Pharmacology, Biochemistry and Behavior 122 (2014) 173–181

Contents lists available at ScienceDirect

Pharmacology, Biochemistry and Behavior

journal homepage: www.elsevier.com/locate/pharmbiochembeh

Antiallodynic and antihyperalgesic activity of 3-[4-(3-trifluoromethyl-phenyl)-piperazin-1-yl]-dihydrofuran-2-one compared to pregabalin in chemotherapy-induced neuropathic pain in mice

Kinga Sałat [a,*], Agnieszka Cios [b], Elżbieta Wyska [b], Robert Sałat [c], Szczepan Mogilski [a], Barbara Filipek [a], Krzysztof Więckowski [d], Barbara Malawska [d]

[a] Department of Pharmacodynamics, Chair of Pharmacodynamics, Jagiellonian University, Medical College, Medyczna 9, 30-688 Cracow, Poland
[b] Department of Pharmacokinetics and Physical Pharmacy, Jagiellonian University, Medical College, Medyczna 9, 30-688 Cracow, Poland
[c] Department of Fundamental Engineering, Faculty of Production Engineering, Warsaw University of Life Sciences, Nowoursynowska 164, 02-787 Warsaw, Poland
[d] Department of Physicochemical Drug Analysis, Chair of Pharmaceutical Chemistry, Jagiellonian University, Medical College, Medyczna 9, 30-688 Cracow, Poland

ARTICLE INFO

Article history:
Received 26 November 2013
Received in revised form 14 March 2014
Accepted 30 March 2014
Available online 12 April 2014

Keywords:
Chemotherapy-induced neuropathic pain
Dihydrofuran-2-one
Pain threshold
Pharmacokinetics
Pregabalin
Transient Receptor Potential channels

ABSTRACT

Background: Anticancer drugs — oxaliplatin (OXPT) and paclitaxel (PACLI) cause painful peripheral neuropathy activating Transient Receptor Potential (TRP) channels. Here we investigated the influence of 3-[4-(3-trifluoromethyl-phenyl)-piperazin-1-yl]-dihydrofuran-2-one (LPP1) and pregabalin on nociceptive thresholds in neuropathic pain models elicited by these drugs. Pharmacokinetics of LPP1 and its ability to attenuate neurogenic pain caused by TRP agonists: capsaicin and allyl isothiocyanate (AITC) were also investigated.

Methods: Antiallodynic and antihyperalgesic effects of intraperitoneally administered LPP1 and pregabalin were tested in the von Frey, hot plate and cold water tests. The influence of LPP1 on locomotor activity and motor coordination was assessed using actimeters and rotarod. Serum and tissue concentrations of LPP1 were measured using the HPLC method with fluorimetric detection.

Results: In OXPT-treated mice LPP1 and pregabalin dose-dependently reduced tactile allodynia (41–106% and 6–122%, respectively, $p < 0.01$). At the dose of 10 mg/kg LPP1 attenuated cold allodynia. In PACLI-treated mice LPP1 and pregabalin reduced tactile allodynia by 12–63% and 8–50%, respectively ($p < 0.01$). Both drugs did not affect cold allodynia, whereas pregabalin (30 mg/kg) attenuated heat hyperalgesia (80% *vs.* baseline latency time; $p < 0.01$). No motor impairments were observed in LPP1 or pregabalin-treated neuropathic mice in the rotarod test, while severe sedation was noted in the locomotor activity test.

LPP1 reduced pain induced by capsaicin (51%; $p < 0.01$) and AITC (41%; $p < 0.05$). The mean serum concentration of LPP1 measured 30 min following i.p. administration was 7904.6 ± 1066.1 ng/ml. Similar levels were attained in muscles, whereas brain concentrations were 62% lower. Relatively high concentrations of LPP1 were also determined in the cerebrospinal fluid and the sciatic nerve.

Conclusions: LPP1 and pregabalin reduce pain in OXPT and PACLI-treated mice. This activity of LPP1 might be in part attributed to the inhibition of TRPV1 and TRPA1 channels, but also central mechanisms of action cannot be ruled out.

1. Introduction

The International Association for the Study of Pain (IASP) defines neuropathic pain as pain initiated or caused by a primary lesion or dysfunction of the nervous system (Backonja, 2003). This type of pain is typically characterized by allodynia — pain hypersensitivity evoked by innocuous stimuli, and hyperalgesia — a decrease in pain threshold resulting in an increased response to a normally painful stimulus. Numerous factors have been identified as potential mechanisms underlying the development of neuropathic pain: mechanical nerve injuries, viral infections or treatment with anticancer drugs — platinum-based drugs (cisplatin and oxaliplatin — OXPT), vincristine and paclitaxel (PACLI), which induce toxic neuropathies both in humans and in animal models (Fallon, 2013; Gilron et al., 2005).

Although analgesic adjuvants (antidepressants and anticonvulsant drugs) provide sufficient efficacy in many pain syndromes, they have proven relatively weaker effect in the alleviation of neuropathic pain. In view of this, neuropathic pain still remains intractable and its treatment

Abbreviations: AITC, allyl isothiocyanate; CSF, cerebrospinal fluid; OXPT, oxaliplatin; PACLI, paclitaxel; ROS, reactive oxygen species; rpm, rotations per minute; TRP channels, Transient Receptor Potential channels.

* Corresponding author. Tel./fax: +48 12 620 55 30.
E-mail address: salat.kinga@gmail.com (K. Sałat).

http://dx.doi.org/10.1016/j.pbb.2014.03.025
0091-3057/

is a challenging endeavor (Fallon, 2013). It is estimated that the resistance to conventional analgesic pharmacotherapy is present in approximately 40% of neuropathic patients (Finnerup et al., 2005; Gagnon et al., 2003).

In the available literature there are a limited number of reports regarding the efficacy of analgesic drugs in the prevention or attenuation of pain in patients treated with OXPT or PACLI. For this reason, there is a strong need for extended studies on available analgesics, as well as searching for novel analgesic-active compounds endowed with a greater analgesic efficacy as compared to currently used drugs in these neuropathic pain syndromes which result from anticancer drug-induced toxicity.

Recently we have shown that 3-[4-(3-trifluoromethyl-phenyl)-piperazin-1-yl]-dihydrofuran-2-one (LPP1) is a very efficacious antiallodynic, antihyperalgesic and analgesic compound in the mouse model of streptozotocin-induced neuropathic pain (Sałat et al., 2013a). We have also demonstrated that this compound can potentiate the antiallodynic effect of pregabalin in diabetic neuropathic animals (Salat and Salat, 2013). In the present study to further assess its antiallodynic and antihyperalgesic efficacy in rodents we use OXPT and PACLI neuropathic pain models to establish the influence of LPP1 and pregabalin (a reference drug) on mechanical and thermal (heat and cold) nociceptive thresholds in mice. The potential sedative and motor-impairing properties of both compounds in neuropathic animals are also investigated.

To gain an insight into the possible mechanisms underlying the antiallodynic and antihyperalgesic activities of LPP1, its antinociceptive activity is assessed in allyl isothiocyanate (AITC) and capsaicin neurogenic pain models. It is well known that so called Transient Receptor Potential channels (TRP channels) for which AITC and capsaicin are selective agonists (TRPA1 and TRPV1 channels, respectively) play a pivotal role as sensors for cold, mechanical (TRPA1 channels) and heat (TRPV1 channels) stimuli in chemotherapy-induced neuropathic pain models (Hara et al., 2013; Materazzi et al., 2012; Nassini et al., 2011; Sałat et al., 2013b). Hence, in this study we use antagonists of these channels — capsazepine (a TRPV1 antagonist) and A-967079 (a TRPA1 antagonist) as reference drugs to confirm the role of TRPV1 and TRPA1 in OXPT- and PACLI-induced neuropathies. To better understand the observed pharmacological responses, concentrations of LPP1 in serum and target tissues are measured.

2. Materials and methods

The behavioral measures were scored by trained observers blind to experimental conditions.

2.1. Animals

Adult male Albino Swiss (CD-1) mice weighing between 18 g and 25 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 had free access to food and water before experiments. The ambient temperature of the room and humidity were kept consistent throughout all the tests. For the behavioral experiments the animals were selected in a random way. Each group consisted of 8–10 animals/dose, and each mouse was used only once. The experiments were performed between 8 a.m. and 3 p.m. Immediately after the assay the animals were killed by cervical dislocation. All the procedures were approved by the Local Ethics Committee of the Jagiellonian University in Cracow (ZI/595/2011).

2.2. Chemicals used in behavioral tests

LPP1 was synthesized at the Department of Physicochemical Drug Analysis, Chair of Pharmaceutical Chemistry, Jagiellonian University in Cracow. The synthesis of this compound was described previously (Sałat et al., 2009). Pregabalin was provided by Tocris Bioscience (Germany). For the assays both LPP1 and pregabalin were prepared in 0.9% saline (Polfa Kutno, Poland) and were injected intraperitoneally (i.p.) 30 min before the behavioral test. Control animals were given 0.9% saline (i.p.) 30 min before testing.

OXPT (Cayman Chemicals, USA) was prepared in a 5% glucose solution (Polfa Kutno, Poland) and was administered as a single i.p. dose of 10 mg/kg. PACLI, capsaicin, capsazepine and A-967079 were purchased from Sigma Aldrich (Poland). To induce neuropathy PACLI was dissolved in ethanol (100%) at 10% of the final desired volume and vortexed for 2 min. An equal volume of Cremophor EL (10% of the final volume, Sigma Aldrich, Poland) was then added and the mixture was vortexed again for 10 min. Prior to the injection, ice-cold saline (80% of the final volume) was added to make up a final volume and the solution was maintained on ice during dosing. Capsaicin, capsazepine and A-967079 were dissolved in ethanol (100%) at 5% of the final desired volume and then 0.9% saline was added. This mixture was vortexed for 10 min. AITC was diluted in corn oil (Sigma Aldrich, Poland) to obtain a 0.1% solution.

2.3. OXPT-induced neuropathic pain

2.3.1. Development of cold allodynia

The development of cold allodynia in response to OXPT was assessed in the cold water test 2 h (acute allodynia) and 7 days (late allodynia) after OXPT injection. In order to compare the cold nociceptive threshold of vehicle-treated mice and OXPT-treated animals the paw withdrawal latencies in response to cold stimulation of hind paws (water bath maintained at 4 °C) were estimated (Sałat et al., 2013a).

2.3.2. Influence on cold allodynia

After the establishment of baseline latencies for each OXPT-treated mouse, the animals were i.p. pretreated with the test compounds: LPP1, pregabalin, TRP channel antagonists or vehicle. 30 min later they were observed until paw withdrawal. A cut-off time of 30 s was established to avoid paw tissue damage. The mice not responding within 30 s were removed from the water and assigned a score of 30 s. The reaction time was measured 2–3 times, with an interval of at least 15 min between the 2 measurements to obtain 2 consecutive values that differed by no more than 10%. Between 2 measurements the hind paws were immediately dried with cellulose paper to avoid paw cooling. Final results were expressed as percent maximal possible effect (%MPE) according to the following formula:

$$\%\text{MPE} = [(\text{mean post} - \text{drug latency} - \text{mean pre} - \text{drug latency})/ (30\ \text{s} - \text{mean pre} - \text{drug latency})] \times 100\%.$$

2.3.3. Influence on tactile allodynia

Hypersensitivity to mechanical stimuli (tactile allodynia) was assessed using the electronic von Frey unit (Bioseb, France) supplied with a single flexible filament applying increasing force (from 0 to 10 g) against the plantar surface of the hind paw of the mouse. The nocifensive paw withdrawal response automatically turned off the stimulus and the mechanical pressure that evoked the response was recorded.

On the day of the experiment, the mice were placed individually in test compartments with a wire mesh bottom and were allowed to habituate for 1 h. After the habituation period, in order to obtain baseline values, each mouse was tested 3 times alternately in each hind paw, allowing at least 30 s between each measurement. Then the mice were i.p. pretreated with the test compounds or vehicle and 30 min later the animals were tested again and mean values for each mouse were obtained (Sałat et al., 2013a).

2.3.4. Influence on heat nociceptive threshold

Heat hyperalgesia was assessed in the hot plate test according to the procedure previously described (Sałat et al., 2013a). Briefly, after the

establishment of baseline latencies to pain reaction, the animals were i.p. pretreated either with the test compounds or vehicle. 30 min later, the animals were placed on the hot plate apparatus (Hot Plate 2A Type Omega, Poland). This apparatus has an electrically heated surface and is supplied with a temperature-controller that maintains the temperature at 55–56 °C. The time until the animal licked its hind paws or jumped was recorded by means of a stop-watch. In this assay a cut-off time was established (45 s) to avoid tissue damage, and mice not responding within 45 s were removed from the apparatus and assigned a score of 45 s.

2.4. PACLI-induced neuropathic pain

To induce painful peripheral neuropathy PACLI was injected i.p. as a single dose (6 mg/kg), (Materazzi et al., 2012). The development of cold allodynia in response to PACLI was measured according to the same protocol as described in Subsection 2.3.1. The reduction of latency time to pain reaction in PACLI-treated animals compared to non-treated mice was an indicator of neuropathy. The antiallodynic/antihyperalgesic activities of LPP1, pregabalin, capsazepine and A-967079 (reference drugs administered i.p.) in PACLI-treated neuropathic mice were assessed using von Frey, cold water and hot plate tests as described in Subsections 2.3.2–2.3.4.

2.5. Influence on motor impairments — the rotarod test

The test was performed according to the method recently described (Salat et al., 2012a). Briefly, naïve and neuropathic 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 i.p. pretreated with the investigated compounds or vehicle. Then, the animals were tested on the rotarod revolving at 6, 18 or 24 rpm. Motor impairments, defined as the inability to remain on the rotating rod for 1 min, were measured at each speed and were expressed as mean time spent on the rotating rod.

2.6. Influence on locomotor activity

The locomotor activity test was performed using activity cages (40 × 40 × 31 cm) supplied with I.R. horizontal beam emitters (Activity Cage 7441, Ugo Basile, Italy) connected to a counter for the recording of light-beam interrupts. 30 min before the experiment the mice were i.p. pretreated with LPP1 or pregabalin (each at 150 mg/kg), then being individually placed in the activity cages in a sound-attenuated room. The number of light-beam crossings was counted in each group during the next 30 min in 6-min intervals.

2.7. Influence on TRPV1 and TRPA1-mediated nociception

2.7.1. Capsaicin test

After the adaptation period (15 min), 1.6 μg of capsaicin dissolved in 20 μl of a mixture containing 0.9% saline and ethanol (5% of the final volume) was injected intraplantarly (i.pl.) in the ventral surface of the right hind paw of the mouse. The test compounds were administered i.pl. 15 min before capsaicin. Control animals received the vehicle. The animals were observed individually for 5 min following capsaicin injection. The amount of time spent on licking, biting or lifting the injected paw was recorded with a chronometer and was considered as an indicator of nociception (Sałat et al., 2009).

2.7.2. AITC-induced pain

After the adaptation period (15 min), 20 μl of 0.1% AITC was injected i.pl. in the ventral surface of the right hind paw of the mouse. The test compounds were administered i.pl. 15 min before AITC. Control animals received the vehicle. The animals were observed individually for 20 min following AITC injection. The amount of time spent on licking, biting or lifting the injected paw was recorded with a chronometer and was considered as an indicator of nociception (Zhao et al., 2012).

2.8. Pharmacokinetic study

Male Albino Swiss (CD-1) mice weighing 18–25 g received a single i.p. dose of LPP1 (30 mg/kg of body weight) dissolved in 0.9% saline. 30 min later these animals were sacrificed by decapitation under light anesthesia (Thiopental sodium, Polfa Kutno, Poland; 70 mg/kg, i.p.). The blood was collected and allowed to clot at room temperature. Serum was obtained by centrifugation at 1500 ×g for 10 min at 4 °C. Mouse brains, quadriceps muscles, cerebrospinal fluid (CSF), and sciatic nerves were harvested and the samples were stored at −80 °C until assayed.

2.8.1. Analytical method

Concentrations of LPP1 in serum, CSF and tissues were measured by a high performance liquid chromatography (HPLC) method. Brains, muscles, and sciatic nerves were weighed and homogenized in 50 mM PBS buffer pH = 7.4 (1:4, w/v) with a tissue homogenizer TH220 (Omni International Inc., Warrenton, VA, USA). Serum (100 μl), CSF (10 μl), and tissue homogenates (100–200 μl) were deproteinized with a mixture of ice-cold methanol and acetonitrile (1:1, v/v). After vigorous vortex-mixing for 1 min, the samples were centrifuged at 8000 ×g for 10 min at 4 °C (EBA 12 R, Hettich, Germany). The supernatants were transferred into HPLC autosampler vials and 10–50 μl aliquots were injected onto the HPLC system.

The HPLC system (Merck-Hitachi LaChrom Elite, Japan) consisted of an L-2130 pump, an L-2200 autosampler, an L-2350 column oven, and an L-2480 fluorescence detector. EZChrome Elite 4.0 (Merck-Hitachi, Japan) software was used for data acquisition. All analyses were performed on a 250 × 4.6 mm LiChrospher®100 RP-18 column (Merck, Darmstadt, Germany) maintained at 40 °C, protected with a guard-column (4 × 4 mm) with the same packing material. The mobile phase consisted of 0.1 M potassium dihydrogen phosphate containing 0.01 M tetrabutylammonium hydrogen sulfate (pH 6.4) and acetonitrile (75:25, v/v). The flow rate was set to 1 ml/min and the fluorescence detector was set at an excitation wavelength of 260 nm and an emission wavelength of 365 nm.

Under these conditions, the retention time of LPP1 was 11.40 min. No interfering peaks were observed in the chromatograms. The calibration curves constructed by plotting peak area *versus* concentrations of LPP1 were linear in the tested concentration ranges. The assay was reproducible with low intra- and inter-day variations (coefficient of variation less than 10%). The limit of quantification was 10 ng/ml in serum and CSF, and 50 ng/g in tissues.

2.9. Data analysis

The analysis of the results obtained in behavioral tests was provided by GraphPad Prism Software (ver. 5, San Diego, CA, USA). The results were statistically evaluated using paired Student's t-test or one-way analysis of variance (ANOVA), followed by Dunnett's *post hoc* comparison. Repeated measures ANOVA and Bonferroni *post hoc* comparison were applied for the statistical evaluation of time-courses of the development of cold allodynia in OXPT-treated and PACLI-treated animals, and for the evaluation of the results obtained in the locomotor activity test. In every case $p < 0.05$ was considered significant.

The log-probit method (Litchfield and Wilcoxon, 1949) was applied to establish median effective doses (ED_{50}) for LPP1 and pregabalin in the

von Frey test. Here the ED_{50} value is defined as the dose of the investigated compound that elevates the paw withdrawal threshold (*i.e.* the dose that diminishes the pain reaction) by 50% as compared to baseline value.

3. Results

3.1. OXPT-induced neuropathic pain

3.1.1. OXPT-treated mice exhibit early- and late-phase cold allodynia

2 h after OXPT injection the mice had significantly reduced pain sensitivity threshold in response to cold stimulus (59% *vs.* non-treated littermates, $p < 0.001$). This reduction of pain threshold was maintained and 7 days after OXPT injection the pain threshold was reduced by 48% ($p < 0.001$) as compared to non-treated mice (Fig. 1a).

3.1.2. LPP1 attenuates cold allodynia in OXPT-treated mice

2 h after OXPT injection LPP1 at the dose of 10 mg/kg was able to attenuate cold allodynia. For this dose the %MPE value was 24.6% ($p < 0.05$). Other doses tested had no effect. At this time point pregabalin at 1, 10 and 30 mg/kg and capsazepine at 30 mg/kg did not demonstrate antiallodynic properties, either. A relatively weak but statistically significant antiallodynic effect was observed for A-967079 at the dose of 30 mg/kg. Its %MPE was 12.84% ($p < 0.05$; Table 1).

7 days after OXPT injection neither LPP1, nor pregabalin (both tested at 1, 10 and 30 mg/kg) demonstrated antiallodynic properties in the cold water test. For doses: 1, 10 and 30 mg/kg the %MPE ranged from 2.2% to 10.7% for pregabalin, and from 3.4% to 9.3% for LPP1. At this time point only A-967079 prolonged the latency time to pain reaction, and its %MPE was 16.7% ($p < 0.05$). Capsazepine had no effect (5.1%).

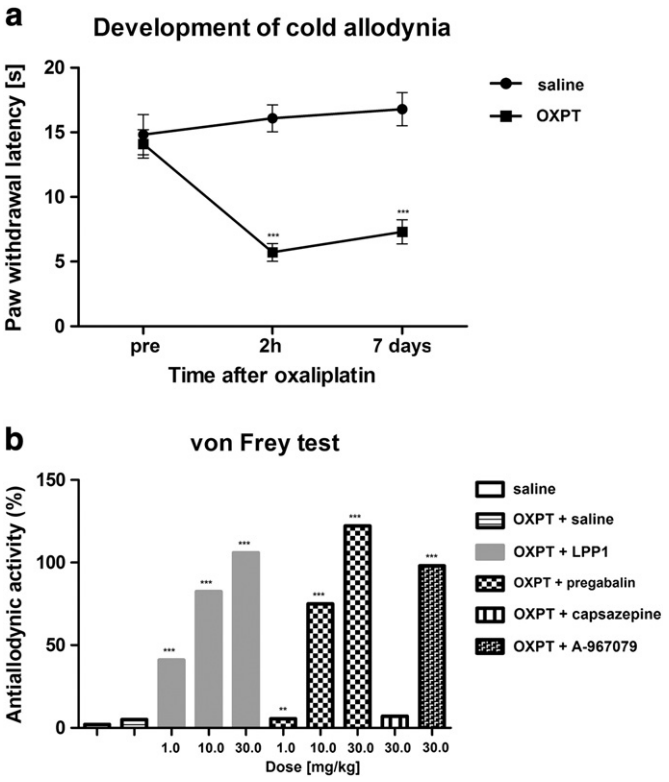

Fig. 1. (**a**) Time courses of the development of OXPT-induced cold allodynia investigated in the cold water test in mice. Statistical analysis: repeated measures analysis of variance (ANOVA), followed by Bonferroni *post hoc* comparison. Results compared to naïve mice: ***$p < 0.001$. Drug effect: $F[1,174] = 54.88$, $p < 0.0001$; time effect: $F[2,174] = 5.17$, $p < 0.01$; interaction: $F[2,174] = 11.10$, $p < 0.0001$. (**b**) Antiallodynic effect of LPP1, pregabalin, capsazepine and A-967079 in the von Frey test in OXPT-treated mice. Results are shown as % antiallodynic activity of the test compounds compared to the respective baseline values in each group. Statistical analysis: paired Student's t-test. Significance *vs.* baseline paw withdrawal threshold: **$p < 0.01$; ***$p < 0.001$. Saline group — mice not treated with OXPT.

3.1.3. LPP1 and pregabalin attenuate tactile allodynia in OXPT-treated mice

In animals not treated with OXPT the mean force that caused paw withdrawal was 3.0 ± 0.08 g. In OXPT-treated mice a statistically significant reduction of pain sensitivity threshold was observed (1.7 ± 0.05 g; $p < 0.001$ *vs.* before OXPT treatment). In neuropathic animals LPP1 in a dose-dependent manner elevated pain sensitivity threshold (Fig. 1b) between 41% and 106% ($p < 0.001$). Pregabalin at doses 1–30 mg/kg was also able to elevate the nociceptive threshold for mechanical stimulation (6%–122% *vs.* baseline values; $p < 0.01$; Fig. 1b). In this test the compound LPP1 was less efficacious but more potent than pregabalin. ED_{50} values of both LPP1 and pregabalin were 1.6 mg/kg and 4.4 mg/kg, respectively. TRP channel antagonists tested in this assay as reference drugs — capsazepine and A-967079, elevated pain sensitivity threshold by 7% and 98%, respectively. For A-967079 this effect was statistically significant at $p < 0.001$ (Fig. 1b).

3.1.4. OXPT-treated mice demonstrate prolonged latency time to heat-induced pain responses

In control animals that were not treated with OXPT the mean latency time to nocifensive response was 10.6 ± 0.8 s. The treatment with OXPT resulted in a prolongation of latency time to heat-induced pain responses. As shown in Table 2 the baseline latencies to pain reaction in OXPT-treated mice ranged from 12.3 ± 0.5 s to 21.2 ± 2.5 s. LPP1 at 1 mg/kg and 30 mg/kg prolonged the latency time to pain reaction in OXPT-treated mice by 31% ($p < 0.01$) and 144% ($p < 0.001$), respectively, whereas pregabalin was active only at 30 mg/kg (41%, $p < 0.05$) and it was much less efficacious than LPP1. Capsazepine (30 mg/kg) prolonged the latency time to pain reaction by 44% ($p < 0.01$), while A-967079 had no effect in this assay (Table 2).

3.2. PACLI-induced neuropathic pain

3.2.1. PACLI-treated mice exhibit early- and late-phase cold allodynia

In naïve mice (*i.e.*, mice not treated with PACLI) the mean latency time to pain reaction in response to cold was 15.3 ± 2.2 s. 2 h later in non-treated animals the latency time to pain reaction was 16.1 ± 1.6 s and 7 days later 18.0 ± 1.7 s. In PACLI-treated mice the latencies were significantly shorter: 5.06 ± 0.7 s ($p < 0.001$ *vs.* non-treated subjects; 2 h after PACLI) and 2.8 ± 0.4 s ($p < 0.001$ *vs.* non-treated subjects; 7 days after PACLI; Fig. 2a).

3.2.2. LPP1 and pregabalin have no effect on cold allodynia in PACLI-treated animals

In the cold water test LPP1 at doses 1, 10 and 30 mg/kg had no influence on cold nociceptive threshold either 2 h after PACLI injection (%MPEs for these doses were: 5.6%, 8.3% and 9.2%, respectively) or 7 days later (%MPEs for these doses were: 2.7%, 4.1% and 6.3%, respectively). Pregabalin at the same doses did not demonstrate antiallodynic properties, either. 2 h after PACLI its %MPEs were: 6.2%, 3.9%, and 7.1%, respectively, and 7 days later the %MPE values were: 8.4%, 9.5% and 8.8%, respectively for 1, 10 and 30 mg/kg. Capsazepine at 30 mg/kg had no effect in this test, while for A-967079 (30 mg/kg) an antiallodynic effect was observed (%MPE was 20.7%, $p < 0.05$ 2 h after PACLI, and 23.2%, $p < 0.05$, 7 days later).

3.2.3. LPP1 and pregabalin attenuate tactile allodynia in PACLI-treated animals

The mean paw withdrawal force in animals not treated with PACLI was 3.01 ± 0.03 g. The treatment with PACLI caused a severe reduction of the mean paw withdrawal threshold (1.7 ± 0.01 g; $p < 0.001$ *vs.* before PACLI). In these neuropathic mice the test compound LPP1

Table 1
Antiallodynic activity of the test compounds in the cold water test in OXPT-treated mice measured 2 h after OXPT injection.

Compound	Dose [mg/kg]	Baseline latency time to pain reaction [s] ± SEM	Post-drug latency time to pain reaction [s] ± SEM	%MPE
LPP1	1.0	8.05 ± 1.6	10.36 ± 1.6	10.5
	10.0	6.94 ± 2.1	12.61 ± 3.0	24.60*
	30.0	3.49 ± 1.1	5.05 ± 1.5	6.21
Pregabalin	1.0	10.42 ± 1.6	11.60 ± 1.5	6.03
	10.0	5.65 ± 2.9	5.69 ± 2.7	0.16
	30.0	5.41 ± 1.5	5.73 ± 1.6	1.30
Capsazepine	30.0	5.01 ± 1.3	5.50 ± 1.4	1.96
A-967079	30.0	4.93 ± 0.6	8.15 ± 1.1	12.84*

Results are shown as mean latency time (±SEM) to pain reaction in response to cold stimulus (4 °C) and as %MPE; n = 8–10. Antiallodynic activity of the test compounds administered i.p. was compared to baseline values of latency time in each group. Statistical analysis: paired Student's t-test. Significance *vs.* respective baseline values.
* $p < 0.05$.

dose-dependently elevated the nociceptive threshold for mechanical stimulation by 12–63% (*vs.* baseline value; $p < 0.01$). Its ED_{50} was 17.4 mg/kg. At the same doses pregabalin was less efficacious than LPP1 and its antiallodynic efficacy ranged from 8% to 50% (Fig. 2b). In PACLI-treated mice capsazepine did not influence the pain threshold (3% *vs.* baseline value), while A-967079 inhibited tactile allodynia by 55% ($p < 0.001$; Fig. 2b).

3.2.4. PACLI-treated animals develop heat hyperalgesia which is attenuated by pregabalin

The injection of PACLI resulted in the development of heat hyperalgesia in mice. This was indicated by a statistically significant decrease in latency time to pain reaction in response to heat stimulus in PACLI-treated animals compared to their non-treated littermates (8.2 ± 0.9 s *vs.* 12.8 ± 0.8 s; $p < 0.01$). LPP1 was not able to elevate the heat sensitivity threshold in this assay. Pregabalin was effective only at 30 mg/kg. This dose prolonged the latency time to pain reaction by 80% ($p < 0.01$ *vs.* baseline latency time). In this assay capsazepine demonstrated a statistically significant activity as it attenuated heat hyperalgesia by 63% ($p < 0.01$). A-967079 was not effective.

3.3. At doses effective in neuropathic pain LPP1 and pregabalin do not cause motor deficits

In the rotarod test neither OXPT, nor PACLI caused motor deficits in mice. At analgesic-active doses both LPP1 and pregabalin did not demonstrate any motor impairing properties in neuropathic mice, either (Fig. 3).

3.4. High doses of LPP1 and pregabalin significantly reduce locomotor activity of neuropathic mice

In mice a single injection of OXPT or PACLI caused a reduction of pain threshold but did not induce a significant decrease of locomotor activity as compared to non-treated littermates. At doses that were tested for the antiallodynic and antihyperalgesic effects (1–30 mg/kg) neither LPP1, nor pregabalin caused a profound decrease of OXPT- or PACLI-treated animals' locomotor activity. In contrast to this, a statistically significant and complete abolition of locomotor activity was observed in OXPT- and PACLI-treated neuropathic mice treated with LPP1 at 150 mg/kg. Similar results were obtained when pregabalin at this dose was administered to neuropathic mice (Fig. 4).

3.5. Intraplantar injection of LPP1 attenuates capsaicin- and AITC-evoked neurogenic pain

LPP1 administered intraplantarly (60 μg/20 μl) was tested for its ability to attenuate nocifensive responses induced by capsaicin. In vehicle-treated mice the duration of capsaicin-induced pain behavior was 49.0 ± 5.9 s. LPP1 reduced this nociceptive reaction by 52.4% ($p < 0.001$ *vs.* control). This activity was similar to that of a TRPV1 antagonist, capsazepine (8 μg/20 μl), while a TRPA1 antagonist, A-967079 had no activity in this test (Fig. 5a).

The same concentration of LPP1 was tested for its antinociceptive activity in the neurogenic pain model induced by AITC. In control animals the duration of the licking response was 114.1 ± 14.2 s. The pretreatment with LPP1 reduced AITC-evoked nociceptive responses by 48.1% ($p < 0.05$ *vs.* control). This activity was similar to that of a selective TRPA1 antagonist A-967079 (47.9%, $p < 0.05$; used as 20 μg/20 μl), (Fig. 5b).

3.6. Pharmacokinetics of LPP1

As shown in Fig. 6 the concentrations of LPP1 measured 30 min following i.p. administration of this compound at the dose of 30 mg/kg were the highest in murine serum (7904.59 ± 1066.08 ng/ml) and muscles (7898.85 ± 933.23 ng/g). Brain concentrations were lower at this time point as reflected by the value of brain-to-serum ratio that equaled to 0.38. Relatively high concentrations of LPP1 were observed

Table 2
Antinociceptive activity of the test compounds in the hot plate test in OXPT-treated mice.

Compound	Dose [mg/kg]	Baseline latency time to pain reaction [s] ± SEM	Post-drug latency time to pain reaction [s] ± SEM	Activity (%)
LPP1	1.0	16.9 ± 1.3	22.1 ± 1.3**	30.8
	10.0	17.4 ± 1.7	20.2 ± 1.4	16.1
	30.0	13.4 ± 1.1	32.7 ± 3.5***	144.0
Pregabalin	1.0	21.2 ± 2.5	22.0 ± 1.9	3.8
	10.0	13.4 ± 1.2	14.7 ± 1.4	9.7
	30.0	12.3 ± 0.5	17.3 ± 1.9*	40.7
Capsazepine	30.0	13.3 ± 1.1	19.2 ± 0.9**	44.4
A-967079	30.0	13.3 ± 1.0	13.6 ± 0.7	2.3

Results are shown as mean latency time (±SEM) to pain reaction in response to heat stimulus (55 °C); n = 8–10. Antinociceptive activity of the test compounds compared to baseline values of latency time in each group. Statistical analysis: paired Student's t-test. Significance *vs.* respective baseline values.
* $p < 0.05$.
** $p < 0.01$.
*** $p < 0.001$.

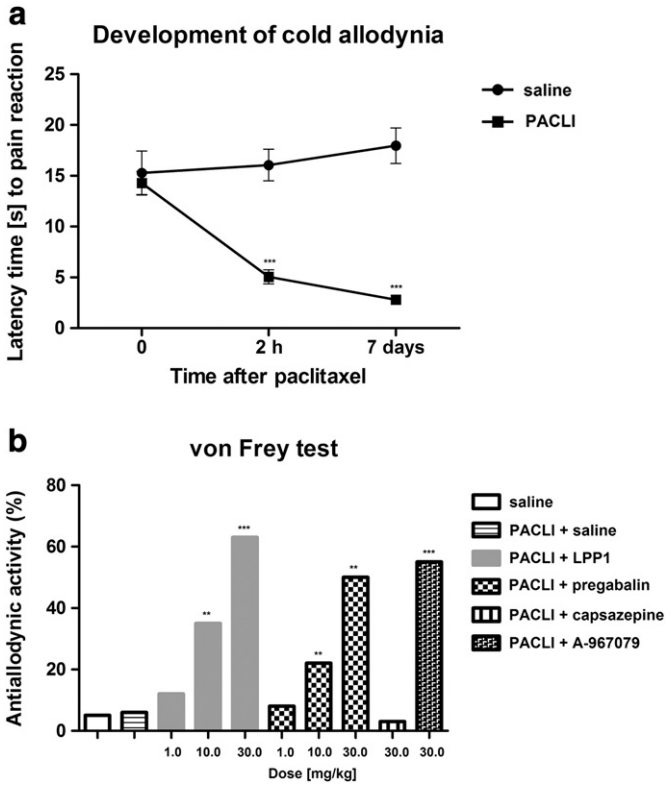

Fig. 2. (**a**) Time courses of the development of PACLI-induced cold allodynia investigated in the cold water test in mice. Statistical analysis: repeated measures analysis of variance (ANOVA), followed by Bonferroni *post hoc* comparison. Results compared to naïve mice: ***$p < 0.001$. Drug effect: $F[1,202] = 61.26$, $p < 0.0001$; time effect: $F[2,202] = 6.22$, $p < 0.01$; interaction: $F[2,202] = 13.32$, $p < 0.0001$. (**b**) Antiallodynic effects of LPP1, pregabalin, capsazepine and A-967079 in PACLI-treated mice evaluated in the von Frey test. Results are shown as % antiallodynic activity of the test compounds compared to baseline values in each group. Statistical analysis: paired Student's t-test. Significance *vs.* baseline values of paw withdrawal threshold: **$p < 0.01$; ***$p < 0.001$. Saline group — mice not treated with PACLI.

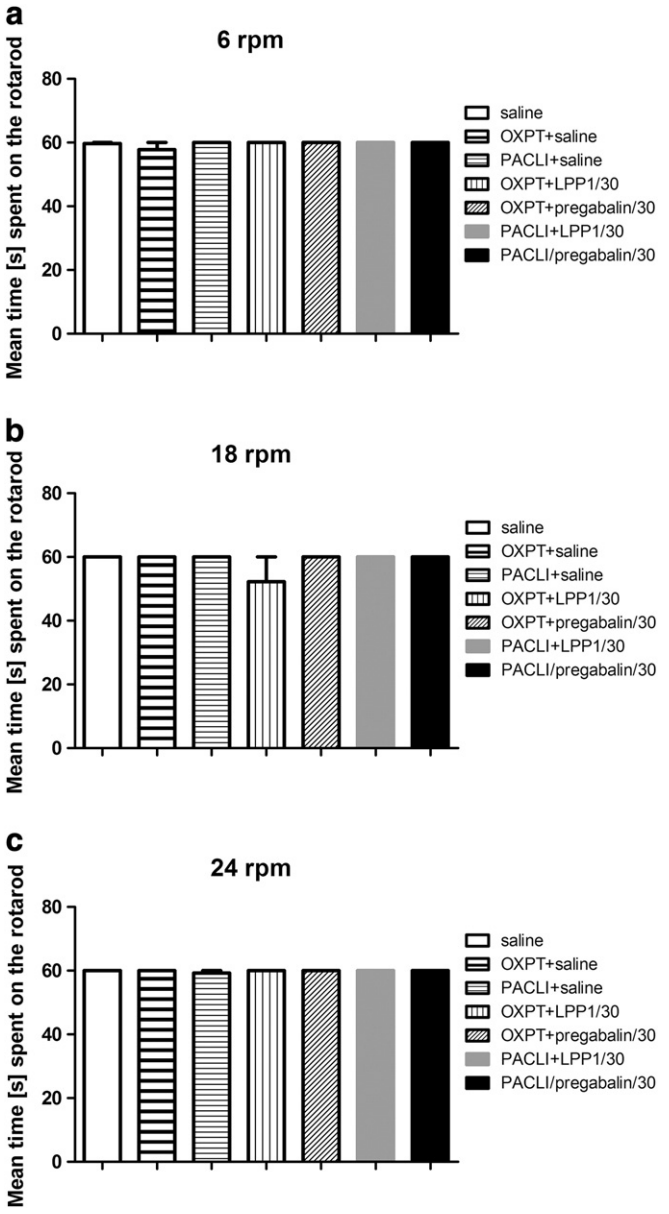

Fig. 3. Influence of LPP1 and pregabalin (each at 30 mg/kg) on motor coordination in OXPT-treated and PACLI-treated mice measured using rotarod revolving at 6 rpm **(a),** 18 rpm **(b)** or 24 rpm **(c)**. Statistical analysis: one-way ANOVA, followed by Dunnett's *post hoc* comparison. $F[6,35] = 0.9499$; $p > 0.05$ (6 rpm), $F[6,35] = 1.000$; $p > 0.05$ (18 rpm), $F[6,35] = 1.000$; $p > 0.05$ (24 rpm). Results compared to saline-treated group: $p > 0.05$.

in the CSF and the sciatic nerve (1339.21 ± 140.40 ng/ml and 954.94 ± 297.75 ng/g, respectively), indicating good penetration of this compound to the possible site of action.

4. Discussion

In the present study in mouse models of neuropathic pain induced by OXPT or PACLI antiallodynic and antihyperalgesic properties of a novel biologically active compound LPP1 have been investigated and compared to that of pregabalin. Previously we proved the effectiveness of LPP1 in rodent models of acute pain, local anesthesia (Sałat et al., 2009; Salat et al., 2012b) and diabetic neuropathic pain induced by streptozotocin (Sałat et al., 2013a). We also demonstrated that this compound could potentiate the antiallodynic effect of pregabalin in diabetic neuropathic mice (Salat and Salat, 2013). A strong cell membrane stabilizing activity (local anesthetic activity) and the antioxidant capacity have been demonstrated for LPP1 previously (Sałat et al., 2009, 2014; Salat et al., 2012b). Although these properties might contribute to its antinociceptive effect, they cannot fully explain its activity observed *in vivo*. Searching for other mechanisms underlying the analgesic activity of LPP1, and in order to determine the site of this compound's action its pharmacokinetics was studied, and its effect on neurogenic pain induced by capsaicin, a selective TRPV1 agonist and AITC, a selective TRPA1 agonist has been assessed.

In the recent years much attention has been paid to the role of TRP channels as peripheral sensors of nociception. TRPV1 and other members of this channel family, including TRPA1 and TRPM8, are abundantly situated within peripheral sensory nerve endings. TRPV1 is a detector of heat, acidity and is sensitive *inter alia* to capsaicin and other vanilloids (Hara et al., 2013; Sałat et al., 2013b). TRPA1 is implicated in the detection of cold, mechanical and some chemical stimuli, such as AITC or oxidative stress byproducts (Sałat et al., 2013b).

We demonstrated that the intraplantar injection of LPP1 attenuated nocifensive behavior evoked by capsaicin acting at TRPV1, and AITC acting at TRPA1. Similar effects were shown for capsazepine (a TRPV1 inhibitor) in the capsaicin test and A-967079 (a TRPA1 antagonist) in the AITC test. This fact indicates that the TRPV1 and TRPA1 channels might be at least in part implicated in the antinociceptive activity of LPP1. Previously demonstrated antioxidant capacity of LPP1 in some *in vitro* (Salat et al., 2012b) and *ex vivo* assays (Sałat et al., 2013a, 2014)

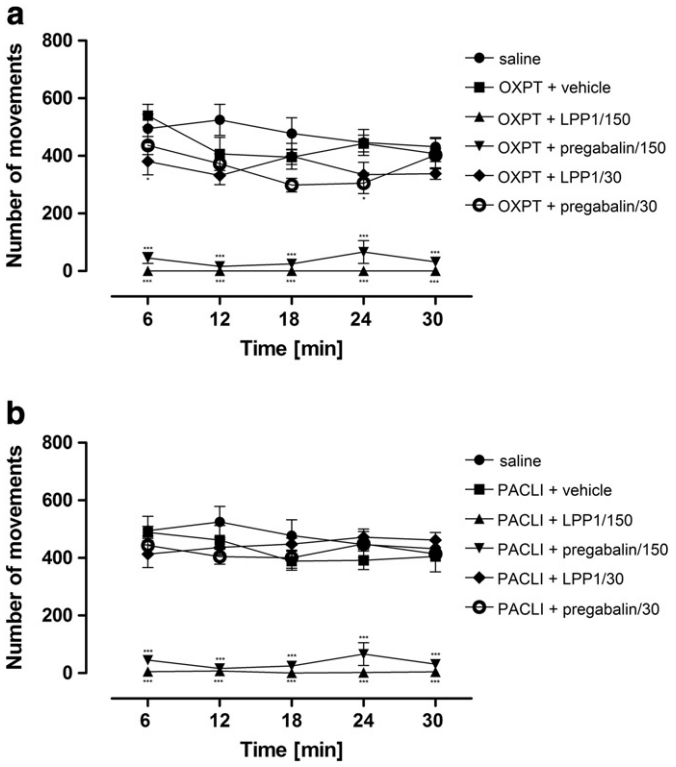

Fig. 4. Influence of LPP1 and pregabalin on locomotor activity in OXPT-treated mice **(a)** and in PACLI-treated mice (**b**). Values are shown as the mean number of light-beam crossings (±SEM). LPP1, pregabalin and the vehicle (0.9% saline) were administered i.p. 30 min before the assay. Statistical analysis of data was performed using repeated measures analysis of variance (ANOVA), followed by Bonferroni multiple comparison. Results compared to OXPT- or PACLI-treated control at the same time points: $^{*}p < 0.05$, $^{***}p < 0.001$. OXPT neuropathic pain: LPP1 — Drug effect: $F[3,80] = 47.48$, $p < 0.0001$; time effect: $F[4,80] = 3.93$, $p < 0.01$; interaction: $F[12,80] = 2.58$, $p < 0.01$. Pregabalin — Drug effect: $F[3,80] = 44.73$, $p < 0.0001$; time effect: $F[4,80] = 6.11$, $p < 0.001$; interaction: $F[12,80] = 2.90$, $p < 0.01$. PACLI neuropathic pain: LPP1 — Drug effect: $F[3,80] = 57.11$, $p < 0.0001$; time effect: $F[4,80] = 2.27$, $p > 0.05$; interaction: $F[12,80] = 2.70$, $p < 0.01$. Pregabalin — Drug effect: $F[3,80] = 49.56$, $p < 0.0001$; time effect: $F[4,80] = 3.01$, $p < 0.05$; interaction: $F[12,80] = 1.73$, $p > 0.05$.

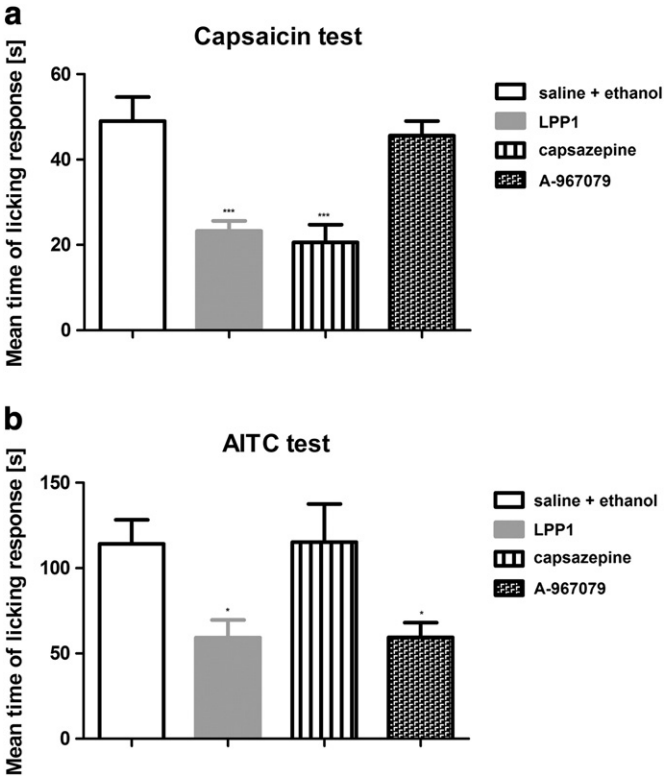

Fig. 5. Antinociceptive activity of LPP1 and the reference compounds: capsazepine and A-967079 administered intraplantarly in the capsaicin test **(a)** and AITC test (**b**). Results are shown as mean duration of the licking/biting response (±SEM) in vehicle-treated and drug-treated groups. Statistical analysis: one-way analysis of variance (ANOVA), followed by Dunnett's *post hoc* comparison. Capsaicin test: $F[3,26] = 12.39$, $p < 0.0001$; AITC test: $F[3,24] = 4.714$, $p < 0.05$. Results compared to control animals: $^{*}p < 0.05$, $^{***}p < 0.001$.

is in line with this finding. Reactive oxygen species (ROS) which gate TRPA1 are able to evoke nociceptive responses in neurogenic inflammation through TRPA1-mediated mechanisms (Sałat et al., 2013b). It seems therefore plausible that the observed antioxidant capacity of LPP1 might contribute to its antinociceptive effect in the AITC test. The lack of complete inhibition of the neurogenic response to AITC in our study could be due to several reasons, including a relatively high concentration of AITC used. Earlier Kwan et al. (2006) showed that neurogenic responses to high concentrations of AITC were reduced but not abolished in knock-out mice, which suggests that high levels of AITC can also influence other targets beyond TRPA1.

In the present study we observed a very prominent effect of LPP1 on tactile allodynia in two models of neuropathic pain induced by OXPT and PACLI. In contrast to this, the influence of LPP1 on cold allodynia and heat hyperalgesia were either transient or completely absent. OXPT and PACLI are two anticancer drugs with very distinct mechanisms of their therapeutic activity. OXPT induces the formation of DNA crosslinks 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 mechanical and cold allodynia (Nassini et al., 2011; Renn et al., 2011) but not heat hyperalgesia (Xiao et al., 2012). PACLI is a microtubule-targeting drug which is also able to produce a chronic, distal and bilaterally symmetrical peripheral neuropathy that is accompanied by neuropathic pain (Hara et al., 2013; Materazzi et al., 2012).

Numerous mechanisms underlying the influence of OXPT on cold and mechanical allodynia have been suggested. They include the alterations in intracellular signaling pathways and impaired regulation of ion channel activity. The role of TRPV1, TRPM8 (Gauchan et al., 2009), TRPA1, Acid-Sensing Ion Channel 3, P_2X_3 purinergic receptor stimulation (Nassini et al., 2011; Zhao et al., 2012) and generation of ROS (Nassini et al., 2011) are also taken into consideration.

Recently, it has been shown that mechanical allodynia and cold allodynia in rodents treated with OXPT are mediated by TRPA1 stimulation (Nassini et al., 2011; Zhao et al., 2012). Zhao et al. (2012) demonstrated that a single dose of OXPT induced acute cold hypersensitivity

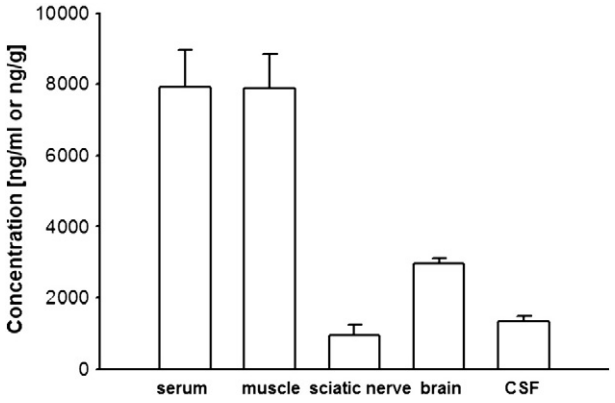

Fig. 6. Serum and tissue concentrations of LPP1 measured 30 min following i.p. administration of this compound at the dose of 30 mg/kg. The values presented are mean ± SD; n = 4.

associated with an enhanced responsiveness of TRPA1 but not TRPM8 or TRPV1. In the present research the involvement of TRPA1 in tactile and cold allodynia has been confirmed using A-967079 which attenuated both these phenomena. In line with other studies, the role of TRPV1 has not been elucidated.

Although the precise mechanism of TRPA1 contribution to OXPT-induced mechanical and cold allodynia is not clear, its role as a sensor for electrophilic, reactive compounds, such as ROS is well established (Nassini et al., 2011). Due to its antioxidant capacity LPP1 might indirectly inhibit TRPA1 resulting in the profound attenuation of OXPT-evoked early-phase hypersensitivity to cold. The mechanisms underlying this dose- and time-dependent response of LPP1-treated mice to cold stimulus are not clear and they require further studies. The lack of efficacy of LPP1 in the late phase of cold allodynia is difficult to explain but this phenomenon might be mediated by other than TRPA1 mechanisms which are not influenced by LPP1.

Beyond the role in the detection of cold stimuli, the TRPA1 channel is also an important contributor to mechanical allodynia (Nassini et al., 2011; Sałat et al., 2013b). Previously it was shown that a single dose of OXPT produced mechanical and cold allodynia in rats, and this effect was selectively abolished by a TRPA1 antagonist, HC-030031 (Nassini et al., 2011). The antiallodynic effect of A-967079 observed in OXPT-treated mice is consistent with the results obtained by other authors (Zhao et al., 2012) and is strong evidence for the participation of TRPA1 in this phenomenon. In OXPT-treated mice LPP1 and pregabalin caused a significant elevation of mechanical nociceptive threshold in the von Frey test. In the case of LPP1 the inhibition of TRPA1 and its aforementioned antioxidant capacity might in part explain the effect observed *in vivo*, while the effect of pregabalin is due to its calcium channel blocking activity (Saif et al., 2010).

It is generally assumed that in OXPT-treated mice heat hypersensitivity is not observed (Xiao et al., 2012). In line with this, in our research the animals treated with OXPT had prolonged baseline latencies to pain reaction in the hot plate test as compared to their non-treated littermates. This might suggest the development of heat hypoalgesia in neuropathic animals, probably due to OXPT-evoked nerve injuries within those nerve fibers which are responsible for the transmission of heat stimuli. In this context, it is difficult to interpret the apparent antinociceptive activity of LPP1 shown in the hot plate test, despite the fact that earlier we demonstrated a significant antinociceptive activity of LPP1 in the hot plate assay in naïve animals (Sałat et al., 2009).

The antiallodynic and antihyperalgesic properties of LPP1 were observed in PACLI-treated mice. A single dose of PACLI caused a significant reduction of the cold nociceptive threshold 2 h and 7 days after its administration. Neuropathic animals showed also signs of heat and mechanical hypersensitivity which is in line with results obtained for this pain model by other authors (Authier et al., 2009; Pascual et al., 2010; Xiao et al., 2012).

Numerous mechanisms underlying PACLI-induced neuropathy have been suggested. Recently Chen et al. (2011) and Materazzi et al. (2012) have shown a partial involvement of TRPA1 in PACLI-evoked mechanohypersensitivity (tactile allodynia) and cold hypersensitivity. PACLI-induced tactile allodynia was only partially attenuated by HC-030031, a TRPA1 antagonist, whereas a combination of HC-030031 with HC-067047 (a TRPV4 antagonist) completely abated this mechanical hypersensitivity (Materazzi et al., 2012). In our research the potential role of TRPA1 in cold and mechanical hypersensitivity in PACLI-treated mice was also confirmed. A-967079 but not capsazepine significantly elevated mechanical and cold nociceptive thresholds of neuropathic animals as compared to their baseline response values.

It is assumed that the treatment with PACLI is associated with ROS generation. Antioxidants inhibit PACLI-induced decreases in cell viability, and increases of reactive oxygen species in the cells and they are able to completely prevent PACLI-induced tactile allodynia (Flatters et al., 2006). An interesting issue has been shown in our study regarding the link between the effect of LPP1 on TRPA1 and the antioxidant capacity of this compound. It has been recently demonstrated that glutathione inhibits TRPA1 and this results in the attenuation of PACLI-evoked sensory neuropathy (Materazzi et al., 2012). We have recently shown that a single dose of LPP1 increases the level of glutathione in the neural tissue (Sałat et al., 2014). This effect might in part explain why LPP1 inhibits TRPA1 thus contributing to the restoration of mechanical nociceptive threshold in neuropathic animals.

LPP1 was not able to restore cold nociceptive threshold in PACLI-treated mice. This lack of the activity might be explained as follows: cold-evoked nociception in this model is mediated not only by TRPA1 (Pevida et al., 2013). Numerous other biologically active molecules on which LPP1 has no effect participate in it. Hence, LPP1 is not efficacious enough to restore cold sensitivity threshold in neuropathic mice. It is worth noting that pregabalin was not able to restore physiological cold sensitivity threshold in PACLI-treated mice, either. An earlier study by Peng et al. (2012) showed its efficacy in the attenuation of cold allodynia induced by docetaxel, a drug similar to PACLI, in rats but this activity of pregabalin was proven after a chronic treatment.

The increased TRPV1 expression in the paw skin of PACLI-treated rats and the sensitization of TRPV1 channels — main contributors to noxious heat-induced pain have been demonstrated by Hara et al. (2013). Other authors showed that the intraperitoneal administration of a TRPV1 antagonist in PACLI-treated animals attenuated thermal hyperalgesia without any effect on mechanical allodynia (Chen et al., 2011). In our study PACLI-treated mice developed heat hyperalgesia which was not influenced either by LPP1, or A-967079, although it was attenuated by pregabalin and capsazepine. This activity of capsazepine confirms the involvement of TRPV1 in heat hyperalgesia in PACLI-treated mice. The lack of LPP1 effect requires further studies. The effect of pregabalin on PACLI-induced heat hyperalgesia can be explained in relation to its calcium channel-blocking activity.

It is well known that both the onset and intensity of drug action depend on its concentration at the biophase. This, in turn, is related to the rate of drug absorption and distribution to target tissues. Following i.p. administration, LPP1 attained high concentrations both in serum and all tissues that may be considered to be the possible site of action of this compound. Due to the fact that LPP1 reaches relatively high concentrations both in muscles and brain tissue, from a pharmacokinetic point of view both peripheral and central mechanisms of action may contribute to its pharmacological activity observed in neuropathic pain models. It is worth noting that the penetration of LPP1 to the CNS might not only be responsible for the analgesic effect, but it may also induce sedation and decrease of animals' locomotor activity as observed at high doses of this compound.

Concluding, our present research shows that LPP1 and pregabalin very effectively attenuate tactile allodynia in OXPT and PACLI models of neuropathic pain with less significant impact on cold and heat nociceptive thresholds. At doses that attenuate neuropathic pain these compounds are devoid of motor impairing or sedative properties, so the effects observed in pain tests are not false positive results. High doses of LPP1 and pregabalin strongly affect the locomotor activity of neuropathic animals. The distinct effect of LPP1 on tactile allodynia and heat hyperalgesia could be due to the fact that different nerve fibers are involved in the transmission of both types of stimuli. Tactile allodynia is mediated through large-diameter afferent A-β fibers, whereas heat hyperalgesia is mediated through small-diameter, unmyelinated C-fibers (Pascual et al., 2010). Hence, LPP1 seems to influence A-β fibers, and this is in line with our earlier studies regarding this compound (Salat et al., 2012b). To some extent the antiallodynic activity of LPP1 can be attributed to its inhibitory effect on TRP channels involved in the detection of mechanical, cold and some chemical nociceptive stimuli but the pharmacokinetic analysis confirms the possibility of both peripheral and central mechanisms of action of this compound.

Acknowledgments

This study was financially supported by the Jagiellonian University grant K/ZDS/003329. The authors wish to thank M.Sc. Magdalena Działo, M.Sc. Katarzyna Zwolak and M.Sc. Alexandra Cachia for their technical assistance in the experiments.

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