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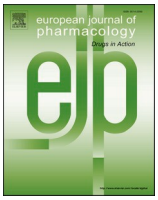

# Effects of sub-chronic, *in vivo* administration of sigma non-opioid intracellular receptor 1 ligands on platelet and aortic arachidonate cascade in rats

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ABSTRACT

Platelets regulate cell-cell interactions and local circulation through eicosanoids from arachidonic acid. Sigma non-opioid intracellular receptor 1 (sigma-1 receptor) expressed in platelets and endothelial cells can regulate intracellular signalization. Our aim was to examine the influence of sub-chronic, *in vivo*-administered sigma-1 receptor ligands 2-morpholin-4-ylethyl 1-phenylcyclohexane-1-carboxylate (PRE-084); *N*-benzyl-2-[(1S)-6,7-dimethoxy-1,2,3,4-tetrahydroisoquinolin-1-yl]ethan-1-amine; dihydrochloride, a new compound (*(S)*-L1); and *N*-[2-[4-methoxy-3-(2-phenylethoxy)phenyl]ethyl]-*N*-propylpropan-1-amine (NE-100) on the *ex vivo* arachidonic acid metabolism of the platelets and aorta of male rats. The serum level of sigma-1 receptor ligands was determined by liquid chromatography-mass spectrometry. Sigma-1 receptor and cyclooxygenase gene expression in the platelets were determined by a reverse transcription-coupled quantitative polymerase chain reaction. The eicosanoid synthesis was examined using a radiolabeled arachidonic acid substrate and enzyme-linked immunosorbent assay. We confirmed the absorption of sigma-1 receptor ligands and confirmed that the ligands were not present during the *ex vivo* studies, so their acute effect could be excluded. We detected no changes in either sigma-1 receptor or cyclooxygenase mRNA levels in the platelets. Nevertheless, *(S)*-L1 and NE-100 increased the quantity of cyclooxygenases there. Both platelet and aortic eicosanoid synthesis was modified by the ligands, although in different ways. The effect of the new sigma-1 receptor ligand, *(S)*-L1, was similar to that of PRE-084 in most of the parameters studied but was found to be more potent. Our results suggest that sigma-1 receptor ligands may act at multiple points in arachidonic acid metabolism and play an important role in the control of the microcirculation by modulating the eicosanoid synthesis of the platelets and vessels.

## 1. Introduction

Platelets are small, non-nucleated cells with a short lifespan (7–9 days) and specific structure (dense tubular system, different granules, lysosomes, mitochondria, and numerous adhesion molecules). This complex structure allows their involvement in hemostasis, cell-cell interaction (monocytes, endothelial cells, and lymphocytes), inflammatory processes, natural and acquired immunity, and regulation of the microcirculation (Gremmel et al., 2016; Koupenova et al., 2018; Mancuso and Santagostino, 2017; van der Meijden and Heemskerk, 2019).

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Bioactive lipid mediators, i.e. eicosanoids (prostaglandins, thromboxane, and lipoxygenase products), synthesized by platelets participate in these processes (Yeung et al., 2018). Eicosanoids, which regulate cellular and vascular function, are generated from free arachidonic acid released from phospholipids in the cell membrane by phospholipase $A_2$ in the presence of ionized calcium by cyclooxygenases (prostaglandin G/H synthases, EC1.14.99.1; COXs), lipoxygenases (EC1.13.11; LOXs), and specific synthases (Paes et al., 2019). Sigma non-opioid intracellular receptor 1 (the sigma-1 receptor), which modulates several cellular functions (Su et al., 2016; Aishwarya et al., 2021; Wang et al., 2021), is expressed on endothelial cells (Amer et al., 2013) and platelets (Váczi et al., 2021). The beneficial effects of sigma-1 receptor agonists have been described in different pathological conditions, such as sepsis (Rosen et al., 2019), ischemic-reperfusion injury (Gao et al., 2018), stroke (Nardai et al., 2020), and cardiovascular diseases (Tagashira et al., 2013). The expression of the sigma-1 receptor gene (*Sigmar1*) has been shown to reduce inflammatory cytokine production (Rosen et al., 2019) and to play a protective role against diabetic complications (Ha et al., 2012).

We aimed to investigate the effects of the sigma-1 receptor ligands on platelet and aortic eicosanoid synthesis in the rats. We hypothesized that sigma-1 receptor ligands administered sub-chronically *in vivo* (daily for one week) can modulate the *ex vivo* arachidonic acid metabolism of the platelets and aorta when the ligand is not currently present. For our studies, we chose two well-characterized ligands and one new sigma-1 receptor ligand. PRE-084 was initially identified as a selective agonist of the sigma-1 receptor (Su et al., 1991), which regulates cytokine production in stroke (Allahtavakoli and Jarrott, 2011), reduces microglial activation in traumatic brain injury, and has protective effects in neurogenic inflammation (Dong et al., 2016) and in the maintenance of the endothelial barrier (Motawe et al., 2020). NE-100 is an antagonist of the sigma-1 receptor (Okuyama and Nakazato, 1996), which has been shown to block sigma-1 receptor agonist (SA4503)–induced thoracic aortic vasodilation (Tagashira et al., 2013). The third ligand, *(S)*-L1, is a recently synthesized new compound, which binds with high affinity to the sigma-1 receptor (Dvorácskó et al., 2021). The serum level of the sigma-1 receptor ligands was determined by liquid chromatography-mass spectrometry (LC-MS) before the *ex vivo* analysis. The eicosanoid synthesis was examined using radiolabeled substrate and enzyme-linked immunosorbent assay. The effect of sigma-1 receptor ligands not only depends on the quantity and activity of the enzymes but can also be influenced by the levels of mRNA of genes coding the sigma-1 receptor and COX (*Ptgs*), which originate from megakaryocytes and are stored in the platelets. To confirm this process, the gene expression of the sigma-1 receptor and COXs in the platelets was also determined.

## 2. Materials and methods

### 2.1. Docking protocol

The PRE-084 and *(S)*-L1 were docked to the target, which is chain C from the X-ray structure of the sigma-1 receptor (PDB ID: 6DK1), using the Glide program from the Schrödinger package (Schrödinger, 2019). Prior to docking, the necessary fields were calculated on a uniform, 1 Å-spaced grid inside a cube with ~32 Å-long edges. The grid centers were located at the center of the ligands from the X-ray structures used. Compounds were prepared for docking with Ligprep (Schrödinger, 2019) and used in an extra precision (XP) docking protocol (Schrödinger, 2019) using the OPLS3e force-field (Roos et al., 2019) for parametrization.

### 2.2. Animals

The animal experiments were performed under the protocol approved by the Ethics Committee for the Protection of Animals in Research at the University of Szeged, Hungary (Permit No. X./238/2019.). All experiments were carried out in accordance with the Guide for the Care and Use of Laboratory Animals published by the U.S. National Institutes of Health. 15-week-old male Wistar (*Rattus norvegicus*) rats were used in this study. All the animals were maintained in a room, in 12-h dark/12-h light cycles, at constant temperature (23 ± 1 °C), with free access to standard laboratory food (SAFE® 132, SAFE, Augy, France) and water *ad libitum*. As required by guidelines and the ethics permit, we placed enrichment devices (e.g. cylinders and cubes) in the rats' cages.

Sample size calculation was performed for the cyclooxygenase metabolism. The estimation was based on the one-way analysis of variance (ANOVA) hypothesis test. We supposed the effect size to be at least 0.6, statistical power $1 - \beta = 0.8$ and significance level $\alpha = 0.05$. The calculation resulted in a sample size of nine per group, with 36 animals in total. Sample size calculation was performed with G*Power version 3.9.1.7 software.

The rats (n = 36) were randomly divided into four groups to examine the effects of the sigma-1 receptor ligands on the eicosanoid synthesis. Therefore, the subgroups generated were as follows: (1) the control/vehicle-treated group; (2) animals treated with PRE-084 (2-morpholin-4-ylethyl 1-phenylcyclohexane-1-carboxylate; hydrochloride; MedChem Express USA); (3) those treated with *(S)*-L1 (*N*-benzyl-2-[(1*S*)-6,7-dimethoxy-1,2,3,4-tetrahydroisoquinolin-1-yl]ethan-1-amine dihydrochloride), a novel high affinity sigma-1 receptor ligand screened in silico from the in-house compound library of the Institute of Pharmaceutical Chemistry, University of Szeged (Dvorácskó et al., 2021)); and (4) those treated with NE-100 (*N*-[2-[4-methoxy-3-(2-phenylethoxy)phenyl]ethyl]-*N*-propylpropan-1-amine hydrochloride; Tocris Bioscience, Bristol, UK), each comprising nine rats.

We studied the basal and terminal (immediately before *ex vivo/in vitro*) fasting blood glucose levels and body weight of all the groups. Serum glucose was determined from blood obtained from rat tails using a D-count blood glucose meter and an Ideal test strip. Before the *ex vivo/in vitro* eicosanoid synthesis studies (terminal phase), platelet counts and serum levels of the sigma-1 receptor ligands were determined in all the rats from blood taken from the abdominal aorta. The absolute platelet count ($10^{10}$) required for the determination of *Sigmar1* and *Ptgs* mRNA could only be obtained by dividing the total number of platelets from each group of animals into three parts. That is, the platelets used for the mRNA assay also came from nine rats but from pooled samples.

### 2.3. In vivo treatment of the rats with sigma non-opioid intracellular receptor 1 ligands

#### 2.3.1. Treatment protocols

The animals were treated with the sigma-1 receptor ligands. All sigma-1 receptor ligand-treated animals received 3 mg/kg body weight of sigma-1 receptor ligand (PRE-084 or *(S)*-L1 or NE-100) intraperitoneally, dissolved in 0.9% sodium chloride. The vehicle-treated animals received a 0.9% sodium chloride solution intraperitoneally. Treatment in all cases was carried out once a day for a week. The dose of sigma-1 receptor ligands was determined based on a previous publication describing the effects of a sigma-1 receptor ligand administered intraperitoneally in rats (Nardai et al., 2020).

#### 2.3.2. Determination of serum sigma non-opioid intracellular receptor 1 ligands level

*2.3.2.1. Reagents and chemicals.* All reagents and chemicals were of analytical or LC–MS grade. Acetonitrile (ACN), methanol (MeOH), and water were obtained from VWR Chemicals (Monroeville, PA, USA). Formic acid (FA) and hydrochloric acid were purchased from Fisher Scientific (Portsmouth, NH, USA). Stock solutions were dissolved in water individually at a final concentration of 1 mg/mL. All standard

stock solutions were prepared on ice, divided into 100 μL aliquots, and stored at −80 °C until further use.

*2.3.2.2. Preparation of plasma samples for the LC-MS.* According to the results of our preliminary recovery studies (Supplementary Table S1), 10 and 330 μL ACN (in the assay of (*S*)-L1 and NE-100, respectively) or 330 μL MeOH (in the assay of PRE-084) were added to a 100 μL rat plasma sample. The organic solvents contained 10 μL internal standard (*N*-benzyl-2-[6,7-dimethoxy-1,2,3,4-tetrahydroisoquinolin-1-yl] propan-1-amine) solution with a concentration of 120 nM (in the assay of (*S*)-L1) or 48 nM (in the assay of PRE-084 and NE-100). The mixture was spun for 60 s and allowed to rest for 30 min at −20 °C to support protein precipitation. The supernatant was obtained via centrifugation of the mixture for 15 min at 15,000×*g* at 4 °C. The supernatant was transferred to a new tube. After concentration under vacuum (Savant SC 110 A Speed Vac Plus, Savant, USA), the samples were reconstituted in 100 μL starting eluent, vortexed, and centrifuged. Finally, 2 μL was injected into the LC–MS/MS system for analysis.

*2.3.2.3. The calibration curves for (S)-L1, PRE-084, and NE-100.* The rat plasma calibration standards of *(S)*-L1 (7.81–250 nM), PRE-084, and NE-100 (3.91–125 nM) were prepared by adding the working standard solutions (instead of 10 μL 0.01 M HCL) into a pool of drug-free rat plasma (Supplementary Table S2). The sample preparation procedure described above was followed.

*2.3.2.4. Analysis of the plasma samples by LC-MS/MS.* The quantitative analysis of *(S)*-L1, PRE-084, and NE-100 was conducted after chromatographic separation using tandem mass spectrometry. An ACQUITY I-Class UPLC™ liquid chromatography system (Waters, Manchester, UK) comprising Binary Solvent Manager, Sample Manager-FL, and Column Manager connected to a Q Exactive™ Plus Hybrid Quadrupole-Orbitrap Mass Spectrometer (Thermo Fisher Scientific, San Jose, CA, USA) equipped with a heated electrospray ion source (HESI-II) was used for the analysis. Gradient chromatographic separation was performed at room temperature on a Kinetex EVO C18 column (Phenomenex; 100 Å, 50 mm × 2.1 mm, particle size 2.6 μm) protected by a C18 guard column (Phenomenex, Torrance, CA, USA) using 0.1% (v/v) aqueous FA as solvent A and ACN containing 0.1% (v/v) FA as solvent B (Supplementary Table S3).

The calibration curve was shown to be linear over the concentration range discussed above. The parallel reaction monitoring (PRM) data acquisition mode was selected with a quantitative mass spectrometric analysis of *(S)*-L1, PRE-084, and NE-100 using MS/MS. The optimal fragmentation conditions and collision energies of each analyte were identified to achieve the best precursor/product transition for quantitation and maximize sensitivity. The mass spectrometer was used in positive mode with the following parameters of the HESI-II source: spray voltage at 3.5 kV, capillary temperature at 253 °C, aux gas heater temperature at 406 °C, sheath gas flow rate at 46 L/h, aux gas flow rate at 11 L/h, sweep gas flow rate at 2 L/h, and S-lens radio frequency level at 50.0 (source auto-defaults). PRM mode was used for quantification by monitoring the transitions of the quantifier and qualifier ions (Supplementary Table S4).

A divert valve placed after the analytical column was programmed to switch flow onto the mass spectrometer only when analytes of interest were eluted from the column (1.5–3.0 min) to prevent excessive contamination of the ion source and ion optics. The washing procedures of the auto sampler before and after injecting samples were programmed to avoid carryover of the analytes. The UHPLC system was checked using MassLynx 4.1 SCN 901 (Waters). The mass spectrometer, data acquisition, and data processing were checked with Xcalibur™ 4.1 (Thermo Fisher Scientific).

### 2.4. Separation of the platelets

Blood was drawn from the rats 20 h after the last injection of the sigma-1 receptor ligands had been administered. Under anesthesia (Euthasol®/pentobarbital-Na/30 mg/kg body weight i.p.), blood was drawn from the abdominal aorta of the rats with a thick needle and diluted (1:2) with phosphate buffer (pH 7.4) containing ethylenediaminetetraacetic acid (EDTA, 5.8 mM) and glucose (5.55 mM). The platelets were separated by differential centrifugation (Mezei et al., 1997, 2000). The platelet-rich plasma was collected after the whole blood had been centrifuged at 200 g for 10 min at room temperature. The platelets were sedimented from the supernatant by centrifugation at 2000*g* for 10 min. The pellet was contaminated with red blood cells; therefore, the erythrocytes were lysed with hypoosmotic ammonium chloride (0.83%, 9 parts) containing EDTA (0.02%, 1 part) over 15 min. The platelets were then washed twice with phosphate buffer pH 7.4 containing 5.8 mM EDTA and 5.55 mM glucose, and centrifuged at 2000 g for 10 min at room temperature to remove the ammonium chloride and the erythrocyte residues/debris. The absolute platelet count was determined before the second centrifugation. After the last centrifugation, the platelets were re-suspended ($2.5 \times 10^8$ platelets/mL) in serum-free Medium 199 tissue culture (Sigma, St. Louis, MO, USA).

### 2.5. Isolation of the aorta

Under anesthesia (Euthasol®/pentobarbital-Na/30 mg/kg body weight i.p.), the abdominal aorta of the rat was isolated from the branch of the iliac artery to the diaphragm after blood collection. At 4 °C temperature, the connective tissue was removed from the aorta, which was sliced into 1–2 mm thick rings with care so as not to damage the endothelium (Mezei et al., 2015).

### 2.6. Examination of arachidonic acid metabolism

#### 2.6.1. Analysis of eicosanoid synthesis with isotope-labeled arachidonic acid

An *ex vivo* examination of the eicosanoid synthesis of platelets was carried out in Medium 199, which consists of mineral salts, nucleotides, amino acids, vitamins, carbohydrates, and $Ca^{2+}$ in the same quantity as the extracellular concentration but without fibrinogen or other plasma proteins.

The *in vivo* vehicle-treated and sigma-1 receptor ligand-treated (PRE-084-treated, *(S)*-L1-treated, or NE-100-treated) platelets ($2.5 \times 10^8$ platelets/mL in each sample) were pre-incubated at 37 °C for 3 min, while the aortic rings (15 mg wet mass/mL) were pre-incubated for 10 min in Medium 199 (Merck). The enzyme reaction examined started after pipetting the tracer substrate 1-$^{14}$C-arachidonic acid (3.7 kBq, 0.172 nM in each sample; American Radiolabeled Chemicals, Inc., St. Louis, MO, USA) into the incubation mixture. The enzyme reaction was stopped by bringing the pH to 3 with formic acid after 13 min of incubation in the case of the platelet samples and after 30 min of incubation in the case of the aortic samples.

The samples were extracted with ethyl acetate, and the organic phases were evaporated. The residues were reconstituted in ethyl acetate and quantitatively applied to silica gel G thin-layer plates (Kieselgel G 60/DC-Fertigplatten, Merck, Art. 5721).

The plates were developed to a distance of 16 cm in the organic phase of ethyl acetate:acetic acid:2,2,4-trimethylpentane:water (110:20:30:100) using overpressure thin-layer chromatography (Chrompress 25, Labor MIM, Hungary) (Abdel-Halim et al., 1980; Kis et al., 1999; Cryer, 2009; Mezei et al., 2015).

A semi-quantitative analysis of labeled arachidonic acid metabolites was performed with a BIOSCAN AR-2000 Imaging Scanner (Eckert & Ziegler Radiopharma, Berlin, Germany) using Win-Scan 2D Imaging Software. The radio-labeled products of arachidonic acid were identified with unlabeled authentic standards (Mezei et al., 1997). Assuming that

the exogenously administered labeled arachidonic acid used as a tracer is converted in the same way as the endogenous source, our method allows measurement of the relative quantity of various prostanoids (Mezei et al., 2015).

#### 2.6.2. Analysis of eicosanoid synthesis by enzyme-linked immunosorbent assay (ELISA)

The course of this procedure is the same as that of the examination of radioactive arachidonic acid metabolism (see above), but, in this case, the radioactive-labeled substrate is not added to the incubation mixture. After incubation, the incubation mixture, which contained either platelets or aortic rings, was placed at −80 °C and stored at this temperature until the assay was performed. Before performing the ELISA test, the samples were lyophilized and the lyophilizates were resuspended in the 310 μL 0.9% NaCl solution as the original incubation medium. The prepared samples were centrifuged at 3000 rpm for 10 min to remove cell debris. Subsequently, the quantity of cyclooxygenase 1 (COX-1; sensitivity: 0.285 ng/mL; assay range: 0.3–60 ng/mL) and cyclooxygenase 2 (COX-2; sensitivity: 0.446 ng/mL; assay range: 0.5–150 ng/mL) was determined in the rat platelets and aorta using ELISA kits (Shanghai Sunred Biological Technology Co. Ltd, PRC) in compliance with the protocols attached. The optical absorbance of the samples was measured at 450 nm using a STAT FAX 2100 ELISA plate reader (Awareness Technology, Inc., Palm City, FL, USA). Based on the calibration curves, the respective concentrations of the metabolites and enzymes under examination were determined with the SPSS 22.0 software.

#### 2.6.3. Determination of sigma non-opioid intracellular receptor 1 and COX-1 and -2 gene expression with a reverse transcription-coupled quantitative polymerase chain reaction (RT-qPCR)

Rat platelet samples were homogenized in TRI Reagent (Sigma-Aldrich, USA). Then RNA from each sample was transcribed to complementary DNA using a High Capacity cDNA Reverse Transcription Kit (Applied Biosystems, USA) according to the manufacturer's protocol based on random priming. The RT-qPCR was performed with a TaqMan Gene Expression Master Mix (Life Technologies, USA) in a Bio-Rad C1000 Touch Thermal Cycler (Bio-Rad Laboratories, USA). Inventoried TaqMan Gene Expression Assays (Life Technologies, USA) were the following: *Sigmar1* - Rn00578590_m1; *Ptgs1* - Rn00566881_m1; *Ptgs2* - Rn01483828_m1; glyceraldehyde 3-phosphate dehydrogenase (*Gapdh*) - Rn01749022_g1. After heat activation at 95 °C for 3 min, the cycling conditions were the following: denaturation for 30 s at 95 °C and amplification for 30 s at 60 °C (40 cycles). qPCR data were analyzed with Bio-Rad CFX Maestro software (Bio-Rad Laboratories, USA). In all the samples, the transcript level of a gene was normalized to an endogenous control gene (*Gapdh*, $\Delta Ct = Ct_{gene} - Ct_{Gapdh}$). Then ΔΔCt was calculated in comparison with the relative expression of the target genes in the vehicle-treated control groups. Fold changes were calculated using the $2^{-\Delta\Delta Ct}$ formula.

### 2.7. Statistical analysis

The physical and laboratory parameters of the animals, the eicosanoid synthesis results of ELISA, and the labeled arachidonic acid substrate were assessed by ANOVA followed by Bonferroni's post hoc test. The results of RT-qPCR tests were assessed by ANOVA followed by Dunnett's post hoc test. A difference at a level of $p < 0.05$ was considered statistically significant. The results are expressed as means ± SD. The statistical analysis was performed by SPSS version 22.0 (IBM Corp. Released 2013. IBM SPSS Statistics for Windows, Version 22.0. Armonk, NY: IBM Corp.)

## 3. Results

### 3.1. Molecular modeling of the docking of the selected ligands to sigma non-opioid intracellular receptor 1

In a recent publication, we described a verified virtual screening protocol to identify novel sigma-1 receptor ligands. The sigma-1 and sigma-2 receptor–binding affinities of the highest-ranked compounds were measured in competitive radioligand binding. As a result of this study, we identified several new sigma-1 receptor ligands, among them compound *(S)*-L1 (*N*-benzyl-2-[(1*S*)-6,7-dimethoxy-1,2,3,4-tetrahydroisoquinolin-1-yl]ethan-1-amine; dihydrochloride, see Fig. 1A), which possesses high binding affinity ($K_i = 11 \pm 3$ nM) to sigma-1 receptor and moderate sigma-1/sigma-2 receptor selectivity (Dvorácskó et al., 2021).

The chemical structure of PRE-084, NE-100, and *(S)*-L1 is presented in Fig. 1A. Every ligand fits the early pharmacophore model developed by Glennon (Glennon et al., 1994). It includes the following structural elements: a basic amine site that is necessary for the formation of an electrostatic bond with Glu172 of the receptor. It is typically flanked by two different hydrophobic groups. The hydrophobic parts are mostly aromatic and/or rigid heterocyclic sites. The binding modes of these ligands are compared in Fig. 1B. The binding pose of NE-100 was extracted from the X-ray structure of the ligand-bound sigma-1 receptor (PDB ID: 6DK0). The binding pose of the other two ligands was obtained with Glide XP docking (Schrödinger, 2019) to the *apo* receptor based on chain C of PDB structure 6DK1.

According to Schmidt et al. (2018), the main difference between agonist and antagonist binding is the presence of the interaction of the ligand with the C-terminal helix shown in the foreground of Fig. 1B. The cyclohexane ring of PRE-084 is the only moiety that can interact with this helix (Schmidt et al., 2018). The *(S)*-L1 and the sigma-1 receptor antagonist NE-100 have very similar binding poses (link to the animation sequence should be inserted here).

### 3.2. Physical and laboratory parameters of the animals

The significant increase in terminal body weight of the animals compared to basal levels correlates with the physiological, time-proportional increase in body weight. No significant difference in terminal body weights was observed between the sigma-1 receptor ligand-treated and the vehicle-treated rat groups. Age- and gender-matched, slightly elevated blood glucose levels in the animal groups are within the reference values published by Charles River Laboratories (Charles River, 2008) (Table 1).

At the end of the *in vivo* experiment (terminal phase), we detected no statistically significant difference in the platelet counts between the groups of animals (Table 1).

### 3.3. Concentration of the sigma non-opioid intracellular receptor 1 ligands in rat plasma

#### 3.3.1. In the preliminary experiments

Prior to the *in vivo* administration of the sigma-1 receptor ligands, we monitored the kinetic changes in the plasma levels of PRE-084, *(S)*-L1, and NE-100 administered intraperitoneally as a single dose in the male rats (Supplementary Figs. S1–S3). A significant elevation in plasma levels was detected 1 h after the administration of PRE-084 and 30 min after the administration of *(S)*-L1 and NE-100. The ligand concentration rapidly decreased, and the quantity of sigma-1 receptor ligands was insignificant or undetectable 20 h after the injection.

#### 3.3.2. In the terminal phase of the in vivo experiment

Plasma concentrations of the sigma-1 receptor ligands were determined in all of the rats 20 h after the last ligand injection immediately before the *ex vivo* studies. The plasma concentrations of the sigma-1

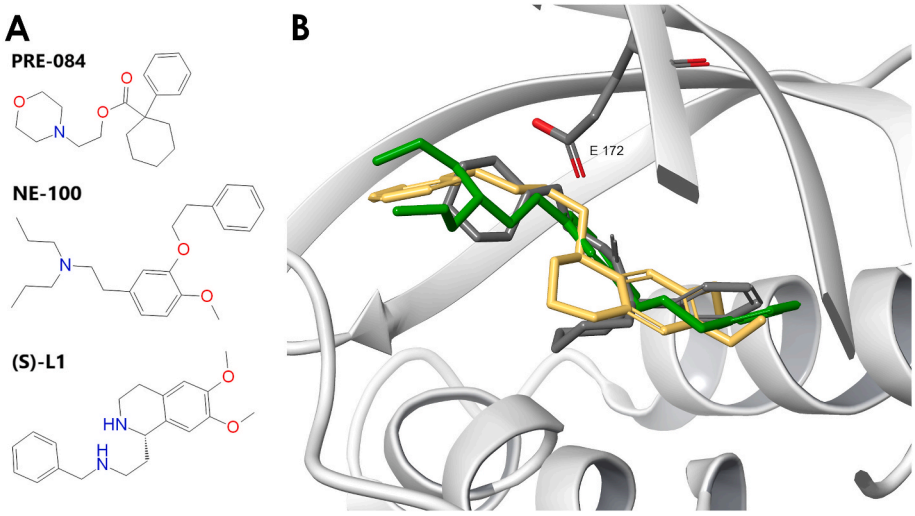


**Fig. 1.** (**A**) Chemical structure of the sigma non-opioid intracellular receptor 1 ligands PRE-084, NE-100, and *(S)*-L1. (**B**) Binding pose of the agonist PRE-084 (gray), antagonist NE-100 (green), and *(S)*-L1 (yellow). The binding pose of NE-100 was extracted from the X-ray structure of the ligand-bound sigma non-opioid intracellular receptor 1 (PDB ID: 6DK0). The binding pose of the other two ligands was obtained with Glide XP docking to the *apo* receptor extracted from chain C of PDB structure 6DK1. To superimpose the binding pockets of 6DK0 and 6DK1, we used the backbone atoms of residues 170–176 and 197–217. (link to the animation sequence should be inserted here).

**Table 1**
Body weight, fasting serum glucose levels, and platelet number of the animals.

| Rats | Body weight (g) | | Serum glucose (mM) | | Platelet ($10^9$/L) |
|---|---|---|---|---|---|
| | *Basal* | *Terminal* | *Basal* | *Terminal* | *Terminal* |
| **Vehicle** | 364 ± 22 | 471 ± 31*** | 6.5 ± 0.2 | 7.3 ± 0.9 | 587 ± 86 |
| **PRE-084** | 337 ± 27 | 445 ± 11*** | 6.4 ± 0.2 | 7.9 ± 0.4 | 685 ± 38 |
| ***(S)*-L1** | 375 ± 21 | 460 ± 14*** | 6.7 ± 0.2 | 8.2 ± 0.4 | 589 ± 71 |
| **NE-100** | 337 ± *20* | 466 ± 19*** | 6.8 ± 0.3 | 6.3 ± 0.3 | 713 ± 137 |

Data is shown as mean ± SD; n = 9–9 rats/group. We determined the basal body weights and fasting (12 h) blood glucose levels of the animals injected with the vehicle or sigma-1 receptor ligands. ANOVA, Bonferroni test, ***$p < 0.001$ terminal body weights were compared to their own basal body weights.

receptor ligands were below the limit of detection or quantification 20 h after the last dose (Table 2).

### 3.4. Effects of the sigma non-opioid intracellular receptor 1 ligands on the *Sigmar1* mRNA level in the rat platelets

The mRNA levels of *Sigmar1* were detected in the rat platelet samples using RT-qPCR. The *Sigmar1* mRNA showed a low concentration level in the rat platelets. Although the Ct values were high in most of the cases, we considered them reliable because the RT-qPCR was performed with TaqMan assays. The daily administration of the sigma-1 receptor ligands for one week did not change the level of *Sigmar1* mRNA in the platelets (Fig. 2).

**Table 2**
Concentration of the sigma non-opioid intracellular receptor 1 ligands in rat plasma. Samples were taken 20 h after the last dose (3 mg/bwkg/day for seven days).

| Analyte | Concentration (M) |
|---|---|
| **PRE-084** | < LOQ |
| ***(S)*-L1** | 5.64 ± 0.32 |
| **NE-100** | < LOD |

Data is shown as mean ± SD; n = 9–9 rats/group; LOQ: limit of quantification; LOD: limit of detection.

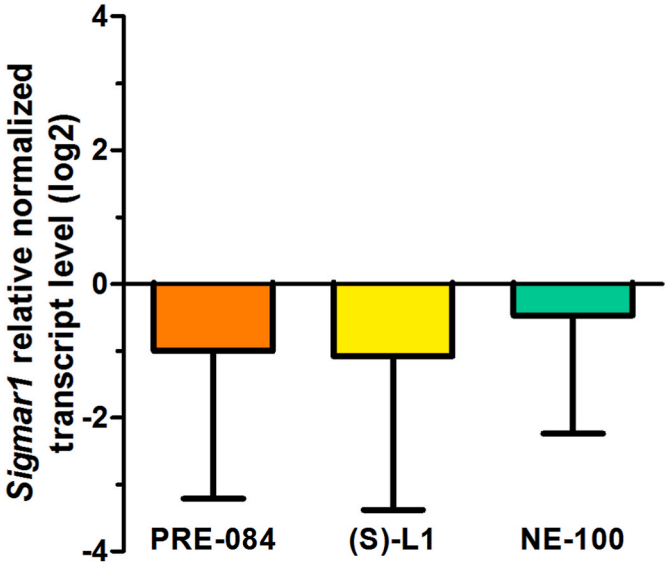


**Fig. 2.** Relative normalized transcript levels of *Sigmar1* in the platelet samples from the rats treated with the sigma non-opioid intracellular receptor 1 ligands. The rats were treated with 3 mg/bwkg/day PRE-084, *(S)*-L1, or NE-100 for seven days. The vehicle-treated group received saline solution. The transcript levels of *Sigmar1* in the rat platelet samples were first normalized to their own endogenous control mRNA levels (*Gapdh*) and then to the similarly normalized mRNA levels in the vehicle-treated animal groups. Fold changes were calculated using the $2^{-\Delta\Delta Ct}$ formula. Mean ± SD, n = 3 samples pooled from nine rats, ANOVA, Dunnett test.

### 3.5. Analysis of the ex vivo arachidonic acid metabolism in the rats

#### 3.5.1. Effects of the sigma non-opioid intracellular receptor 1 ligands on the ex vivo eicosanoid synthesis of the platelets

*3.5.1.1. Application of radioactive arachidonic acid substrate to study platelet eicosanoid synthesis.* The total quantity of the radioactive cyclooxygenase metabolites (COX; sum of 6-keto prostaglandin $F_{1\alpha}$/6-k-$PGF_{1\alpha}$, which is a stable metabolite of prostacyclin; prostaglandin $F_{2\alpha}$/$PGF_{2\alpha}$; prostaglandin $E_2$/$PGE_2$; prostaglandin $D_2$/$PGD_2$; thromboxane $B_2$/$TxB_2$, which is a stable metabolite of $TxA_2$; and 12-L-hydroxy-5,8,10-heptadecatrienoic acid/12-HHT) in the **rat platelets** was significantly reduced by both PRE-084 and *(S)*-L1, while it was elevated by NE-100 (Table 3).

**Table 3**
Arachidonic acid metabolism in the rat platelets.

| RATS | COX (cpm) | LOX (cpm) | COX + LOX (cpm) | COX/LOX |
|---|---|---|---|---|
| **Vehicle** | 1425 ± 63 | 3132 ± 291 | 4649 ± 137 | 0.44 ± 0.01 |
| **PRE-084** | 1271 ± 84* | 3437 ± 100** | 4726 ± 51 | 0.37 ± 0.01*** |
| ***(S)*-L1** | 1146 ± 84*** | 3428 ± 122** | 4568 ± 170 | 0.33 ± 0.01*** |
| **NE-100** | 1897 ± 76*** | 2788 ± 111*** | 4685 ± 93 | 0.68 ± 0.02*** |

**cpm**: count per minute; **COX**: total quantity of radioactive cyclooxygenase metabolites synthesized from arachidonic acid in platelets; **LOX**: total quantity of radioactive lipoxygenase metabolites synthesized from arachidonic acid in platelets. All data are represented as mean ± SD. n = 9–9 rats/group. ANOVA, Bonferroni test, *$p < 0.05$, **$p < 0.03$, ***$p < 0.01$ compared to the vehicle-treated groups.

While the total quantity of radioactive LOX metabolites was raised by PRE-084 and *(S)*-L1, it was lowered by NE-100, thus indicating an opposite effect on the total quantity of radioactive COX and LOX metabolites. The sigma-1 receptor ligands did not change the total quantity of radioactive arachidonic acid metabolites (COX + LOX). The COX/LOX ratio was significantly reduced in the PRE-084 or *(S)*-L1 groups, while it was increased by NE-100 compared to the vehicle-treated rat platelets (Table 3).

In the **platelets**, the synthesis of $TxB_2$ (stable metabolite of $TxA_2$, the main vasoconstrictor and platelet aggregator product) and the production of $PGD_2$ (vasodilator and platelet anti-aggregator metabolite) were significantly decreased in the PRE-084 and *(S)*-L1 groups, while they were increased in the NE-100 group as compared to the vehicle-treated samples (Fig. 3).

In the **platelets**, the quantity of vasoconstrictor and platelet aggregator radioactive cyclooxygenase metabolites (CON: the sum of $PGF_2\alpha$ and $TxB_2$) was reduced by PRE-084 and *(S)*-L1 but raised by NE-100 (Table 4). The quantity of vasodilator and anti-aggregator radioactive cyclooxygenase metabolites (DIL: the sum of $PGE_2$ and $PGD_2$) was lowered by PRE-084, but it was boosted by NE-100. We can see a significant reduction in the ratio of the CON and DIL metabolites in the *(S)*-L1 ligand group (Table 4).

*3.5.1.2. Cyclooxygenase enzyme levels in the platelets detected by ELISA.* We detected both COX-1 and COX-2 enzymes **in the platelets** by ELISA. The concentration of COX-2 in the rat platelets was higher than that of COX-1. The level of the COX-1 and COX-2 enzymes was elevated by all of the sigma-1 receptor ligands used but in a different manner. The largest total concentration of these enzymes (COX-1+COX-2) was detected in the *(S)*-L1 ligand group (Table 5).

**Table 4**
Vasoconstrictor, platelet aggregator (CON) and vasodilator, platelet anti-aggregator (DIL) radioactive COX metabolites in the rat platelets.

| RATS | CON (cpm) | DIL (cpm) | CON/DIL |
|---|---|---|---|
| **Vehicle** | 840 ± 34 | 354 ± 26 | 2.43 ± 0.21 |
| **PRE-084** | 763 ± 66** | 319 ± 26** | 2.37 ± 0.12 |
| ***(S)*-L1** | 478 ± 71*** | 356 ± 80 | 1.41 ± 0.43*** |
| **NE-100** | 1103 ± 100*** | 356 ± 80 | 2.19 ± 0.3 |

**cpm**: count per minute; **CON**: the sum of $PGF_{2\alpha}$ and $TxB_2$; **DIL**: the sum of $PGE_2$ and $PGD_2$. All data are represented as mean ± SD. n = 9–9 rats/group. ANOVA, Bonferroni test, **p < 0.03, ***p < 0.01 compared to the vehicle-treated groups.

**Table 5**
Effect of the sigma non-opioid intracellular receptor 1 ligands on the quantity of COX-1 and COX-2 enzymes in the rat platelets determined by ELISA.

| RATS | COX-1 (ng/mL) | COX-2 (ng/mL) | COX-1+COX-2 (ng/mL) | COX-1/COX-2 |
|---|---|---|---|---|
| **Vehicle** | 4.37 ± 1.3 | 5.89 ± 1.9 | 10.59 ± 3.3 | 0.77 ± 0.14 |
| **PRE-084** | 5.5 ± 1.1 | 6.99 ± 2.2 | 12.87 ± 3.0 | 0.72 ± 0.09 |
| ***(S)*-L1** | 6.17 ± 0.9** | 11.52 ± 0.9*** | 17.89 ± 1.0*** | 0.51 ± 0.09** |
| **NE-100** | 6.39 ± 0.8*** | 10.43 ± 1.33*** | 16.81 ± 2.0*** | 0.62 ± 0.06* |

**COX-1**: type 1 cyclooxygenase; **COX-2**: type 2 cyclooxygenase. All data are represented as mean ± SD. n = 9–9 rats/group. ANOVA, Bonferroni test, *$p < 0.05$, **$p < 0.03$, ***$p < 0.01$ compared to the vehicle-treated groups.

*3.5.2. Effects of sigma non-opioid intracellular receptor 1 ligands on the ex vivo eicosanoid synthesis of the aorta*

*3.5.2.1. Application of the radioactive arachidonic acid substrate for the study of aortic eicosanoid synthesis.* The quantity of radioactive COX metabolites in the **rat aorta** was significantly reduced by PRE-084, while the quantity of radioactive LOX products was significantly lower in the *(S)*-L1 group, compared to the vehicle-treated group (Table 6).

The quantity of radioactive COX and LOX metabolites in the **rat aorta** was significantly elevated in the NE-100 group compared to the vehicle-treated samples. The total quantity of radioactive arachidonic acid metabolites (COX + LOX) was significantly lower in the PRE-084 and *(S)*-L1 groups but higher in the NE-100 sigma-1 receptor antagonist group compared to the vehicle-treated rat aorta. The COX/LOX ratio of the radioactive arachidonic acid metabolites in the rat aorta significantly increased in the *(S)*-L1 group compared to the vehicle-treated animals (Table 6).

In the **rat aorta**, the synthesis of 6-k-$PGF_{1\alpha}$ (stable metabolite of prostacyclin and the main vasodilator and platelet anti-aggregator product of the aorta) was significantly increased by *(S)*-L1 and NE-100

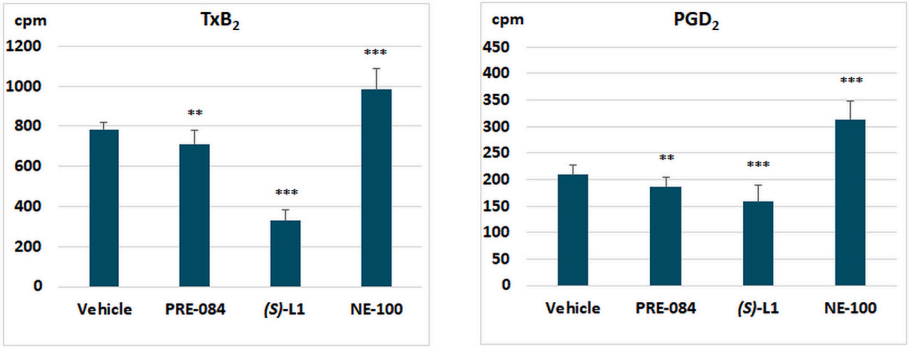


**Fig. 3.** The effects of the sigma non-opioid intracellular receptor 1 ligands on the different radioactive cyclooxygenase metabolites in the rat platelets. The y-axis represents the isotope activity in count per minute (cpm). All data are represented as mean ± SD. n = 9–9 rats/group. ANOVA, Bonferroni test, **$p < 0.03$, ***$p < 0.01$ compared to the vehicle-treated groups.

**Table 6**
Arachidonic acid metabolism in the rat abdominal aorta.

| RATS | COX (cpm) | LOX (cpm) | COX + LOX (cpm) | COX/LOX |
|---|---|---|---|---|
| **Vehicle** | 915 ± 215 | 551 ± 178 | 1466 ± 346 | 1.75 ± 0.4 |
| **PRE-084** | 717 ± 157* | 421 ± 87 | 1094 ± 153** | 1.8 ± 0.7 |
| ***(S)*-L1** | 826 ± 133 | 305 ± 67*** | 1132 ± 137* | 2.86 ± 0.8*** |
| **NE-100** | 1443 ± 202*** | 833 ± 51*** | 2277 ± 247*** | 1.73 ± 0.2 |

**cpm**: count per minute; **COX**: total quantity of radioactive cyclooxygenase metabolites synthesized from arachidonic acid in the abdominal aorta; **LOX**: total quantity of radioactive lipoxygenase metabolites synthesized from arachidonic acid in the abdominal aorta. All data are represented as mean ± SD. n = 9–9 rats/group. ANOVA, Bonferroni test, *p < 0.05, **p < 0.03, ***p < 0.01 compared to the vehicle-treated groups.

and reduced by PRE-084, compared to the vehicle-treated sample. The production of $TxB_2$ in the rat aorta was significantly lowered by both PRE-084 and *(S)*-L1 but raised by NE-100 (Fig. 4).

PRE-084 significantly decreased the quantity of DIL radioactive COX metabolites, while NE-100 increased the quantity of both DIL and CON radioactive COX metabolites in the rat aorta compared to the vehicle-treated group. Overall, NE-100 lowered the CON/DIL ratio of radioactive COX metabolites compared to the vehicle-treated group (Table 7).

*3.5.2.2. Cyclooxygenase enzyme levels in the aorta measured by ELISA.* We detected a higher concentration of COX-2 than COX-1 (Table 8) in the rat aorta.

PRE-084 and *(S)*-L1 increased the aortic COX-1 and COX-2 concentrations, with a higher elevation observed for COX-1 (Table 8). NE-100 reduced the COX-2 level, therefore, and shifted the balance between the enzymes to COX-1. The most effective sigma-1 receptor ligand was *(S)*-L1, which increased the level of COX-1 and COX-2 (Table 8).

### *3.6. Effects of sigma non-opioid intracellular receptor 1 ligands on the Ptgs1 mRNA level in the rat platelets*

The daily administration of sigma-1 receptor ligands for one week did not change the level of *Ptgs1* mRNA in the rat platelets (Fig. 5). Interestingly, the effect of *(S)*-L1 was different in the case of the *Sigmar1* and *Ptgs1* mRNA levels: an effect similar to the sigma-1 receptor agonist PRE-084 was observed for the *Sigmar1* mRNA level, while a similar effect of the sigma-1 receptor antagonist NE-100 was seen for the *Ptgs1* mRNA concentration in the rat platelets. We measured but could not detect *Ptgs2* mRNA by RT-qPCR in the platelet samples using 40 cycles.

**Table 7**
Vasoconstrictor, platelet aggregator (CON) and vasodilator, platelet anti-aggregator (DIL) radioactive COX metabolites in the rat aorta.

| RATS | CON (cpm) | DIL (cpm) | CON/DIL |
|---|---|---|---|
| **Vehicle** | 539 ± 128 | 242 ± 28 | 2.22 ± 0.4 |
| **PRE-084** | 459 ± 115 | 206 ± 14*** | 2.28 ± 0.59 |
| ***(S)*-L1** | 533 ± 93 | 236 ± 35 | 2.30 ± 0.44 |
| **NE-100** | 813 ± 139*** | 477 ± 74*** | 1.72 ± 0.24*** |

**cpm**: count per minute; **CON**: the sum of $PGF_{2\alpha}$ and $TxB_2$; **DIL**: the sum of 6-k-$PGF_{1\alpha}$ (which is a stable metabolite of prostacyclin), $PGE_2$, and $PGD_2$. All data are represented as mean ± SD. n = 9–9 rats/group. ANOVA, Bonferroni test, *p < 0.05, ***p < 0.01 compared to the vehicle-treated groups.

**Table 8**
Effect of sigma non-opioid intracellular receptor 1 ligands on the COX-1 and COX-2 enzyme levels in the rat aorta as determined by ELISA.

| RATS | COX-1 (ng/mL) | COX-2 (ng/mL) | COX-1+COX-2 (ng/mL) | COX-1/COX-2 |
|---|---|---|---|---|
| **Vehicle** | **3.70** ± 1.0 | **9.1** ± 2.1 | **13.27** ± 1.88 | **0.39** ± 0.14 |
| **PRE-084** | **5.84** ± 1.1*** | **10.84** ± 0.41 | **16.66** ± 1.65 | **0.54** ± 0.08 |
| ***(S)*-L1** | **8.41** ± 0.7*** | **13.86** ± 0.9*** | **21.89** ± 0.97*** | **0.61** ± 0.09* |
| **NE-100** | **4.37** ± 0.9 | **5.49** ± 0.75*** | **10.31** ± 0.14 | **0.76** ± 0.03 |

**COX-1**: type 1 cyclooxygenase; **COX-2**: type 2 cyclooxygenase. All data are represented as mean ± SD. n = 9–9 rats/group. ANOVA, Bonferroni test, *p < 0.05, ***p < 0.01 compared to the vehicle-treated groups.

## 4. Discussion

In this study, we investigated the effects of sub-chronic, *in vivo*-administered sigma-1 receptor ligands on platelet and aortic eicosanoid synthesis in healthy male rats. For our studies, we selected the sigma-1 receptor ligands PRE-084, NE-100, and *(S)*-L1 based on their binding strength to the receptor and their binding position in the sigma-1 receptor binding pocket (Fig. 1). Treatment duration was determined by platelet lifespan (one week) (Thon and Italiano, 2012; Cimmino and Golino, 2013). We confirmed that all the sigma-1 receptor ligands administered intraperitoneally entered the circulation, but their 30 min serum levels showed a large variation, which may indicate a difference in their absorption rate. The difference observed in the time-dependent analysis of the serum levels of the ligands (Figs. S1–S3) could be explained by their distinct metabolism and excretion. None of the ligands we studied at the dose used in our studies and for the duration involved caused metabolic changes in the rats that resulted in a change in body weight or blood glucose levels (over the time period). No effect

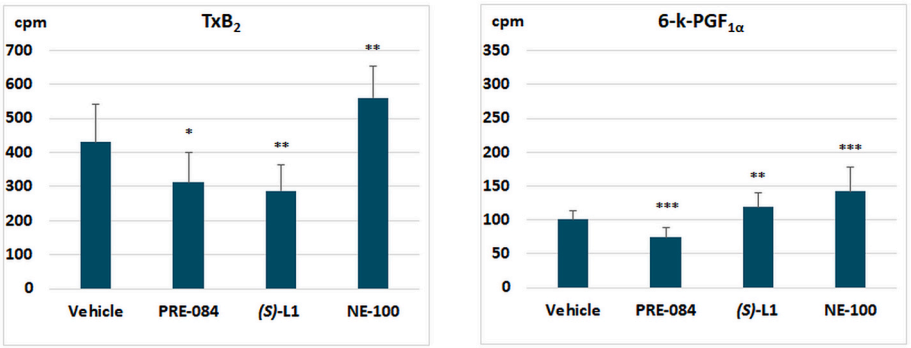


**Fig. 4.** The effects of the sigma non-opioid intracellular receptor 1 ligands on the different radioactive cyclooxygenase metabolites in the rat aorta. The y-axis represents the isotope activity in count per minute (cpm); **6-k-$PGF_{1\alpha}$**: 6-keto prostaglandin $F_{1\alpha}$; **$TxB_2$**: thromboxane $B_2$. All data are represented as mean ± SD. n = 9–9 rats/group. ANOVA, Bonferroni test, *p < 0.05, **p < 0.03, ***p < 0.01 compared to the vehicle-treated groups.

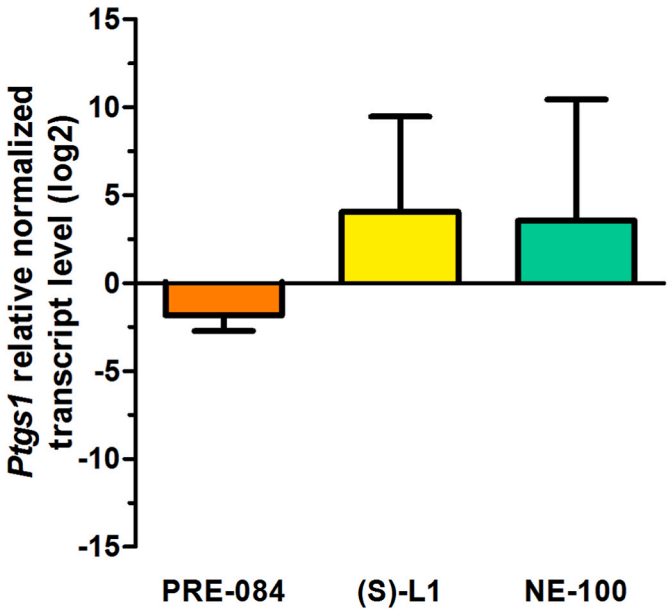


**Fig. 5.** Relative normalized transcript levels of *Ptgs1* in the rat platelet samples treated with sigma non-opioid intracellular receptor 1 ligands. The rats were treated with 3 mg/bwkg/day PRE-084, *(S)*-L1, or NE-100 for seven days. The vehicle-treated group received only saline solution. The transcript levels of *Ptgs1* in the rat platelet samples were first normalized to their own endogenous control mRNA levels (*Gapdh*) and then to the similarly normalized mRNA levels in the vehicle-treated animal groups. Fold changes were calculated using the $2^{-\Delta\Delta Ct}$ formula. Mean ± SD, n = 3 samples pooled from nine rats, ANOVA, Dunnett test.

of sub-chronic treatment was observed on platelet formation with sigma-1 receptor ligands, although this is not relevant in the present study, as their eicosanoid synthesis was monitored at standard platelet counts ($2.5 \times 10^8$ platelets/μL) (Table 1).

In order to rule out the direct effects of sigma-1 receptor ligands, our *ex vivo/in vitro* studies were started 20 h after the last ligand treatment, when the serum levels of ligands were already below the limit of detection or quantification (Table 2). Thus, the changes in arachidonic acid metabolism induced by the sigma-1 receptor ligands in the platelets and aorta and detected *ex vivo* may be due to changes in platelet and aortic function induced by the ligands *in vivo*. The sigma-1 receptor ligands we tested also affected rat platelet and aortic arachidonic acid metabolism in different ways (Tables 3 and 6).

**In the rat platelets**, none of the ligands induced changes in either sigma-1 receptor or COX mRNA levels; that is, the difference in arachidonic acid metabolism cannot be explained by the effects of the ligands on either sigma-1 receptor or cyclooxygenase enzyme transcription (Figs. 2 and 5). No change in COX enzyme concentration was detected in the rat platelets by ELISA after *in vivo* treatment with the PRE-084 ligand (Table 5). Therefore, a decrease in the total quantity of arachidonic acid metabolites formed by the cyclooxygenase pathway may be explained by inhibition of the COX enzyme or by a decrease in the absolute or relative quantity of free arachidonic acid substrate (Table 3). The generation of arachidonic acid metabolites is dependent on the presence of sufficient quantity of free arachidonic acid, which may be influenced by membrane phospholipid composition, phospholipase activity, and $[Ca^{2+}]_i$ levels. Sigma-1 receptor is known to be able to alter all these factors (Hayashi, 2019; Paes et al., 2019; Penke et al., 2018; Starr and Werling, 2002). No drop in the total quantity of arachidonic acid metabolites (COX + LOX) was observed with PRE-084, so an absolute reduction in free arachidonic acid due to higher phospholipid re-acylation can be ruled out. The decrease in the total quantity of COX metabolites could have been caused by a relative substrate decrease due to direct inhibition of cyclooxygenase and/or increased lipoxygenase activity, as supported by the drop in the COX/LOX ratio (Table 3). While no data are available for the direct action of sigma-1 receptor ligands on COX activity, a recent lipidomics study found a downregulation of the COX pathway due to bufotenin, a sigma-1 receptor ligand (Wang et al., 2021).

In contrast to the PRE-084 ligand, both *(S)*-L1 and NE-100 induced a similar increase in COX enzyme levels in the rat platelets (Table 5). However, *(S)*-L1 reduced the formation of COX metabolites, whereas NE-100 boosted it (Table 3). The higher COX enzyme levels and lower COX metabolite levels suggest that the PRE-084 and *(S)*-L1 ligands may have different effects on the translation and synthesis of COX enzymes, as well as on enzyme activity and metabolite production. The effects of *(S)*-L1 and NE-100 were the opposite not only on COX product formation but also on that of LOX (Table 3). In parallel with the elevation of the arachidonic acid substrate, the expression and/or activity of COX enzymes might be stimulated. Of the two COX isoforms, COX-1 is constitutively expressed in various tissues, but the expression of COX-2 is mostly inducible. Although the platelets are non-nucleated cells, they can *de novo* synthesize COX-1 from the cytoplasmic mRNA that originates from the megakaryocyte (Evangelista et al., 2006). In a previous report, COX-2 mRNA and protein were detected in human platelets, but in a significantly smaller quantity than COX-1 (Hu et al., 2017). However, under our present experimental conditions, we were unable to detect COX-2 (*Ptgs2*) mRNA in rat platelets by 40-cycle RT-qPCR. This may be explained by the limited pool of mRNA transcripts from megakaryocytes that can be used for protein (e.g. COX-2) synthesis, so increased arachidonic acid metabolism may lead to depletion of intracellular reserves (mRNA, enzyme pool, and $[Ca^{2+}]_i$).

The effect of sigma-1 receptor ligands on platelet arachidonic acid metabolism is likely to occur not only through the modulation of COX and LOX but also through the modulation of specific enzymes. This is suggested by the fact that the administration of *(S)*-L1 in the rats shifted the ratio of CON/DIL COX products in the platelets (Table 4). The drop in the CON/DIL ratio in the platelets was induced by *(S)*-L1 by lowering the formation of CON products (Table 4 and Fig. 3). The synthesis of the main platelet cyclooxygenase metabolite, the vasoconstrictor- and platelet-aggregating thromboxane, was reduced much more by our new ligand, *(S)*-L1, than by PRE-084 (Fig. 3).

Under physiological conditions, platelet and endothelial cell arachidonic acid metabolism is known to differ significantly. Platelets primarily produce vasoconstrictor thromboxane, which induces platelet aggregation, and the endothelium primarily produces vasodilator prostacyclin, which inhibits it; however, under physiological conditions, these products are in balance and thus ensure physiological microcirculation (Csányi et al., 2007; Mezei et al., 2017; Mitchell and Kirkby, 2019).

The effects of the sigma-1 receptor ligands we studied on arachidonic acid metabolism in the **rat aorta** showed a significant difference compared to their effects on platelets. In contrast to platelets, the total quantity of eicosanoids formed by the aorta via the lipoxygenase pathway was decreased by *(S)*-L1, whereas NE-100 increased it. Sigma-1 receptor ligands induced a change in the aorta that was in the same in direction as that in the platelets but smaller in total quantity of COX metabolites (Tables 3 and 6). These changes can be explained by changes in both absolute and relative arachidonic acid substrate quantity. The drop in the total quantity of arachidonic acid metabolites (COX + LOX) by *(S)*-L1 and the increase due to NE-100 suggest that sigma-1 receptor ligands may have affected arachidonic acid release from phospholipids, i.e. the absolute quantity of substrate. On the other hand, the shift in the ratio of cyclooxygenase to lipoxygenase metabolites (COX/LOX) in the aorta due to *(S)*-L1 may suggest that the ligand affected the two enzyme systems differentially, resulting in a relative substrate deficiency (Table 6). Although the effects of the ligands on the total quantity of platelet and aortic cyclooxygenase metabolites were similar (Tables 3 and 6), their effects on the ratio of cyclooxygenase-derived vasoconstrictor and platelet aggregator (CON) and

vasodilator, platelet aggregation inhibitor cyclooxygenase metabolites (DIL) (CON/DIL) were significantly different. *(S)*-L1 in the platelets and NE-100 in the aorta resulted in a decrease in CON/DIL, whereas the other ligands produced no change (Tables 4 and 7; Figs. 3 and 4). NE-100 lowered the CON/DIL ratio in the aorta despite raising the synthesis of both CON and DIL metabolites (Table 7 and Fig. 4). The synthesis of the major aortic cyclooxygenase metabolite, the vasodilator and anti-aggregator prostacyclin, was enhanced by the new ligand we tested, *(S)*-L1, which was inhibited by PRE-084 (Fig. 4).

The neuroprotective (Penke et al., 2018) and cardioprotective (Lewis et al., 2020) effects of the sigma-1 receptor have already been demonstrated. However, only a fraction of all cardiovascular diseases is of cardiac origin, and macro- and microvascular dysfunction and/or platelet activation are responsible for the development and exacerbation of many diseases. The eicosanoids are *de novo*, locally formed (Evangelista et al., 2006), short half-life (Egan and FitzGerald, 2006; Paes et al., 2019), autocrine-, paracrine-, and endocrine-acting (Paes et al., 2019) arachidonic acid metabolites. Under physiological conditions, the balance of vasoactive and platelet aggregation-mediated cyclooxygenase products synthesized by platelets and vascular endothelium ensures proper microcirculation and tissue perfusion (Mancuso and Santagostino, 2017; Mitchell and Kirkby, 2019). The sub-chronic *in vivo* administration of the PRE-084, *(S)*-L1, and NE-100 sigma-1 receptor ligands in healthy rats modulated both rat platelet and aortic eicosanoid synthesis; however, these effects were cell/tissue-specific. On platelet and aortic eicosanoid synthesis, the effects of our ligands resulted in a predominance of vasodilator and platelet aggregation inhibitory COX eicosanoids. Under our present study conditions, despite the different mode and intensity of action, all three ligands we investigated played a protective role in maintaining the balance of platelet and vascular eicosanoid synthesis, i.e. tissue perfusion and microcirculation. Of the sigma-1 receptor ligands we tested, *(S)*-L1 proved to be the most potent.

## 5. Conclusion

Our results suggest that sigma-1 receptor ligands may act at multiple points in arachidonic acid metabolism. This effect was found to be cell-specific. Our experimental results on eicosanoid synthesis in rat platelets confirm the regulatory role of the sigma-1 receptor in cellular functions.

*(S)*-L1, a readily absorbed and rapidly eliminated/metabolized novel sigma-1 receptor ligand, affected rat platelet and aortic arachidonic acid metabolism in a manner similar to the sigma-1 receptor agonist PRE-084, but in a manner different from the antagonist NE-100.

Sigma-1 receptor ligands may play an important role in the regulation of microcirculation by modulating platelet and vascular eicosanoid synthesis.

## CRediT authorship contribution statement

**Sándor Váczi:** Conceptualization, Methodology, Investigation, Formal analysis, Resources, Data curation, Writing – original draft, preparation, Writing – review & editing, Supervision. **Lilla Barna:** Formal analysis, Investigation, Resources, Writing – original draft, preparation, Visualization, Supervision. **Krisztián Laczi:** Formal analysis, Writing – original draft, preparation. **Ferenc Tömösi:** Writing – original draft, preparation, Visualization, Formal analysis, Resources, Supervision. **Gábor Rákhely:** Writing – original draft, preparation, Visualization, Funding acquisition. **Botond Penke:** Data curation, Visualization, Funding acquisition. **Lívia Fülöp:** Visualization, Funding acquisition. **Ferenc Bogár:** Formal analysis, Visualization, Funding acquisition. **Tamás Janáky:** Data curation, Visualization, Funding acquisition. **Mária A. Deli:** Data curation, Visualization, Funding acquisition. **Zsófia Mezei:** Conceptualization, Methodology, Investigation, Data curation, Writing – original draft, preparation, Writing – review & editing, Supervision, Funding acquisition.

## Declaration of competing interest

None.

All authors of this manuscript have seen and approved the final version of the submitted manuscript. This article is the authors' original work, has not been published previously, and is not intended for publication elsewhere.

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## Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.ejphar.2022.174983.

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