectomy was performed in the left-frontal region above the sensorimotor cortex, centred at 2.0 mm rostral and 1.5 mm left-lateral from the bregma. A steel-guide tube carrying a piston with a diameter of 3 mm and a stroke length of 4 mm, was positioned over the cranial opening. To make a brain injury, the piston was actuated by a 50 g weight sliding down the guide tube from a height of 10 cm. The removed bone flap was then placed back, and the incision was closed. The suture and the surrounding area were disinfected with 5% iodine solution immediately after the application and then daily for the following 6 days [10].

(*E*)-4-[3-(8-bromo-6-methyl-4-oxo-4*H*-chromen-3-yl)-1-cyclohexylallyl]morpholin-4-ium chloride (33b) (Figure 2) was administered intraperitoneally as a freshly prepared aqueous solution (1 mg · kg⁻¹ b.w.) or suspension (10 and 50 mg · kg⁻¹ b.w., stabilized with Tween-80) at 1 h post-TBI and then q.d. for the following 6 days. Intact and control rats were receiving equivolume normal saline at the same dosing regimen.

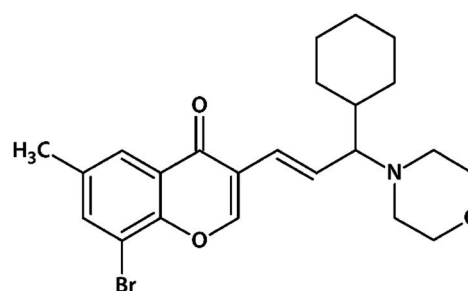


Figure 2. Structural formula of (*E*)-4-[3-(8-bromo-6-methyl-4-oxo-4*H*-chromen-3-yl)-1-cyclohexylallyl]morpholin-4-ium chloride (33b)

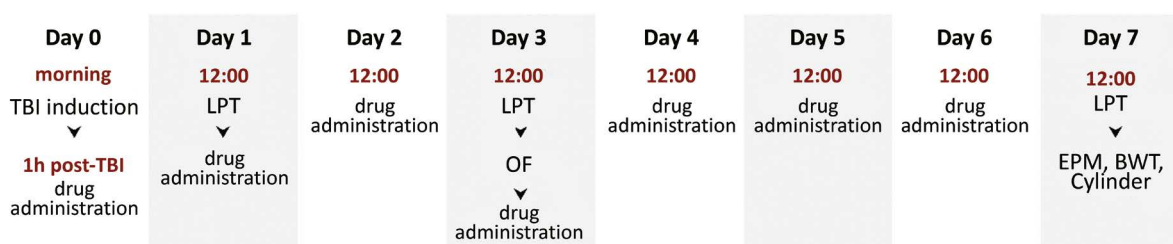


Figure 1. Experimental schedule.

TBI – traumatic brain injury; LPT – Limb Placing test; OF – Open Field; EPM – Elevated Plus Maze; BWT – Beam Walking test

The severity of the neurological deficit was scored on post-TBI days 1, 3, and 7 using Limb Placing test (LPT) to assess the response of the anterior and posterior contralateral limbs to tactile and proprioceptive stimulation in 7 consecutive tasks. Limb function impairment was scored as follows: the rat performed normally, 2 points; the rat performed with a delay of >2 s and/or incompletely, 1 point; and the rat did not respond to the limb stimulation, 0 points. The maximum possible total score was 14 [11].

Locomotion and exploratory behaviour were assessed on post-TBI day 3 in the Open Field (OF) test, recording animal movement in a circular arena (RPC OpenScience Ltd, Russia) on a video camera for 3 min. The video recordings were processed automatically using the VideoMot software (TSE Systems, Germany). Total distance travelled, mean velocity, numbers of visits, freezing episodes, total freezing time, time in centre, numbers of grooming, rearing, and hole-peeping episodes were registered [12].

On post-TBI day 7, the LPT, Elevated Plus Maze (EPM), Cylinder, and Beam Walking (BWT) tests were conducted. The EPM is used to assess general locomotor activity, anxiety-like, and exploratory behaviour. Animal movement in the EPM (RPC OpenScience Ltd, Russia) was recorded on a video camera for 3 min. Time in open (OA) and closed arms (CA), time in centre, number of entries to OA and CA, grooming, rearing, peeing-out (of CA), and head-dipping (in OA) episodes were registered manually [13].

The Cylinder test was conducted to assess forelimb use asymmetry during the examination of a cylindrical setup. The rat was transferred into a transparent plastic cylinder (RPC OpenScience Ltd, Russia) and video recorded for an unlimited time, until at least 10 limb use observations to explore the cylinder were made. The recordings were then rewatched in frame-by-frame mode to assess the independent use of the forelimbs ipsi- and contralateral to the injury, as well as the simultaneous use of both forelimbs to explore the cylinder during a full rear. Percent use of the contralateral forelimb (CL) relative to the total number of limb use observations was calculated as follows [14]:

$$CL = (\text{Contr} + 0.5 \text{ Sim}) / (\text{Ipsi} + \text{Contr} + \text{Sim}) \times 100,$$

where *Contr* is forelimb contralateral to the injury, *Sim*, simultaneous use of both forelimbs, *Ipsi*, forelimb ipsilateral to the injury.

The Beam Walking test (BWT) is used to assess the severity of fore- and hindlimb sensorimotor deficit. The setup (RPC OpenScience Ltd, Russia) consists of two nar-

rowing beams, each 165 cm long, placed one below the other. The lower beam serves as a platform for the animal to place its paws whenever it slips off the upper beam. A black box is placed at the narrow end of the beams as a hiding place and the finish point for the animal. Prior to TBI induction, the rats were trained to perform in the BWT for 3 consecutive days. During the test, animal performance in 3 separate trials was recorded on a video camera. The recordings were then rewatched in frame-by-frame mode to count the number of contralateral limb placement onto the lower beam (i. e., errors), number of paw slips off the upper beam, and the total number of steps. Each parameter was calculated separately for the fore- and hindlimbs contralateral to the injury, then averaged between the 3 trials, and used to calculate the Sensorimotor Deficit Index (SDI; %) as follows [15]:

$$SDI = \text{Errors} + 0.5 \times \text{Paw slips} / \\ / \text{Total number of steps} \times 100$$

The statistical data analysis was performed using GraphPad Prism 8.2 software (GraphPad Software Inc., San Diego, CA, USA). The data was tested for normality using the Shapiro-Wilk *W*-test. For normally distributed data, the significance of differences between group means was tested using one-way ANOVA followed by Tukey's multiple comparison *post hoc* test. Otherwise, the Kruskal-Wallis non-parametric test followed by Dunn's *post hoc* test was used. The data is presented as arithmetic mean (*M*) ± standard error of mean (*m*). For the LPT, the data is presented as median (lower quartile; upper quartile).

RESULTS AND DISCUSSION

Traumatic injury of the motor cortex and the underlying structures resulted in a severe motor impairment leading to the decreased locomotion and exploratory behaviour. In the LPT, injured animals presented with a pronounced fore- and hindlimb motor deficit at post-TBI days 1, 3, and 7. 33b administration did not affect limb function in this test at any of the doses. However, a partial recovery of both fore- and hindlimb function (38.6 % on average) at post-TBI day 7 was noted in the animals that received the compound at 10 mg · kg⁻¹ b.w. (*p* < 0.05) (Table 1).

In the OF, TBI impaired general locomotion and exploratory behaviour, indicated by a 74 % decrease in the number of grooming episodes (*p* < 0.05), and a 96 % decrease in hole-peeping frequency (*p* < 0.01) in controls

compared with intact animals. Notably, control rats also tended to reduce the distance travelled and rearing frequency, and an increase in the time spent in the centre of the field. The allylmorpholine derivative had no significant effect on animal behaviour in this test at any dose given (Figure 3).

Table 1. Effects of the allylmorpholine derivative 33b on animal limb function in the Limb Placing test

Group	Day 1	Day 3	Day 7
Intact	14 (14;14)**	14 (14;14)**	14 (14;14)**
Control	1,5 (0;5)	1,4 (0;5)	3,9 (1;6)
33b 1 mg · kg ⁻¹	0,9 (0;2)	2,0 (0;6)	4,8 (2;7)
33b 10 mg · kg ⁻¹	1,1 (0;5)	2,8 (0;5)	5,4 (3;10)*
33b 50 mg · kg ⁻¹	0,7 (0;4)	1,8 (0;5)	4,9 (0;10)

Note. * $p < 0,05$ vs. Control; ** $p < 0,01$ vs. Control.

In the EPM, the anxiety levels were not statistically different between the injured and intact animals on post-TBI day 7. 33b at 10 mg · kg⁻¹ reduced the number of entries to CA by 74 %, and the number of head dips, by 80 % ($p < 0.05$ for both) (Figure 4).

Despite insufficient statistical significance, TBI rats tended markedly to reduce the use of the forelimb contralateral to the injury in the Cylinder test. Like as in the previous tests, the allylmorpholine derivative had no positive effect on contralateral forelimb function (Figure 5).

In the BWT, TBI animals had 40% higher SDI values for both fore- and hindlimbs compared with intact specimens ($p < 0.01$ for both). 33b did not improve motor function following TBI at any dose given (Figure 6).

Despite several relevant molecular mechanisms of action being confirmed *in vitro* for the CCAM 33b [8], this compound failed to provide neuroprotection at 1, 10, or 50 mg · kg⁻¹ in a rat model of TBI. While BWT

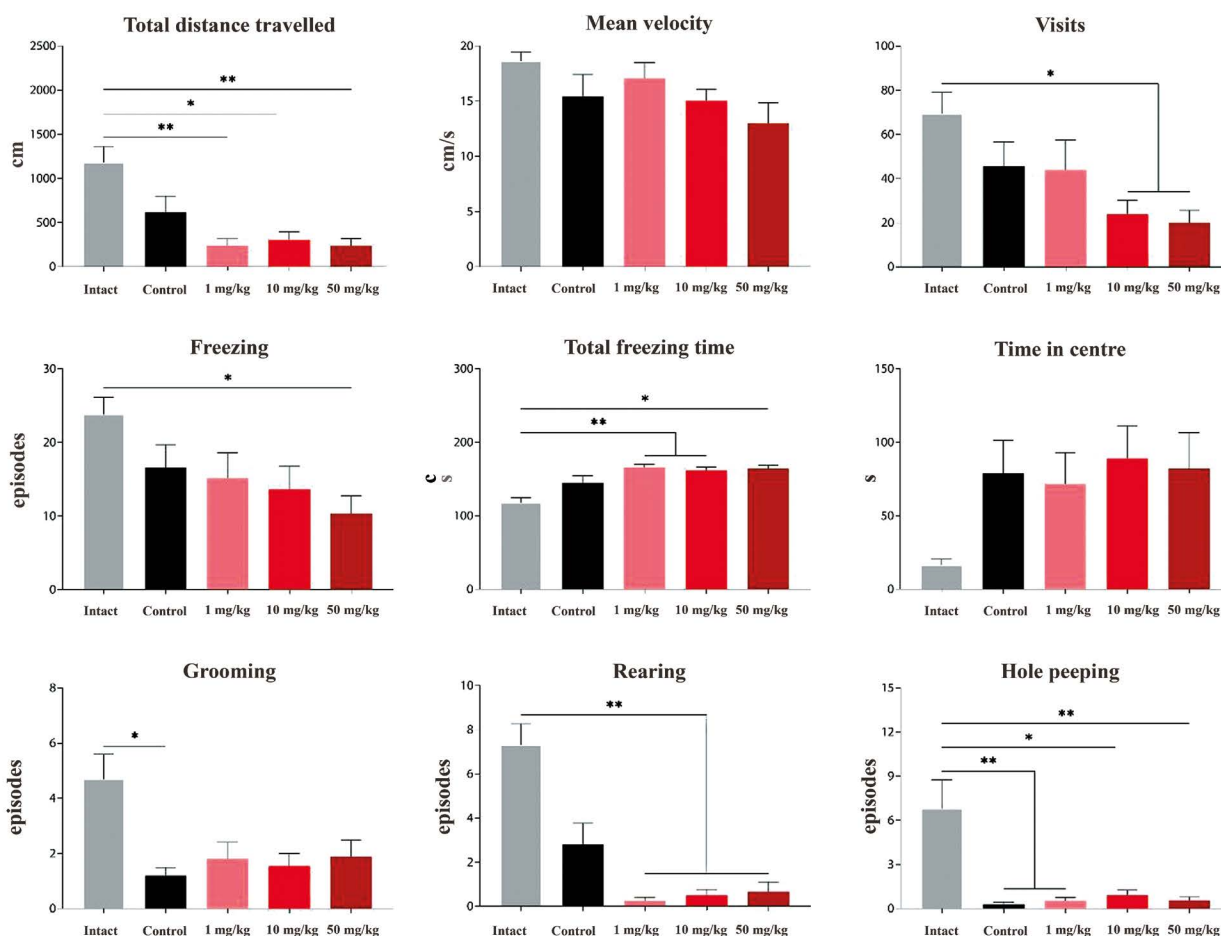


Figure 3. Effects of the allylmorpholine derivative 33b on animal locomotion and exploratory behaviour in the Open Field.

* $p < 0.05$; ** $p < 0.01$

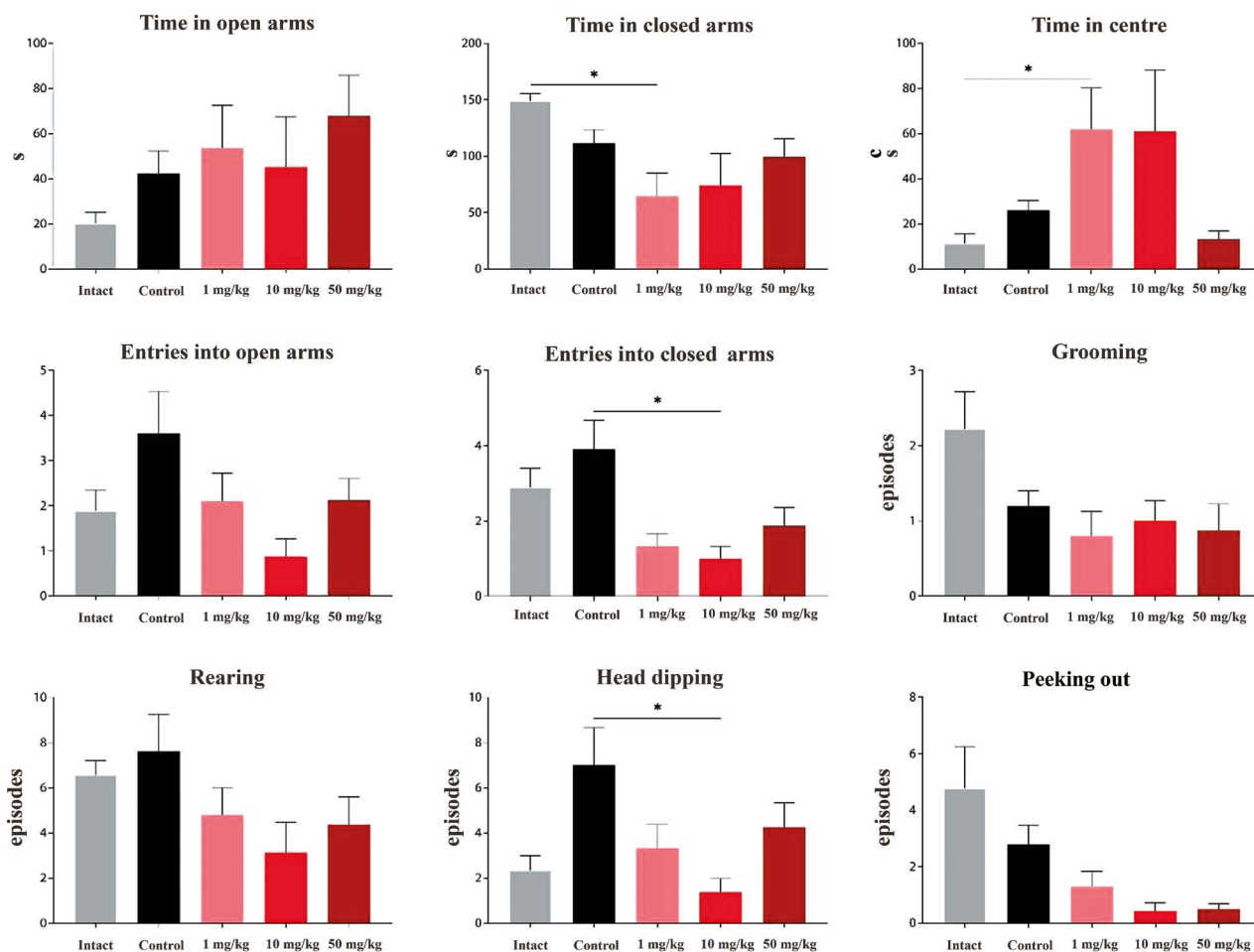


Figure 4. Effects of the allylmorpholine derivative 33b on animal exploratory and anxiety-like behaviour in the Elevated Plus Maze. * $p < 0.05$

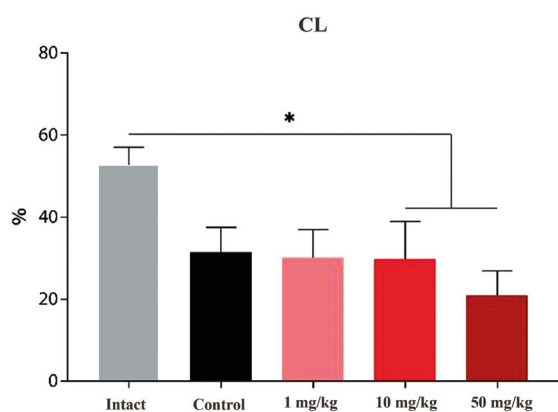


Figure 5. Effects of the allylmorpholine derivative 33b on the function of the forelimb contralateral to the injury in the Cylinder test.

CL – percent use of the contralateral forelimb; * $p < 0.05$

data hinted at some potentially beneficial effects of $10 \text{ mg} \cdot \text{kg}^{-1}$ 33b, they were not confirmed in any other tests, all more objective in nature. The most plausible reason for the absence of the neuroprotective activity

may lie in certain pharmacokinetic limitations of 33b that were not accounted by the experimental design.

The doses used in this work were selected based on the median lethal dose (LD50) value for 33b ($320 \pm 30 \text{ mg} \cdot \text{kg}^{-1}$ intraperitoneally for mice) obtained in the preliminary study. Those doses could be considered potentially effective, since 33b and several other CCAM have been shown to exert central effects in *Danio rerio* in equivalent concentration ranges [16–18].

The selected dosing regimen has previously been deemed appropriate for experimental neuroprotective agents 6-oxo-1-phenyl-2-phenylamino-1,6-dihydropyrimidin-4-ol (mafedine) [19, 20], and [(2E)-4-hydroxy-4-oxobut-2-enoyloxy]-N,N-diethylethanaminium} butanedioate (FDES) [10], but it might be not suitable for the CCAM. Additionally, the intraperitoneal bioavailability of 33b might have been insufficient due to its poor solubility in water, and low systemic absorption from coarse,

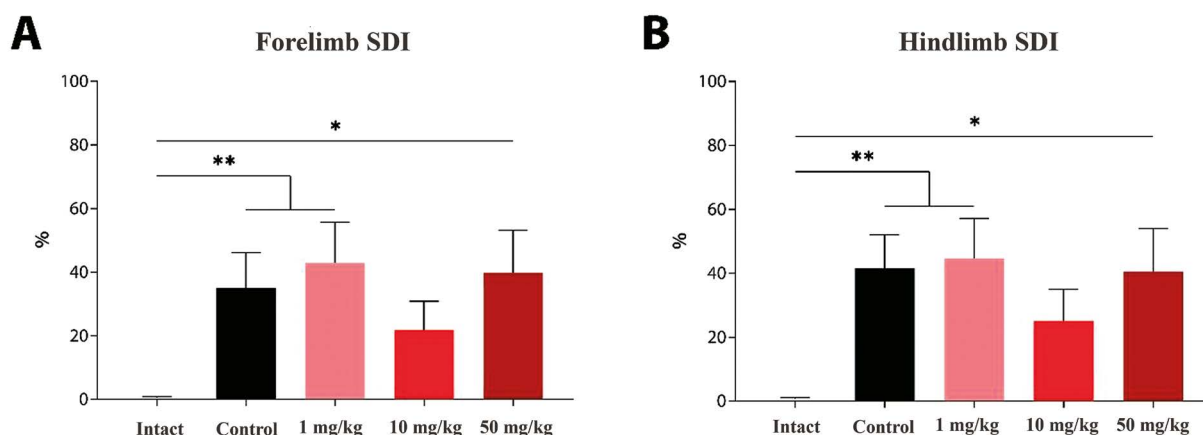


Figure 6. Effects of the allylmorpholine derivative 33b on the function of the forelimb (A) and hindlimb (B) contralateral to the injury in the Beam Walking test.

SDI – Sensorimotor Deficit Index; * $p < 0.05$; ** $p < 0.01$

borderline stable suspensions. For example, different routes of administration (intraarterial or intranasal), dosing regimens, and dosage forms (true solutions) were used for investigational morpholine derivatives AK295 [21] and LM11A-31 [22], which were able to provide neuroprotection in rat models of TBI.

On the other hand, insufficient crossing of the blood-brain barrier (BBB) appears to be unlikely in the case of compound 33b. Several *in silico* studies have found the morpholine moiety to increase lipophilicity and facilitate drug permeation through the BBB [23–26]. TBI itself is associated with the pathologically increased BBB permeability, allowing even macromolecular compounds, i.e., proteins, or nucleic acids, to enter the cerebral circulation at detectable levels [27]. Moreover, 33b could be indirectly proven to cross the BBB by the sedative effect exerted in the EPM in the present study, and by other CCAM at equivalent concentrations in a previous series of screening experiments in zebrafish [16–18].

The failure to observe the expected neuroprotective activity might also be a result of 33b undergoing metabolism *in vivo* and yielding products with different or no pharmacological activity. Among others, the inability to reproduce animal pharmacokinetics is a major drawback of *in vitro* test systems [28]. Because of that, despite high popularity and several undeniable advantages, *in vitro* screening can only be considered as a preliminary stage to provide a rationale for further *in vivo* evaluation of drug efficacy and safety.

CONCLUSION

In the present work, the allylmorpholine derivative 33b was revealed to have no neuroprotective effect in a rat model of TBI, despite having confirmed *in vitro* affinity towards several molecular targets thought to be essential for TBI pathogenesis and posttraumatic recovery. The most plausible reasons for this absence of activity may lie in certain pharmacokinetic issues, mainly, the metabolism of 33b, which were not accounted by the experimental design. Our results emphasize the importance of *in vivo* evaluation of the efficacy and safety of any neuroprotective drug candidates.

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