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Antihyperalgesic Activity of 3-[4-(3-Trifluoromethyl-Phenyl)-Piperazin-1-yl]-Dihydrofuran-2-One in the Oxaliplatin-Induced Cold Hyperalgesia in Mice

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Neuropathic pain is a drug resistant type of chronic pain and there is a strong medical demand to search for novel analgesically active compounds for its alleviation. In the present paper we investigated antihyperalgesic properties of 3-[4-(3-trifluoromethyl-phenyl)-piperazin-1-yl]-dihydrofuran-2-one (LPP1) in a mouse model of toxic polyneuropathy induced by a single intraperitoneal injection of oxaliplatin. Its influence on animals locomotor activity and motor coordination was also assessed using activity cages and rotarod apparatus, respectively. Acute toxicity in neuropathic mice that received LPP1 was assessed, too.

To demonstrate antihyperalgesic properties of LPP1 the cold plate test was used. In this assay the test compound showed antihyperalgesic properties at doses of 1, 10 and 30 mg/kg, and these doses had no influence on animals' locomotor activity or motor performance. A dose of 150 mg/kg completely abolished the animals locomotor activity ($P<0.0001$). The median effective dose (LD50) of LPP1 established in the acute toxicity test was 329.8 mg/kg.

In conclusion, basing on the results obtained it has been shown that LPP1 can be an interesting lead structure in the search for novel analgesics to be used for neuropathic pain syndromes.

Key words: Cold hyperalgesia, cold plate test, mice, oxaliplatin-induced painful toxic neuropathy

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INTRODUCTION

Neuropathic Pain Special Interest Group of the International Association for the Study of Pain (NeuPSIG) defines neuropathic pain (NP) as pain arising from a lesion or dysfunction of the central or peripheral nervous system. Numerous possible causes of this condition comprise metabolic dysfunctions (diabetes mellitus), infectious diseases (herpes simplex infections, human immunodeficiency virus infections), mechanical injuries of nerves or the use of anti-cancer drugs, such as vincristine, taxanes or platinum complexes [cisplatin, oxaliplatin (OXPT)] (Ueda, 2006; Sałat et al., 2014c; Sałat et al., 2014d).

As chronic, non-receptor pain, NP is weakly sensitive to classical analgesic drugs, such as non-steroidal anti-inflammatory drugs or opioids, and it is estimated that 40-50% of patients are resistant to available pharmacotherapy (Watkins et al., 2001). In view of the above, the treatment of NP is still a challenging endeavor and there is a strong medical demand to search for novel analgesically active compounds to be potentially used for pain relief in neuropathic patients.

Previously, we demonstrated that 3-[4-(3-trifluoromethyl-phenyl)-piperazin-1-yl]-dihydrofuran-2-one (LPP1) has significant analgesic, antiallodynic and antihyperalgesic properties in mouse models of acute (Sałat et al., 2012b; Więckowski et al., 2013), tonic (Sałat et al., 2013a) and some neuropathic (Sałat and Sałat, 2013; Sałat et al., 2014a) pain models. In the present research LPP1 is tested for its ability to attenuate cold hyperalgesia evoked by a single administration of OXPT. OXPT is a platinum anti-cancer drug which is frequently used in many malignant tumors. The effective treatment with this drug is often limited because of its adverse effects, among which painful peripheral toxic neuropathy is the most frequent reason for therapy discontinuation (Umapathi et al., 2005). OXPT-induced neuropathy is manifested by hypersensitivity to cold which is very often present in OXPT-treated patients and this effect is also observed in animals that receive this drug. Hence, in this study we assess the ability of LPP1 to reduce cold hyperalgesia in mice. In addition, the effect of LPP1 on locomotor activity and motor coordination of OXPT-treated mice is evaluated and its acute toxicity in neuropathic mice is investigated.

MATERIALS AND METHODS

Animals

Male Albino Swiss (CD-1) mice weighing between 18 g and 22 g were used in the experiments. The animals were kept in groups of 10 mice in cages at room temperature of 22 ± 2° C, under light/dark (12:12) cycle, and they had free access to food and water before the experiments. The ambient temperature of the room and humidity were kept constant throughout the testing. The animals destined for the experiments were randomly selected. Each group consisted of 8-10 animals/dose. Immediately after the assay the animals were euthanized by cervical dislocation. Behavioral measures were scored by a trained observer blind to the experimental conditions. The experiments were performed between 8 a.m. and 3 p.m. All the procedures were approved by the Local Ethics Committee of the Jagiellonian University in Cracow (ZI/595/2011).

Chemicals

LPP1 was synthesized at the Department of Physicochemical Drug Analysis, Chair of Pharmaceutical Chemistry, Jagiellonian University in Cracow. For the behavioral tests it was prepared in 0.9% saline (Polfa Kutno, Poland) and was injected intraperitoneally (i.p.) 30 min before each test. The doses of LPP1 used in *in vivo* assays were chosen on the basis of our previous preliminary data obtained for this compound in other pharmacological tests. Control mice were given 0.9% saline 30 min before testing. OXPT (Cayman Chemicals, USA) was prepared in 5% glucose solution and was administered to mice as a single i.p. dose of 10 mg/kg 3 hours before the first measurement of the development of cold hypersensitivity.

Induction of cold hypersensitivity and measurement of the pain sensitivity threshold

The development of cold hypersensitivity induced by OXPT administration was assessed using the cold plate test. In order to compare the cold nociceptive threshold in vehicle-treated control mice and OXPT-treated control animals, hind paw licking or lifting latencies in response to noxious cold stimulation (temperature of 4°C) were estimated repeatedly 3 hours, then 1, 5, 6 and 7 days after OXPT injection. In this assay the cut off time of 60 s was established to avoid paw tissue damage (Sałat et al. 2013a). For the assessment of antihyperalgesic properties of LPP1 only mice with signs of neuropathy (i.e. OXPT-treated mice that showed reduced latencies of nocifensive reaction in response to cold as compared with vehicle-treated mice) were used. These mice were randomly divided into four groups which were first tested for their baseline latencies of pain responses, and then they were treated with the test compound at doses of 1, 10, 30 or 50 mg/kg, and subjected again to the cold plate test.

The assessment of the cold nociceptive threshold in mice was conducted using the cold plate apparatus (Bioseb, France) according to the method described recently (Osikowicz et al., 2009). Briefly, on the day of the experiment the mice were placed individually on the apparatus supplied with a thermo-controller that was set up to keep the temperature of the plate constant at 4°C. The measurement of the pain reaction (lifting or licking the hind paw) was assessed as described above.

Influence on locomotor activity

The locomotor activity test was performed both in non-neuropathic mice and in OXPT-treated mice using activity cages (40 × 40 × 30 cm, supplied with I.R. beam emitters), (Activity Cage 7441, Ugo Basile, Italy) connected to a counter for the recording of light-beam interrupts. For this test two doses of LPP1 were chosen: 30 mg/kg (the highest dose that was effective in pain tests) and 150 mg/kg (the dose that was expected to induce severe neurological and behavioral impairments). Thirty minutes before the experiment the mice were intraperitoneally pretreated with the test compound or the vehicle, and then they were individually placed in the activity cages in a sound-attenuated room. The animals horizontal and vertical movements (i.e. the number of light-beam crossings) were counted during the next 30 min of the test (Sałat et al., 2013b).

Influence on motor coordination

The test was performed according to the method described by Sałat et al. (2012a) with some minor modifications. The mice were trained daily for 3 days on the rotarod apparatus (Rotarod apparatus, May Commat RR0711, Turkey; rod diameter: 2 cm) rotating at a constant speed of 18 rotations per minute (rpm). During each training session the animals were placed on a rotating rod for 3 min with an unlimited number of trials. The proper experimentation was conducted at least 24 h after the final training trial. On the test day, 30 min before the rotarod test the mice were intraperitoneally pretreated with the test compound (30 mg/kg – the highest analgesically active dose) or the vehicle. Then the animals were tested on the rotarod apparatus revolving at 6, 18, 24 rpm. Motor impairments, defined as the inability to remain on the rod for 1 min, were measured at each speed and were expressed as the mean time spent on the rotating rod.

Assessment of acute toxicity

Acute toxicity was investigated in OXPT-treated mice according to the method described by Litchfield and Wilcoxon (1949). Each experimental group consisted of 6 animals. Total mortality rate was assessed during the 72-hour period. Finally, the median lethal dose (LD_{50} value) was established (Sałat et al., 2012b).

Data analysis

Data analysis of the *in vivo* results was provided by GraphPad Prism Software (v.5, San Diego, California, USA). The numerical results from behavioral tests were expressed as the means ± SEM (standard error of the mean). For the statistical evaluation of the results paired Student's

t-test or one-way analysis of variance (ANOVA), followed by Dunnett's post hoc comparison were used to compare the results obtained in drug-treated and control groups. Repeated measures ANOVA, followed by Bonferroni post hoc comparison was applied for the statistical evaluation of time-courses of the development of cold hyperalgesia and locomotor activity. In every case $P<0.05$ was considered significant. The median lethal dose (LD_{50}) was calculated using the log-probit method (Litchfield and Wilcoxon, 1949).

RESULTS

OXPT-induced cold hyperalgesia

The injection of OXPT resulted in a gradual decrease in the nociceptive threshold in these mice compared with their non-treated littermates (Fig.1). Since the statistically significant reduction in the cold sensitivity threshold was observed in all four experimental groups 3 hours

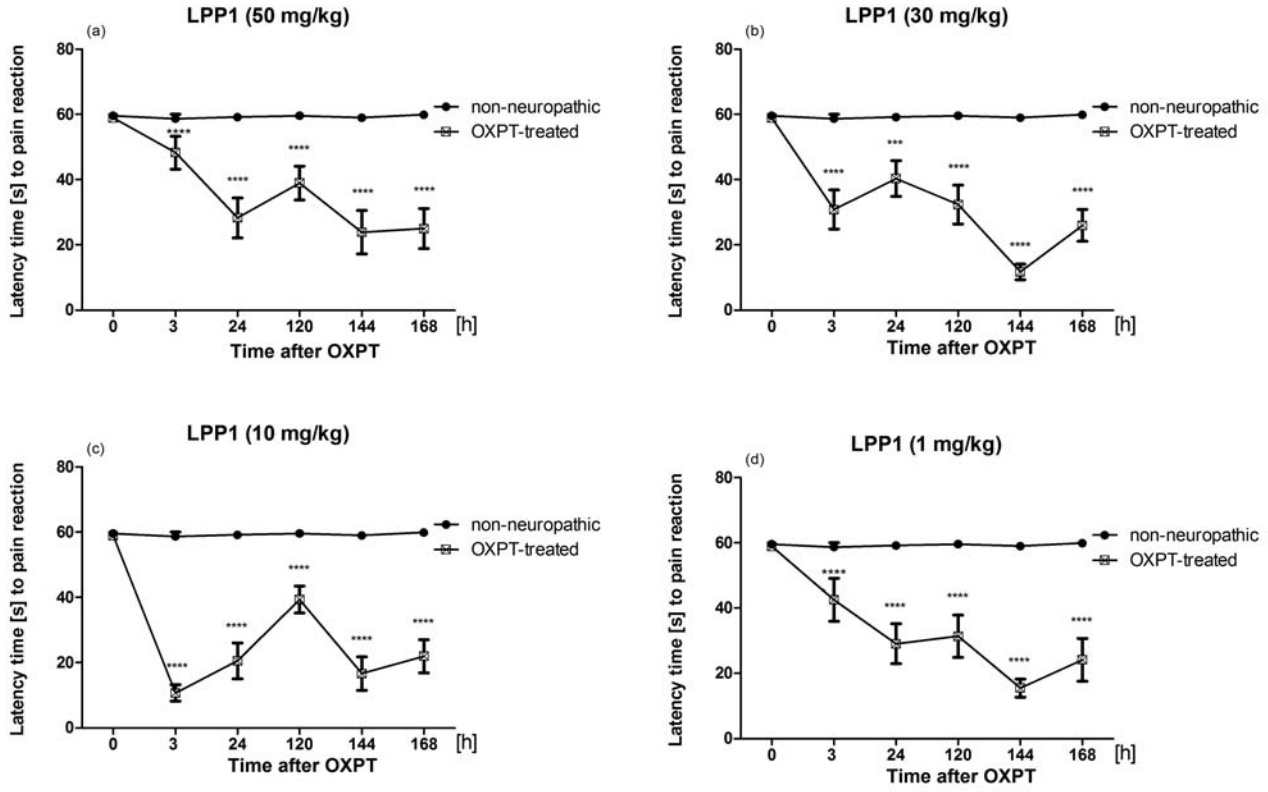

Fig. 1. Development of cold hyperalgesia in response to temperature of 4°C measured using the cold plate test in mice treated with OXPT compared to pain reactivity of non-treated littermates. Results are shown as the mean latency time to pain reaction. Statistical analysis: repeated measure analysis of variance (ANOVA), followed by Bonferroni post hoc comparison. Significance compared to mice not treated with OXPT (non-neuropathic mice): *** $P<0.001$; **** $P<0.0001$.
(a) Development of cold hyperalgesia in mice that subsequently served for the assessment of the antihyperalgesic properties of LPP1 (50 mg/kg) – drug effect: $F[1,90]=55.67$, $P<0.0001$; time effect: $F[5,90]=8.13$, $P<0.0001$; time x drug interaction: $F[5,90]=8.20$, $P<0.0001$.
(b) Development of cold hyperalgesia in mice that subsequently served for the assessment of the antihyperalgesic properties of LPP1 (30 mg/kg) - drug effect: $F[1,18]=135.09$, $P<0.0001$; time effect: $F[5,90]=11.97$, $P<0.0001$; time x drug interaction: $F[5,90]=11.58$, $P<0.0001$.
(c) Development of cold hyperalgesia in mice that subsequently served for the assessment of the antihyperalgesic properties of LPP1 (10 mg/kg) - drug effect: $F[1,18]=217.76$, $P<0.0001$; time effect: $F[5,90]=20.17$, $P<0.0001$; time x drug interaction: $F[5,90]=19.01$, $P<0.0001$.
(d) Development of cold hyperalgesia in mice that subsequently served for the assessment of the antihyperalgesic properties of LPP1 (1 mg/kg) - drug effect: $F[1,18]=82.05$, $P<0.0001$; time effect: $F[5,90]=9.09$, $P<0.0001$; time x drug interaction: $F[5,90]=9.04$, $P<0.0001$.

after OXPT injection, this time point was set up as acute hyperalgesia, while for the measurement of late hyperalgesia the animals responsiveness 7 days after OXPT injection was used. Statistical analyses of data obtained for the development of cold hyperalgesia were as follows: for mice that were subsequently used to assess the antihyperalgesic properties of LPP1 at a dose of 1 mg/kg (Fig.1a) – drug effect: $F[1,18]=82.05$, $P<0.0001$; time effect: $F[5,90]=9.09$, $P<0.0001$; time x drug interaction: $F[5,90]=9.04$, $P<0.0001$. For mice that were used to assess the antihyperalgesic properties of LPP1 at 10 mg/kg (Fig.1b) – drug effect: $F[1,18]=217.76$, $P<0.0001$; time effect: $F[5,90]=20.17$, $P<0.0001$; time x drug interaction: $F[5,90]=19.01$, $P<0.0001$. For mice that subsequently served for the assessment of the antihyperalgesic properties of LPP1 at a dose of 30 mg/kg (Fig. 1c) – drug effect: $F[1,18]=135.09$, $P<0.0001$; time effect: $F[5,90]=11.97$, $P<0.0001$; time x drug interaction: $F[5,90]=11.58$, $P<0.0001$, and for mice that were used to assess the antihyperalgesic properties of LPP1 (50 mg/kg; Fig. 1d) – drug effect: $F[1,90]=55.67$, $P<0.0001$; time effect: $F[5,90]=8.13$, $P<0.0001$; time x drug interaction: $F[5,90]=8.20$, $P<0.0001$.

Antihyperalgesic activity of LPP1

The antihyperalgesic activity of LPP1 at doses of 1, 10, 30 and 50 mg/kg was assessed twice: 3 hours and 7 days after OXPT (Fig. 2). The dose of 1 mg/kg had antihyperalgesic properties 7 days after OXPT (significant at $P<0.05$), but not 3 hours after OXPT (Fig. 2a). The dose of 10 mg/kg elevated the pain threshold only in the acute phase (significant at $P<0.05$; Fig. 2b). The dose of 30 mg/kg showed antihyperalgesic proper-

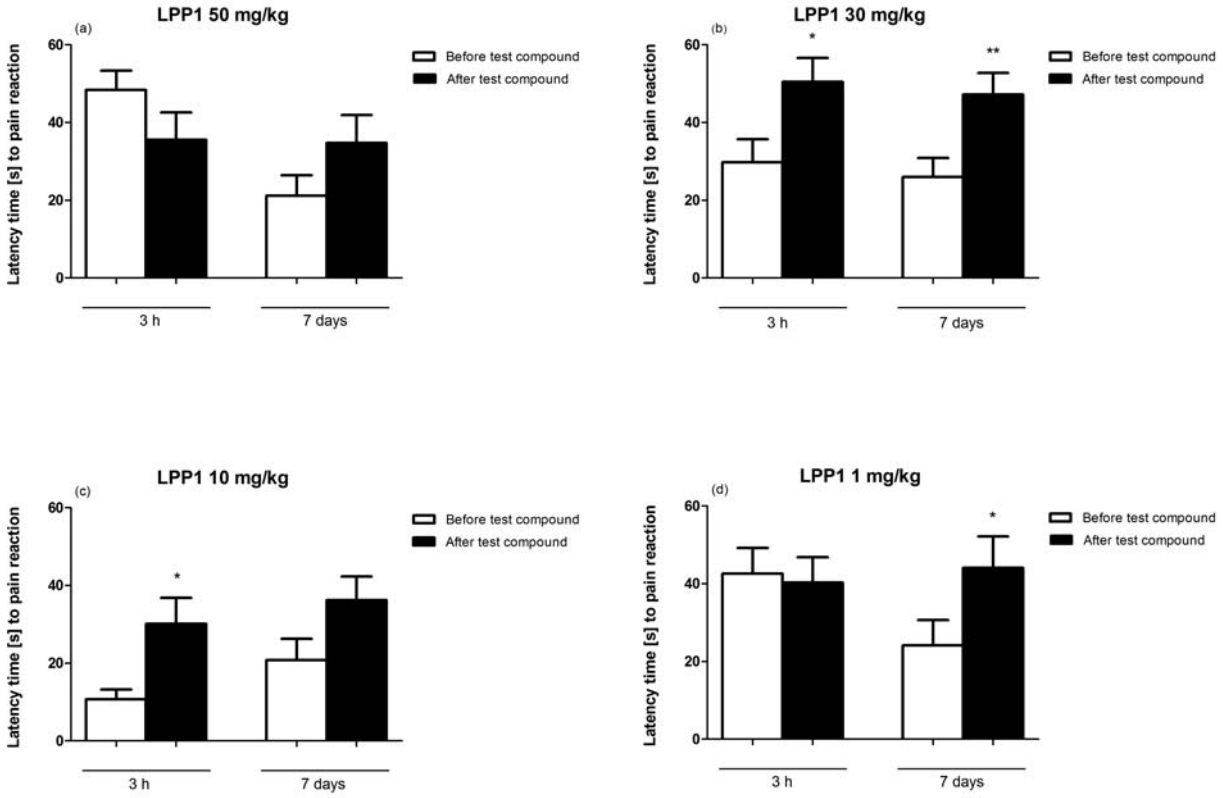

Fig. 2. Antihyperalgesic activity of LPP1 in OXPT-treated neuropathic mice measured using the cold plate test at two time points: 3h and 7 days after OXPT injection. Results are shown as the mean latency time to pain reaction (licking or lifting the hind paw) in response to cold (temperature of 4° C). Statistical analysis: paired Student's t-test: *P<0.05, **P<0.01 (vs. baseline latency of neuropathic mice – i.e., latency before the test compound's administration).

ties 3 hours (significant at P<0.05) and 7 days (significant at P<0.01) after OXPT administration (Fig. 2c). The dose of 50 mg/kg did not demonstrate antihyperalgesic activity either in the acute phase or in the late phase (Fig. 2d).

Influence on locomotor activity

In the locomotor activity test the influence of LPP1 (30 mg/kg and 150 mg/kg) on animals locomotor activity was assessed in neuropathic mice and compared with that of non-neuropathic and OXPT-treated neuropathic controls (Fig. 3). The dose of 30 mg/kg had no effect on animals locomotor activity (data not shown), but the dose of 150 mg/kg completely abolished animals locomotor activity (P<0.0001). The locomotor activity of OXPT-treated control mice was only slightly reduced as compared with non-neuropathic control mice, and these differences were not statistically significant (Fig. 3).

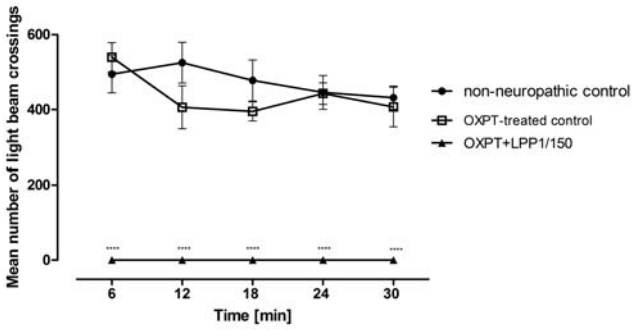

Fig. 3. Influence of LPP1 at the dose of 150 mg/kg on locomotor activity of OXPT-treated mice compared to non-neuropathic and OXPT-treated controls. Results are shown as mean number of light beam crossings in control mice and LPP1-treated mice. Statistical analysis: repeated measure analysis of variance (ANOVA), followed by Bonferroni post hoc comparison. Significance compared to OXPT-treated control mice: ****P<0.0001. Drug effect: F[4,25]=38.17, P<0.0001; time effect: F[4,100]=5.87, P<0.001; time × drug interaction: F[16,100]=2.38, P<0.01.

Influence on motor coordination

In the rotarod test at 6, 18 or 24 rpm LPP1 at a dose of 30 mg/kg did not induce motor coordination impairments in OXPT-treated mice.

Assessment of acute toxicity

To assess acute toxicity of LPP1 its LD_{50} equal to 329.8 mg/kg (254.1-428.0) was established in OXPT-treated neuropathic mice. This value was approximately twice as low as the previously established LD_{50} value for LPP1 in non-neuropathic mice (Sałat et al., 2012b).

DISCUSSION

The aim of the present research was to establish an antihyperalgesic effect of a novel dihydrofuran-2-one derivative, the compound LPP1, in a mouse model of OXPT-induced painful toxic neuropathy. Previously we demonstrated significant antinociceptive properties of LPP1 in rodent models of acute (Sałat et al., 2009; Sałat et al., 2012b), tonic (Sałat et al., 2013a) and diabetic neuropathic pain (Sałat et al., 2013a; Sałat and Sałat, 2013). This compound also showed strong local anesthetic properties in models of local anesthesia in guinea pigs (Sałat et al., 2009), and it had antioxidant capacity *in vitro* and *ex vivo* (Sałat et al., 2012b; Sałat et al., 2014b). These results suggest that both the cell membrane-stabilizing activity and the reactive oxygen species-scavenging activity may contribute to pharmacological effects caused by this compound and observed *in vivo*.

The ability of LPP1 to attenuate OXPT-evoked hyperalgesia was assessed using the cold plate test as OXPT induces hypersensitivity to cold both in patients treated with this drug and in experimental animals. OXPT is an anticancer drug which induces the formation of DNA cross-links causing apoptotic death of dividing cells, but it has also affinity for the peripheral nervous system. In rodents a single injection of OXPT induces painful peripheral neuropathy accompanied by tactile allodynia (pain induced by normally innocuous stimuli) and cold hyperalgesia (Nassini et al., 2011; Renn et al., 2011), but not heat hyperalgesia (Xiao et al., 2012).

At doses of 1-30 mg/kg LPP1 prolonged the latency of nocifensive reaction in response to cold stimulus, which indicates that it has antihyperalgesic properties *in vivo*. Recently, it has been shown that cold hyperalgesia in rodents

treated with OXPT is mediated by the Transient Receptor Potential cation channel subfamily A, member 1 (TRPA1) stimulation (Nassini et al., 2011; Zhao et al., 2012). Zhao et al. (2012) demonstrated that a single dose of OXPT induces cold hypersensitivity associated with enhanced responsiveness of TRPA1 but not with other transient receptor potential channels. Although the precise mechanism of TRPA1 contribution to OXPT-induced cold hyperalgesia is not clear, its role as a sensor for electrophilic, reactive compounds, such as reactive oxygen species, is well established (Nassini et al., 2011). In our earlier research the blockade of TRPA1 as a potential mechanism of action of LPP1 was demonstrated (Sałat et al., 2014a). Due to its antioxidant capacity, LPP1 might also indirectly inhibit TRPA1, resulting in the profound attenuation of OXPT-evoked hypersensitivity to cold.

The mechanisms underlying the observed dose- and time-dependent responses of LPP1-treated mice to cold stimulus are not clear and they require further studies. The lack of efficacy of LPP1 at a dose of 10 mg in the late phase of cold hyperalgesia, and the lack of antihyperalgesic activity of LPP1 at 1 mg/kg in the acute phase are difficult to explain but this observation suggests that early and late phases of OXPT-induced hyperalgesia are mediated by two distinct mechanisms which are not equally sensitive to various doses of this analgesically active compound.

In this research the influence of the compound LPP1 on neuropathic animals locomotor activity was also investigated. For this purpose two doses were chosen – the dose of 30 mg/kg which was the highest analgesically active dose of LPP1, and the dose of 150 mg/kg. In our previous research we observed that doses up to 100 mg/kg had no influence on animals motor skills, while doses higher than 100 mg/kg might induce neurological and behavioral deficits. In the present study for the dose of 150 mg/kg a significant reduction in animals' locomotor activity was demonstrated. This may suggest sedative properties of this compound *in vivo*, but it should be emphasized that this effect was observed only for the dose of 150 mg/kg, and sedation was not observed for analgesically active low doses. This is an important fact as it is well known that compounds with sedative properties may give false positive results in pain tests. Similarly, drug-induced motor deficits may also be responsible for these false positive results. In view of the above, the effect of LPP1 at the analgesically active dose (30 mg/kg) on OXPT-treated animals' motor coordination was assessed using the rotarod test. The results obtained confirmed that this compound did not induce motor impairments at doses that were effective in pain tests.

The studies to assess acute toxicity of LPP1 in OXPT-treated mice resulted in the establishment of the LD_{50} value after i.p. administration which was 329.8 mg/kg. In our earlier research the LD_{50} value in mice without neuropathy was established (748 mg/kg) (Sałat et al., 2012b). In view of the above, it can be concluded that OXPT co-administered with LPP1 increases its toxicity in mice as the LD_{50} value in neuropathic mice is more than twice as low as that found for their non-neuropathic littermates.

To conclude, in this study a significant antihyperalgesic effect of a novel derivative of dihydrofuran-2-one was demonstrated. This compound effectively attenuated cold hyperalgesia induced by OXPT at doses much lower than the doses causing acute toxic effects. This indicates that LPP1 might be a very interesting lead structure in the search for analgesics used in neuropathic patients.

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