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Design and synthesis of *N*-(benzylpiperidinyl)-4-fluorobenzamide: a haloperidol analog that reduces neuropathic nociception via σ_1 receptor antagonism

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ABSTRACT

Aims: Haloperidol is a neuroleptic drug with high affinity towards the σ_1 receptor (σ_1R), acting as antagonist that decreases neuropathic pain, but has CNS side effects. This work describes the design and synthesis of a novel analog *N*-(1-benzylpiperidin-4-yl)-4-fluorobenzamide (**LMH-2**), which produced antihyperalgesic and antiallodynic effects in rats with neuropathy induced by chronic constriction injury of the sciatic nerve (CCI), being more active than gabapentin (The most widely used drug for the treatment of neuropathic pain).

Main methods: LMH-2 was designed as haloperidol analog. Its structure was characterized by spectroscopic (^{1}H and ^{13}C NMR) and spectrometric mass (electronic impact) techniques. Additionally, *in silico* predictions of pharmacokinetic, pharmacodynamic and toxicological properties were obtained, with promising results. A competitive binding assay using radioligands was employed to evaluate the *in vitro* affinity for σ_1R, whereas *in vivo* antihyperalgesic and antiallodynic activities were investigated using Wistar rats with CCI.

Key findings: LMH-2 showed high affinity for σ_1R in an *in vitro* binding assay, with a K_i = 6.0 nM and a high σ_1R/σ_2R selectivity ratio. Molecular docking studies were carried out to determine the binding energy and to analyze **LMH-2**-protein interactions. Through an *in silico* pharmacological consensus analysis, **LMH-2** was considered safe for *in vivo* evaluation. Thus, **LMH-2** had dose-dependent antiallodynic and antihyperalgesic activities; its efficacy was comparable to that of gabapentin, but its potency was 2-times higher than this drug.

Significance: LMH-2 administration produced antihyperalgesic and antiallodynic effects by the antagonism of σ_1R, suggesting its potential use as an analgesic drug for neuropathic pain.

Keywords: *N*-(1-Benzylpiperidin-4-yl)-4-fluorobenzamide, haloperidol, σ_1 receptor, neuropathic pain, allodynia, hyperalgesia.

1. INTRODUCTION

The σ_1 receptor (σ_1R) plays a pivotal role in the transmission of pain. These receptors are in the plasma and subcellular membranes, particularly in the endoplasmic reticulum. It is known that σ_1R plays a modulatory role in intracellular Ca^{2+} signaling and in the activity of some ion channels and several neurotransmitter systems, mainly in the glutamatergic pathway [1,2]. Preclinical evidence supports the modulatory role of σ_1R in nociception, mainly based on the inhibition of σ_1R leads to decreased amplification of pain signaling within the CNS (dorsal spinal cord, thalamus, periaqueductal gray, basolateral amygdala and rostroventral medulla) [3]. The σ_1R KO mice is insensitive or shows attenuated expression of pain behaviors in formalin or capsaicin-induced and neuropathic pain models [4-6]. The antagonism of σ1R leads to decreased amplification of pain signaling within the spinal cord (central sensitization) and the periphery. There is considerable evidence showing that **BD1047**, a potent σ_1R antagonist, blocks nociceptive effects [7-9]. Moreover, this drug might interact with several unknown targets and, thus, interfere with opioid antinociception [10]. However, there is evidence showing that haloperidol, a dopaminergic antagonist used mainly as a neuroleptic drug, decreases neuropathic pain in humans when administered alone [11] or in combination with tramadol [12]. Currently, it has been proposed that the therapeutic efficacy of haloperidol on neuropathic pain involves the blockade of σ_1R, but not via dopamine receptor antagonism [5, 6]. However, haloperidol presented several neurological side effects that restrict its use as a therapeutic agent. Haloperidol has central nervous system effects, particularly extrapyramidal symptoms (catalepsy and motor imbalance) and tardive dyskinesia, sedation, and dulling of cognition [13]. Other adverse effects of the typical antipsychotics include the neuroleptic malignant syndrome, orthostatic hypotension, changes in liver function, anticholinergic and antiadrenergic side effects, sexual dysfunction, and

weight gain. Also, high dose of haloperidol may result in irreversible liver damage [14,15]. These findings provided evidence to consider σ1R antagonists as an innovative and alternative approach for treating pain, especially neuropathic pain but also other sensitizing pain conditions. Therefore, we decided to design a novel analog of haloperidol for its possible use in treating neuropathic pain through the antagonism of the σ_1R, but without the unwanted side effects. Here, we report the design, *in vitro* σ_1R affinity and *in vivo* antiallodynic and antihyperalgesic activities of **LMH-2** after its systemic administration against CCI of the sciatic nerve model in rats.

2. MATERIALS AND METHODS

*2.1. Drug design of **LMH-2***

The previously reported pharmacophore models suggest three major features that are imperative for high binding affinity at σ_1R: I) an ionizable tertiary amino group, II) a mainly hydrophobic region, composed of an aryl ring situated at 6–10 Å from the amino group, and III) a small secondary hydrophobic region at 2.4–3.9 Å from the amine [16,17].

The new chemical entity **LMH-2** was designed as an analog of haloperidol (Figure 1) and fulfills the requirements of reported pharmacophore models for high-affinity σ_1R ligands: I) An ionizable tertiary amine that interacts with the σ_1R through electrostatic interactions, II) a main hydrophobic region situated at 7.035 Å from the amine, and III) a secondary hydrophobic moiety at 3.607 Å from the amine (Figure 2). Both aryl groups can form additional π-π interactions with the σ_1R.

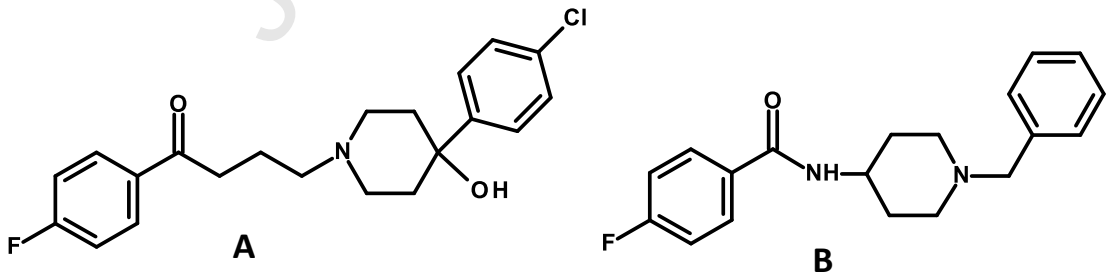

Figure 1. Chemical structures of haloperidol (A) and **LMH-2** (B).

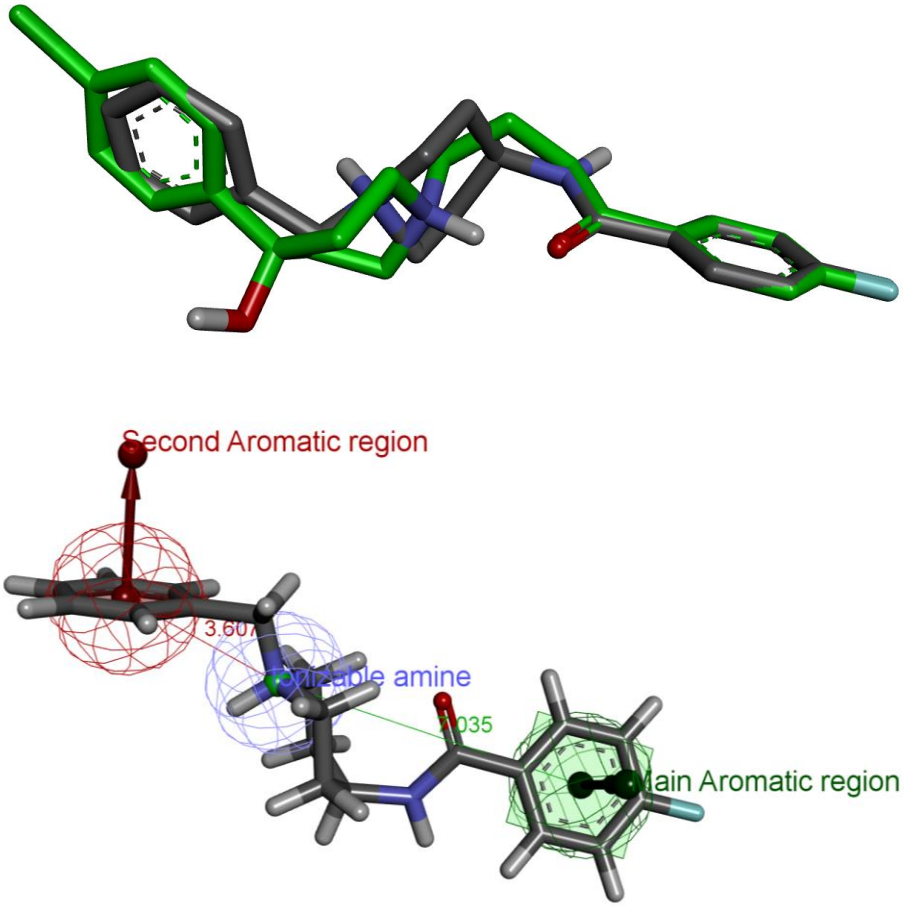

Figure 2. Overlay of haloperidol (green) and **LMH-2** (black) and the pharmacophore pattern found in the σ_1R ligands applied to **LMH-2**

LMH-2 (substituted with a fluorine atom at the 4-position of the benzene ring) follows the Lipinski rule of five, compatible with good pharmacokinetic behavior (Table 1). These physicochemical parameters or properties are similar to those shown by haloperidol and **S1RA,** an advanced σ_1R antagonist, which has shown promising results in phase II clinical trials for neuropathic pain [18].

Table 1. Physicochemical properties of **LMH-2**, haloperidol, and **S1RA**

Compound	MW	clogP	Hydrogen bond acceptors	Hydrogen bond donors	Number of rotatable bonds	Polar surface area
	<500	**<5**	**<10**	**<5**	**<10**	**<60 A^2**
LMH-2	312	3.11	3	1	3	49.4
Haloperidol	375	4.31	3	1	5	36.9
S1RA	337	3.33	5	0	5	36.9

2.2. Biological activity spectra prediction

The *in silico* biological activity spectra calculated for **LMH-2** were obtained using ACD/ToxSuite software, the OSIRIS program (http://www.organic-chemistry.org/prog/peo/) and the admetSAR database (http://lmmd.ecust.edu.cn:8000/). The estimation of the general biological potential of the compound can be performed based on the structural formula, its predicted toxicity, and drug-like pharmacokinetic properties.

2.3. Chemistry

All the starting materials and reagents were obtained commercially from Sigma-Aldrich (St. Louis, MO, USA). Melting points were determined on an SRS EZ Melt MPA120 automated apparatus from Stanford Research Systems and are uncorrected. TLC monitored reactions on 2 x 5 cm precoated silica gel 60 F_{254} plates (Merck KGaA, Darmstadt, Germany) were visualized under 254-365 nm UV light.

The chemical structures of the synthesized compound were confirmed based on their spectral data (^{1}H NMR, ^{13}C NMR spectra, and EIMS). NMR studies were performed on an INOVA-400 MHz instrument. Chemical shifts (δH, δC) and coupling constant values (*J*) are given in ppm and Hz, respectively. A standard reference of TMS (δ_H = 0, δ_C = 0) in $CDCl_3$ and DMSO-d_6 as solvents was used. Mass spectra were recorded on a JEOL JMS-700 instrument (JEOL USA Inc., Peabody, MA, USA).

2.4. Binding assay for the σ_1 receptor

The test compound solutions were prepared by dissolving approximately 10 μmol (usually 2–4 mg) of **LMH-2** in the required amount of DMSO so that a 10 mM stock solution was obtained. To obtain the required test solutions for the assay, the DMSO stock solution was diluted with the respective assay buffer. All binding experiments were carried out in duplicates in 96-well multiplates. The protocol of the σ_1R binding assay was performed with the radioligand [^{3}H]-(+)-pentazocine (22.0 Ci/mmol; Perkin Elmer). The thawed membrane preparation of guinea pig brain cortex (approximately 100 μg of protein) was incubated with various concentrations of **LMH-2**, 2 nM [^{3}H]-(+)-pentazocine, and TRIS buffer (50 mM, pH 7.4) at 37 °C. The nonspecific binding was determined with 10 μM

unlabeled (+)-pentazocine. The K_d-value of (+)-pentazocine is 2.9 nM [19, 20]. The dissociation constant (K_d) describes the affinity of a ligand towards a protein. The ligand-protein affinity is influenced by the noncovalent intermolecular interactions between the two molecules, such as hydrogen bonds, electrostatic interactions, hydrophobic forces, and van der Waals forces. The smaller the dissociation constant, the more strongly the ligand is bound to the protein.

2.5. Pharmacological evaluation in vivo

2.5.1. Animals

Young male Wistar rats of approximately 90-110 g were provided by the bioterium of the Pharmacology Department of the CINVESTAV Coapa. The animals were kept under standard conditions (with 12 h day/night cycles, 25 °C and a humidity of 45-65%). The experiments were carried out in accordance with the official Mexican standard NOM-062-ZOO-1999 and under the technical specifications for the production, care and use of laboratory animals and following a pain model authorized by the IASP and the National Institutes of Health guide for the care and use of Laboratory Animals (NIH Publications No. 8023, revised 1978). Rats were evaluated after surgery at 170-180 g in adult age, the route of administration was subcutaneous (on the dorsum of the rat in the interscapular region), and each experimental group consisted of at least six animals.

2.5.2. Sciatic nerve surgery

The CCI involves placing four ligatures on the sciatic nerve to interrupt neurotransmission, which generates allodynia and hyperalgesia. The animals were anesthetized with ketamine/xylazine (50/12 mg/kg, i.p.) followed by dissection of the biceps femoris. The most proximal part was located at the trifurcation of the sciatic nerve, and approximately 7 mm was ligatured with silk thread (3-0) with a distance of 1 mm between each. Subsequently, the muscle was sutured with chromic catgut absorbable thread (4-0) and the skin with 3-0 silk thread; 15 days after surgery, the presence of allodynia and hyperalgesia behavioral responses was evaluated.

2.5.3. Evaluation of allodynia and hyperalgesia

The evaluation of hyperalgesia was carried out using von Frey filaments, applying a tactile stimulus on the plantar surface of the hind limbs with a filament to exert a force of 15 g. A filament exerting the force of 15 g was used to measure the pain threshold, since filaments of less strength are imperceptible to the sensation of the rats. Each stimulus was applied 10 times with intervals of 3 seconds each. The behavior that reflects hyperalgesia is quantified as the withdrawal latency of the paw stimulated with the filament. For assessing allodynia, the method consisted of applying a cold stimulus; 0.1 mL of acetone was placed on the dorsal surface of the hind limbs. The response of allodynia was quantified as the duration of jerking and licking of the limb during a lapse of 1 minute. These behaviors were analyzed at 0, 30, 60, 90, 120 and 180 minutes after the administration of the compound to be evaluated. To establish the therapeutic window, a screening of the compound to be evaluated was performed using logarithmic doses and evaluating the antiallodynic and antihyperalgesic effects. Subsequently, a dose-response curve was generated for both activities (0.1, 1.0, 3.2, 10.0, 31.6, 100.0 mg/kg, s.c.).

2.6. Statistical analysis

To determine the effect of the compound on the presence of allodynia and hyperalgesia behavioral responses, the effects of each treatment were analyzed using six animals in each group and the results are expressed as the average ± standard error of the mean (S.E.M.). The value of the area under the curve (AUC) was calculated from the respective time course. The AUC for each dose of analyzed compound was calculated using the trapezoidal method. Student´s t-test was used to compare two groups. For the analysis of the nociceptive sensitivity to mechanical stimulation at different time points and under different treatments, one-way ANOVA was used, followed by Dunnett's post hoc test with $p \leq 0.05$ comparison *vs*. vehicle. In the AUC analysis, data were compared using one-way ANOVA followed by Tukey's *post hoc* test ($p \leq 0.05$). In all statistical analysis, a $p < 0.05$ was considered statistically significant.

2.7. Molecular docking approach

Molecular docking predicts the binding energy (ΔG), as well as the orientation and three-dimensional conformation (3D) of the ligand-site binding action, generally in a protein determined by X-ray diffraction or by modeling by

homology when the structure (3D) is unknown [21]. All *in silico* calculations were done with Molecular Operating Environment (MOE, Chemical Computing Group Inc. http://www.chemcomp.com) version 2018.01. MOE is software that strongly supports the design of bioactive molecules through molecular simulation, protein structure analysis, small molecule data processing, and the study of protein and small molecule docking [22].

The crystal structure of the sigma-1 receptor (σ_1R) complexed with *N*-(1-benzylpiperidin-4-yl)-4-iodobenzamide (5HK2) at 3.2 Å resolution was obtained from the Protein Data Bank (http://www.rcsb.org/pdb) [23]. All water molecules were deleted, and the hydrogen atoms and charges were adjusted with the MMFF94 force field from the MOE suite. The 3D structures were built and minimized in MOE, using the same force field as that mentioned above. The docking was performed considering all residues within a 4.5 Å sphere centered on cocrystallized ligand atoms. As a placement function, Alpha Triangle was selected, and the scores were calculated with the Affinity DG function, which measures the enthalpy contribution to the free energy of binding (MOE), in accordance with a validation procedure to reproduce the docking, the same pose of cocrystallized ligand in the crystal structure (RMSD = 0.569 Å) and a score of -6.692 Kcal/mol. For each ligand, 100 conformations were generated, and the top-ranked conformation based on docking score energy was selected for further studies of molecular docking. After molecular docking, we analyzed the best calculated binding poses, and the graphical representations were created as surface maps and ligand interactions in MOE (Figure 3).

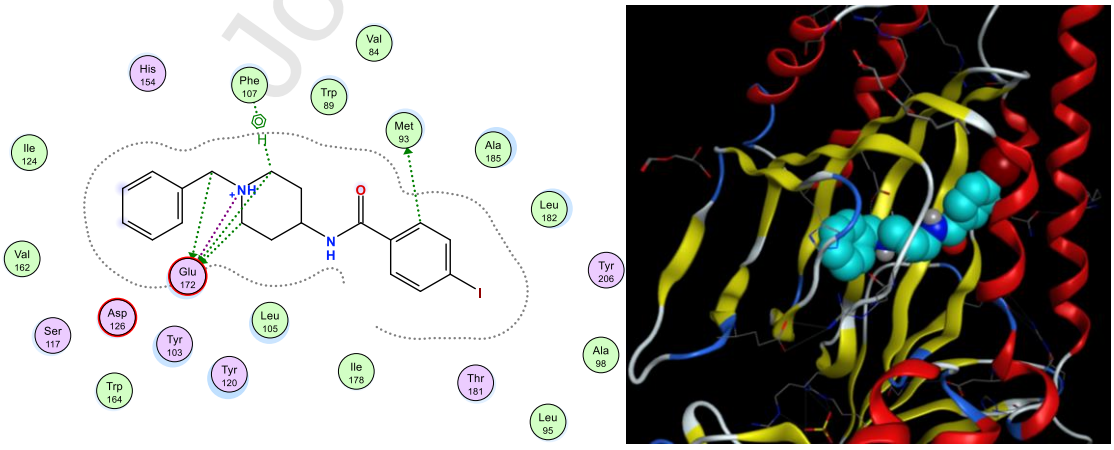

Figure 3. 2D and 3D interaction map of the docking validation of σ_1R cocrystallized with *N*-(1-benzylpiperidin-4-yl)-4-iodobenzamide (5HK2). RMSD = 0.569 Å, Docking Score of -6.692 Kcal/mol.

The cocrystallized ligand *N*-(1-benzylpiperidin-4-yl)-4-iodobenzamide shows several interactions with σ_1R, mainly by electrostatic forces between the ionized amino group and Glu172, Phe107 and Met 93. Additionally, the amino group shows polar interactions with Trp89 (Figure 4).

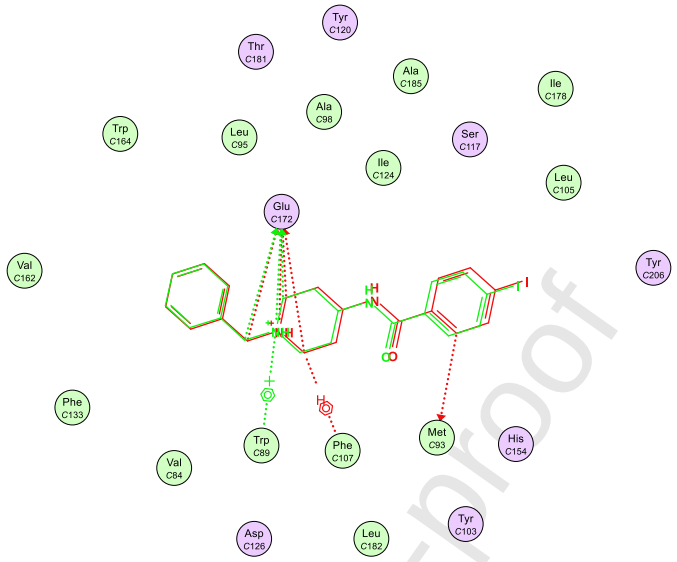

Figure 4. Overlay of cocrystallized ligand and its validated docking posture. Red: cocrystallized, green: validation

3. RESULTS

3.1. In silico toxicology prediction

To calculate the potential toxicity activity of **LMH-2**, we employed OSIRIS property explorer (https://www.organic-chemistry.org/prog/peo/), a computational server that predicts the risk of toxicity of the synthesized compounds on the following criteria: mutagenic, tumorigenic, irritant and reproductive effective. It presents numerical values and color-codes for the different criteria, which are indicated as red, a warning of toxicity; orange for intermediate toxicity; and green for nontoxic. The results for the final compound **LMH-2**, haloperidol, and **S1RA** are shown in Table 2.

Table 2. Toxicological properties and drug-like qualifications of **LMH-2**, haloperidol, and **S1RA** calculated with OSIRIS property explorer.

Compound	Mutagenic activity	Tumorigenic activity	Irritant activity	Effect in the reproduction	Drug likeness	Drug score
LMH-2	●	●	●	●	7.85	0.81
Haloperidol	●	●	●	●	12.32	0.6

S1RA					1.66	0.37

Table 3 shows the theoretical results of absorption and metabolism calculated for **LMH-2, S1RA,** and haloperidol based on a QSAR model by the admetSAR server http://lmmd.ecust.edu.cn/admetsar2 [24].

Table 3. Predictive results of **LMH-2**, haloperidol, and S1RA computed with admetSAR

	LMH-2	**Haloperidol**	**S1RA**
Intestinal absorption	+	+	+
	0.9736	1.000	1.000
Blood-brain barrier	+	+	+
	0.979	0.9465	0.9973
PGp substrate	+	+	-
	0.6047	0.6673	0.5651
CYP2C9 substrate	-	-	-
	0.8617	0.8355	0.7895
CYP2D6 substrate	+	+	-
	0.5497	0.8919	0.5617
CYP3A4 substrate	+	+	+
	0.5079	0.5796	0.7762
AMES toxicity	-	-	+
	0.7433	0.9133	0.5171
Carcinogenic	-	-	-
	0.9218	0.8769	0.8664
hERG inhibition	Weak Inhibitor	Strong Inhibitor	Strong Inhibitor

The complemented toxicological profile prediction of compounds **LMH-2**, **S1RA** and haloperidol was carried out with the ACD/ToxSuite program [25], which reported several results, such as the percentage of the probability of an hERG potassium channel blockage (correlated with cardiotoxicity), the inhibition of CYP450 family isoforms (correlated with drug-drug interactions), as well as acute toxicity (mean lethal dose = LD_{50}) by the oral administration route for the rat or mouse model, according to the category in the Organization for Economic Cooperation and Development (OECD) classification. Predictions of the toxicological profile are reported in Table 4.

Table 4. In silico toxicity profiles predicted for **LMH-2**, haloperidol, and **S1RA** using ACD/ToxSuite software

Compound	% of inhibition or blockage (*Ki* or IC_{50} < 10 μM)						Half maximal lethal dose (LD_{50}, mg/kg, p.o.)		Category OECD
	hERG	CYP450 isoforms							
		3A4	2D6	2C9	2C19	1A2	Mouse	Rat	
LMH-2	14%	5	54	5	5	3	540	930	IV
Haloperidol	90%	5	63	7	6	0	160	180	III
S1RA	66%	26	16	9	5	55	570	960	IV

3.2. Chemistry

Figure 5 shows the synthetic route to obtain **LMH-2**.

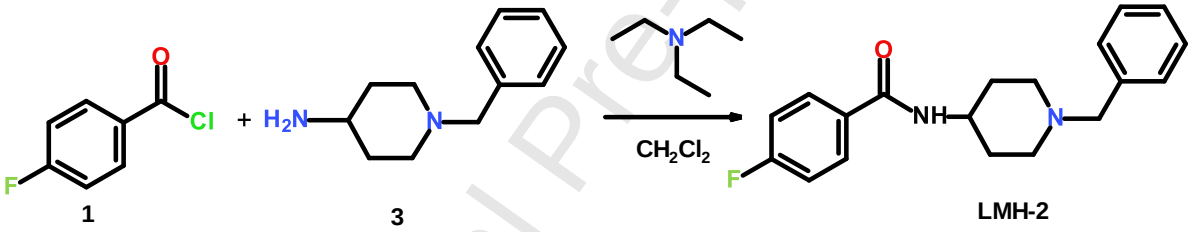

Figure 5. Chemical synthesis of **LMH-2**

*3.2.1. Synthesis of **LMH-2***

Into a 25 mL round bottom flask equipped with magnetic stirring, a gas trap, and a liquid addition funnel, 4-amino-1-benzylpiperidine (15.8 mmol) and triethylamine (17.4 mmol) were slowly added dropwise and stirred with 3 mL of dry dichloromethane (CH_2Cl_2) for 15 minutes. Afterward, the flask was placed in an ice bath (5° C) with the addition of 4-fluorobenzoyl chloride (17.4 mmol) diluted in 5 mL of CH_2Cl_2. The solution was stirred 6 h at room temperature. The reaction was monitored by TLC analysis until completed. The solvent was evaporated to dryness, and the solids were washed with water, filtered and recrystallized from ethanol, obtaining white crystals weighing 0.51 g with a melting point of 160.5-161.9 °C and a yield of 95%.

^{1}H NMR (600 MHz, $CDCl_3$) δ: 1.55 and 2.00 (m, 4H, 2H-9, 2H-11), 2.16 and 2.84 (m, 4H, 2H-8, 2H-12), 3.51 (s, 2H, NCH_2Ph), 3.95-4.02 (m, 1H, H-10), 7.088 (t, 2H, H-3, H-5, J_m=2, J_o=8.6 Hz), 7.23-7.35 (m, 2H, H-2', H-6'), 7.74-

7.76 (m, 2H, H-2, H-6, J_o=8.2 Hz, J_m= 5.2 Hz with the F heteroatom). **^{13}C NMR** (150 MHz, $CDCl_3$) δ: 32.2 (C-9, C-11), 47.2 (C-10), 52.3 (C-8, C-12), 63.0 (C-13), 114.9 (C-4'), 115.5 (d, C-2´, C-6´, $^2J_{C-F}$=22 Hz),127.5 (C-3, C-5), 128.6 (C-2, C-6), 129.2 (d, C-3´, C-5´, $^3J_{C-F}$=7 Hz), 130.9 (C-1'), 138 (C-1), 164.13 (d, C-F, $^1J_{C-F}$=248.5 Hz), 165.7 (C=O). **EIMS** m/z 312.00 (20%, M^+), 295.06 (10%), 82.46 (100%).

3.3. In vitro σ_1R affinity

The σ_1 and σ_2 receptor affinities of **LMH-2** were determined in receptor binding studies employing the radioligands [^{3}H]-(+)-pentazocine and [^{3}H]-di-*o*-tolylguanidine, respectively. These results are given in Table 5, including corresponding reference compounds: haloperidol, di-*o*-tolylguanidine, (+)-pentazocine, and **S1RA**.

Table 5. σ_1 and σ_2 receptor affinities of **LMH-2** and reference compounds

Compound	K_i (nM)		Selectivity
	σ_1	σ_2	σ_1/σ_2
LMH-2	6.0	190	31.6
haloperidol	6.3	78	12
di-*o*-tolylguanidine	89	58	0.7
(+)-pentazocine	5.7	-	-
S1RA	17	>1000	>58

3.4. Molecular docking on the σ_1 receptor

To determine the interaction form of the **LMH-2** with σ_1R, it was docked into the receptor binding site obtained from the Protein Data Bank (PDB ID: 5HK2), as reported by Schmidt et al. [23]. The docked complex with the lowest binding energy was selected (−10.99 kcal/mol). Figure 6 shows a two- and three-dimensional graph of the binding of **LMH-2** to the receptor. The interaction between the protonated amine from the heterocycle and Glu172 was observed, as well as the π–π interaction between Tyr103 and fluorobenzoyl moieties (Figure 6). Both interactions agree with those reported in the literature [23].

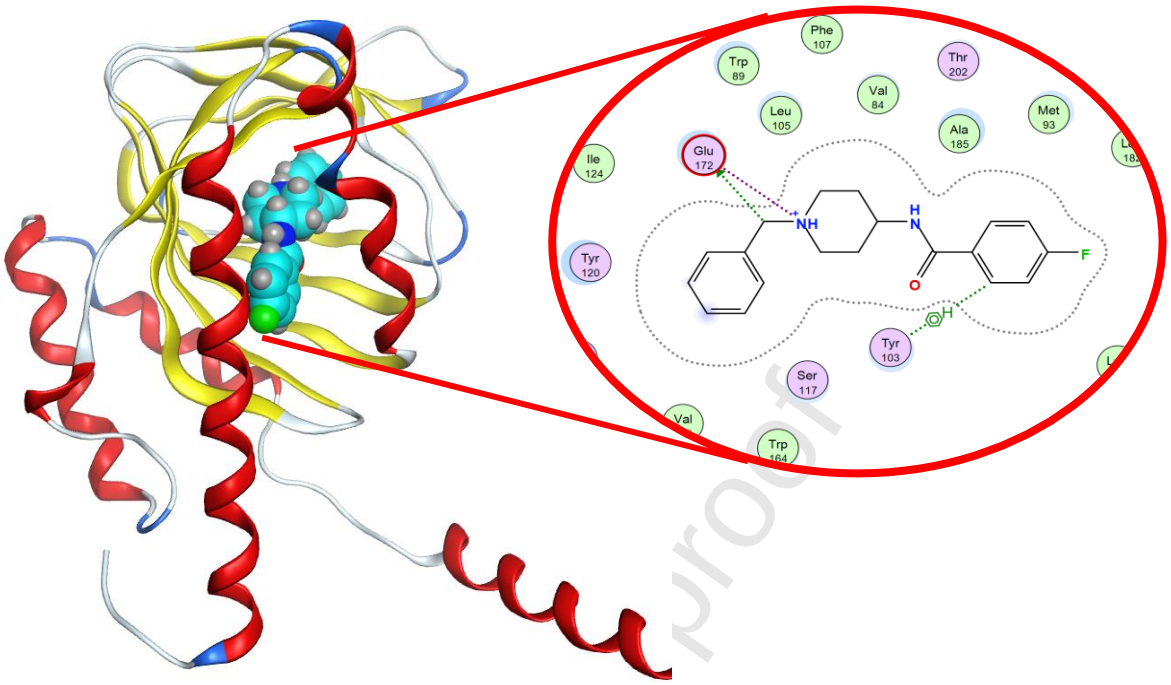

Figure 6. 2D and 3D interaction map of the docking of σ_1R with **LMH-2**.

*3.5. In vivo antiallodynic and antihyperalgesic assay for **LMH-2***

In Figure 7, we observed that the sham rats (receiving surgical incision without the ligature of the nerve) did not increase the amount of time of licking before the application of a cold stimulus (acetone) to the ipsilateral leg. However, rats with CCI and vehicle administration increased the amount of time licking to 18.74 ± 0.74 s. This time increase was considered as a 100% effect [26]. However, in panel B, it is shown that rats with CCI increased the % withdrawal response after a mechanical stimulus was applied via a 15 g von Frey filament (10 stimuli); the number of positive responses in a series of 10 was considered as 100% hyperalgesia.

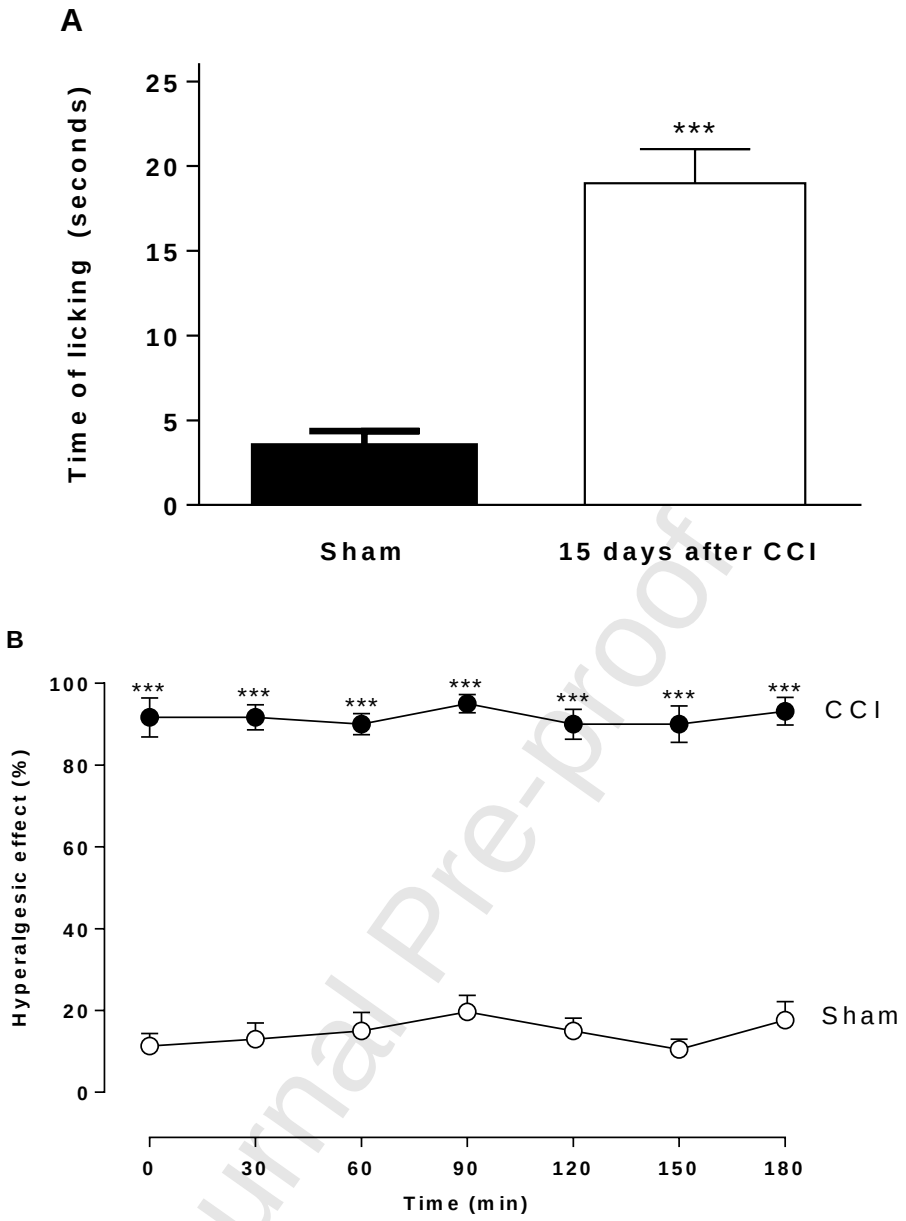

Figure 7. The allodynic effect was determined by ipsilateral application of a cold stimulus with acetone. Allodynia is measured as accumulative time of licking in seconds for 1 min (panel A). The hyperalgesic effect was established with a mechanical stimulus with a 15 g von Frey filament. The response corresponded to the average of 10 stimuli, and the maximum response was considered as 100% (panel B). In both panels, the rats with sciatic nerve ligation (CCI) were compared to the rats undergoing surgery without ligation of the sciatic nerve (Sham). The data are expressed as an average of six rats ± S.E.M. The significant difference was determined by ANOVA Two-Way, multiple comparisons (***$p \leq 0.001$, $F_{1\text{-}60} = 1056$) comparing the CCI *vs.* Sham rats.

Figure 8 shows the dose-response curves of the antiallodynic and antihyperalgesic effects administered at logarithmic doses of **LMH-2** (0.1, 1.0, 3.2, 10.0, 31.6, and 100.0 mg/kg, s.c.) and gabapentin (**GBP)** (5.6, 10.0, 17.8, 31.6, 56.2 and 100.0 mg/kg, s.c.).

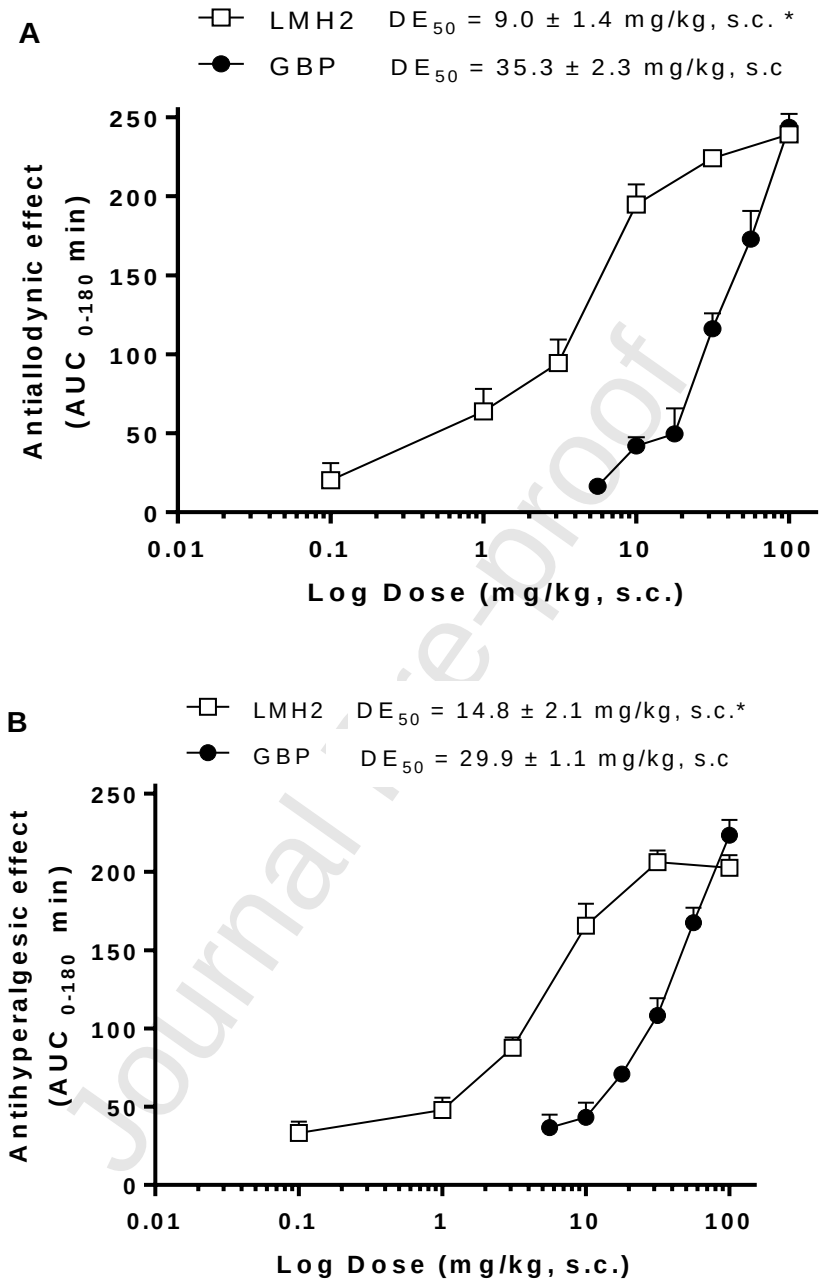

Figure 8. Dose-response curve of the antiallodynic effect (A) and antihyperalgesic (B) effects of the administration of **LMH-2** (0.1-100.0 mg/kg, s.c.) and gabapentin (**GBP**, 5.6-100.0 mg/kg, s.c.). The area under the curve (AUC) for each of the drug was calculated of the time curse (antiallodynic or antihyperalgesic effects *vs* 0-180 min), AUC was determined by trapezoidal rule. Each experimental point is shown as the % response of the average of 6 experimental animals ± S.E.M. The ED_{50} was determined using the linear and logarithmic method, the significant difference between both values was established by Student´s t-test (*p≤0.0.5).

4. DISCUSION

4.1 In silico predictions

In tables 2 and 3, it is observed that **LMH-2** has a better qualification with respect to the standard drugs, in addition to not having mutagenic or tumorigenic properties as does **S1RA,** with mutagenic properties probably conferred by the naphthyl fragment of the molecule. Also **LMH-2** undergoes intestinal absorption in humans and could cross the blood-brain barrier. This fact is of great importance for this project since it is desired that the compounds reach the CNS. However, according to the program data, this compound could be a substrate of the P glycoprotein (unfavorable data for the compounds). This protein is widely distributed in the cells of the intestinal epithelium, and it is responsible for expelling previously absorbed xenobiotics towards the intestinal lumen. It creates an efflux for the expulsion of its substrate that is dependent on ATP. Regarding metabolism, the program suggests that **LMH-2** could be metabolized by the CYP2D6 and CYP3A4 isoforms. It is worth mentioning that it is preferable to have a compound as a substrate of several isoforms or only one isoform. Its metabolism or excretion could be reduced or inhibited by another xenobiotic, increasing possible toxic effects.

In table 4, we can observe that he *in silico* calculation of inhibition for the five main isoforms of CYP450 (3A4, 2D6, 2C9, 2C19 and 1A2) for **LMH-2** were comparable to Haloperidol at relevant clinical concentrations (<10 μM), showing low probabilities of drug-drug interactions and undesirable adverse effects. Also, **LMH-2** had a low probability of being cardiotoxic, with less than 15% of the hERG potassium channel blockage in comparison to the compound **S1RA** and haloperidol, which had a high probability (66-90%) of being cardiotoxic. These results were presented at clinical and relevant concentrations of 10 μM. According to the data established by the program, **LMH-2** falls into category IV established by OECD, whereas haloperidol, which showed probable toxicity in acute administration, was in category III (LD_{50}).

4.2. Chemistry

Compound **LMH-2** was obtained with a high yield (95%) and it chemical structure was confirmed by spectroscopic (^{1}H and ^{13}C Nuclear magnetic resonance) and spectrometric data by electronic impact of the sample.

4.3. In vitro σ_1R affinity

LMH-2 showed promising σ_1R affinity of 6.0 nM, whereas the σ_2R affinity was 190 nM, exhibiting outstanding 31.6-fold selectivity. In competitive radioligand receptor binding studies, a K_i–value of 6.0 nM (pK_i = 8.22) was determined for the σ_1R affinity of **LMH-2**, whereas (+)-pentazocine and haloperidol showed the same σ_1R affinity, with K_i–values of 5.7 and 6.3 nM, respectively. However, **S1RA**, which is in phase II clinical trials, revealed nearly 3-fold lower σ_1R affinity [K_i = 17 nM (pK_i of 7.73)] than **LMH-2** and haloperidol.

4.4. Molecular docking on the σ_1 receptor

The interaction of **LMH-2** with the σ_1 receptor is showed in Figure 6. The contacts between the protonated amine from the piperidine heterocycle and residue Glu172 was observed, as well as the π–π interaction between residue Tyr103 and fluorobenzoyl moiety. For haloperidol, the binding energy (ΔG) calculated by molecular docking was -11.1 Kcal/mol with a predicted K_i of 6.82 nM (experimental K_i = 6.3 nM). In the case of **S1RA**, a 3-D interaction between the ionizable nitrogen of morpholine and the amino acid residue Glu172 was also observed. The binding energy was calculated as -10.76 Kcal/mol and a K_i of 12.14 nM (experimental K_i =17 nM).

*4.5. In vivo antiallodynic and antihyperalgesic assay for **LMH-2***

These results agree with those previously reported, where acetone and the 15 g von Frey filament have been used to generate allodynia and hyperalgesia [21]. It has been documented that the behavioral response observed in this experimental model is generated by a cascade of events that initiate damage to the peripheral nerves, particularly tactile allodynia, which is associated with the ascending fiber system, while the persistence of allodynia and hyperalgesia depends on the descending pathway from the rostral ventral cord. Apparently, the ectopic discharges generated by the nerve ligation that correspond to a

reorganization of ion channels such as Na^+ and Ca^{2+} are responsible for the hypersensitivity generated in the animal [27]. It has also been documented that in this model, an inflammatory phenomenon is generated in which cytokines such as IL-1β and TNF-α [28], as well as NKI and AMPA receptors, participate in nerve degeneration and sensitization at the central level [29, 30]. There is little information related to the participation of σ_1R antagonists in the modulation of neuropathic pain. Some σ_1R antagonist ligands related to the antiallodynic and antihyperalgesic effects have been reported; for example, compound 3-(6',7'-dihydro-1'*H*-spiro [piperidine-4,4'-pyrano [4,3-c]pyrazole]) was evaluated in the capsaicin test, and (+)-MR200 was evaluated in the formalin test [31,32]. A group of perhydroquinoxalines was designed to act on the σ_1R; however, they could not cross the blood-brain barrier, and their antiallodynic and antihyperalgesic effects were attributed to peripheral κ-opioid agonism [33]. Recently, in our working group, we designed the compound 2-(3,4-dichlorophenoxy)-*N*-(2-morpholin-4-yl-ethyl)acetamide, which showed high affinity for the σ_1R (*K*i = 42 nM). This compound showed activity in a model of inflammatory pain at the peripheral and spinal levels, while another of the compounds *N*-(2-morpholin-4-yl-ethyl)-2-(1-naphthyloxy)acetamide showed antihyperalgesic and antiallodynic effects in the CCI model. Because the effects were reversed by the agonists σ_1 (+)-pentazocine and PRE-084, the participation of the σ_1R was assumed [20, 34]. The fact that **LMH-2** possesses antihyperalgesic and antiallodynic effects contributes to the knowledge that antagonists of σ_1R may be of therapeutic utility for the treatment of neuropathic pain. In the present work, it could not be established if the effect of **LMH-2** was reversed by a σ_1R agonist; thus, the true interactions with this receptor remains to be established. However, it has been documented that the antinociceptive activity of haloperidol is due to the activation of σ_1R rather than dopaminergic activity [6]. Since **LMH-2** is a haloperidol analog, it could also interact only at σ_1R [33], but more evidence of its transduction mechanism is required. The most widely used drugs in the clinic for the treatment of neuropathic pain are pregabalin and gabapentin (**GBP**), and these do not eliminate pain. Clinically, they have high antiallodynic and antihyperalgesic efficacies compared with efficacies of other alternatives, such as anticonvulsants, anesthetics, and benzodiazepines [35, 36]. For this reason, in the present work, the antiallodynic

and antihyperalgesic effects of **LMH-2** were compared with those obtained with gabapentin. In Figure 8, the effects of **LMH-2** expressed as AUC were compared with those of gabapentin using the same route of administration (s.c.). It can be established that the effect of **LMH-2** and gabapentin is dose-dependent because there is a significant difference between doses evaluated (one-way ANOVA followed by Tukey´s post hoc ($p \leq 0.05$)), both in the antihyperalgesic and antiallodynic effects.

On the other hand in figure 8, GBP (100 mg/kg, s.c.) showed greater efficacy antihyperalgesic (95.9 ± 3.8%) and antiallodynic (90.23 ± 4.04%); whereas, **LMH-2** at the same dose generated less antihyperalgesic (66.02 ± 6.9%) and antiallodynic (78.37 ± 4.6%) response. However, when comparing the potency of the effects it can be seen that the new designed compound **LMH-2** is more potent than gabapentin because its ED_{50} is lower, both in the antihyperalgesic effect (**LMH-2**, DE_{50} = 14.8 ± 2.1 mg/kg and GBP, DE_{50} = 29.9 ± 1.1 mg/kg) as well in the antiallodynic effect (**LMH-2**, ED_{50} = 9.0 ± 1.4, GBP, ED_{50} = 35.3 ± 2.3 mg/kg) this means that a lower dose is required for **LMH-2** generate the same antihyperalgesic and antiallodynic response as gabapentin in the ICC model. These results together suggest that **LMH-2** is a new chemical entity with potential therapeutic utility in the treatment of neuropathic pain, almost 2 times more potent than gabapentin. It is important to mention that it is necessary to continue exploring the pharmacological activity of this compound, not only in the CCI model but also in other models of nociception. In addition, it is important to characterize its transduction mechanism, as well as determine the adverse effects and the short- and long-term toxicity.

5. CONCLUSIONS

The novel haloperidol analog **LMH-2** was obtained in one step with excellent yield (95%). **LMH-2** showed adequate *in silico* pharmacokinetic behavior and reasonable metabolic and toxicological properties, and low toxicity was calculated with good drug-like qualifications. In the *in vitro* competitive binding assay against σ_1R, **LMH-2** had a K_i-value of 6 nM, being equipotent with haloperidol but three times more potent than S1RA (K_i = 17 nM). Moreover, **LMH-2** displayed favorable σ_1R/σ_2R selectivity. *In vivo*, **LMH-2** produced dose-dependent antihyperalgesic and antiallodynic effects and showed similar

antinociceptive efficacy, but with more potency than gabapentin, in a model of CCI in the rat; thus, it is suggested that this compound could be acting as a σ_1R antagonist. However, it is necessary to further analyze its pharmacological potential as a possible therapeutic alternative for the treatment of neuropathic pain.

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References

[1] E.J. Cobos, J. M. Entrena, F. R. Nieto, C.M. Cendán, E. Del Pozo, Pharmacology and therapeutic potential of sigma(1) receptor ligands. Curr Neuropharmacol. 6 (2008) 344-366.

[2] M.P. Davis, Sigma-1 receptors and animal studies centered on pain and analgesia. Expert Opin Drug Discov. 10 (2015) 885-900.

[3] G. Alonso, V. Phan, I. Guillemain, M. Saunier, A. Legrand, Immunocytochemical localization of the sigma(1) receptor in the adult rat central nervous system. Neuroscience 97(2000) 155–170.

[4] C.M. Cendan, J.M. Pujalte, E. Portillo-Salido, L. Montoliu. J.M. Baeyens Formalin-induced pain is reduced in sigma(1) receptor knockout mice. Eur J Pharmacol. 511 (2005) 73–74.

[5] J.M. Entrena, E.J. Cobos, F.R. Nieto, C.M. Cendán, J.M. Baeyens, E. Del Pozo, Antagonism by haloperidol and its metabolites of mechanical hypersensitivity induced by intraplantar capsaicin in mice: role of sigma-1 receptors. Psychopharmacology 205 (2009) 21-33.

[6] J.V. Espinosa-Juárez, O.A. Jaramillo-Morales, F.J. López-Muñoz, Haloperidol Decreases Hyperalgesia and Allodynia Induced by Chronic Constriction Injury. Basic Clin Pharmacol Toxicol.121 (2017) 471-479.

[7] C. Kibaly, L. Meyer, C. Patte-Mensah, A.G. Mensah-Nyagan, Biochemical and functional evidence for the control of pain mechanisms by dehydroepiandrosterone endogenously synthesized in the spinal cord. FASEB J. 22 (2008) 93-104.

[8] D.H. Roh, H. W. Kim, S. Y. Yoon, H. S. Seo, Y. B. Kwon, K. W. Kim, H.J. Han, A.J. Beitz, H.S. Na, J.H. Lee, Intrathecal injection of the sigma(1) receptor antagonist BD1047 blocks both mechanical allodynia and increases in spinal NR1 expression during the induction phase of rodent neuropathic pain. Anesthesiology. 109 (2008) 879-889.

[9] J.S. Son, Y.B. Kwon, Sigma-1 Receptor Antagonist BD1047 Reduces Allodynia and Spinal ERK Phosphorylation Following Chronic Compression of Dorsal Root Ganglion in Rats.
Korean J Physiol Pharmacol. 14 (2010) 359-64.

[10] C. Sánchez-Fernández, J.M. Entrena, J.M. Baeyens, E.J. Cobos, Sigma-1 Receptor Antagonists: A New Class of Neuromodulatory Analgesics. Adv Exp Med Biol. 964 (2017) 109-132.

[11] Y. Shir, Z. Shenkman, L. Kaplan, Neuropathic pain unrelieved by morphine, alleviated by haloperidol. Harefuah, 118 (1990) 452-454.

[12] S. Negro, A. Martín, M.L. Azuara, Y. Sánchez, E. Barcia, Stability of tramadol and haloperidol for continuous subcutaneous infusion at home. J Pain Symptom Manage. 30 (2005) 192-199.

[13] S.A. Ahmadi, M. Sabahi, R. Haddadi, The effect of acute and repeated administration of buspirone, 8-OHDPAT and fluoxetine on haloperidol-induced extrapyramidal symptoms. Neuropsychopharmacol Hung. 21(2019) 59-68.

[14] H.A. Nasrallah, A.T. Chen, Multiple neurotoxic effects of haloperidol resulting in neuronal death. Ann Clin Psychiatry. 29 (2017) 195-202.

[15] Z. Halici, H. Dursun, O.N. Keles, E. Odaci, H. Suleyman, N. Aydin, E. Cadirci, Y. Kalkan, B. Unal, Effect of chronic treatment of haloperidol on the rat liver: a stereological and histopathological study. Naunyn Schmiedebergs Arch Pharmacol. 379 (2009) 253-61.

[16] R. A. Glennon, Pharmacophore identification for sigma-1 (sigma1) receptor binding: application of the "deconstruction-reconstruction-elaboration" approach. Mini Rev Med Chem. 5 (2005) 927-940.

[17] E. Laurini, V. Da Col, B.Wünsch, S.Pricl, Analysis of the molecular interactions of the potent analgesic S1RA with the σ1 receptor. Bioorg Med Chem Lett. 23 (2013) 2868-2871.

[18] J. Bruna, S. Videla, A. A. Argyriou, R. Velasco, J. Villoria, C.Santos, et al. Efficacy of a novel sigma-1 receptor antagonist for oxaliplatin-induced neuropathy: a randomized, double-blind, placebo-controlled phase IIa clinical trial. Neurotherapeutics 15 (2018) 178–189.

[19] C. Meyer, B. Neue, D. Schepmann, S. Yanagisawa, J. Yamaguchi, E.U. Würthwein, K. Itami, B. Wünsch, Improvement of s1 receptor affinity by late-stage CH-bond arylation of spirocyclic lactones, Bioorg. Med. Chem. 21 (2013) 1844– 1856.

[20] G. Navarrete-Vázquez, A. Austrich-Olivares, B. Godínez-Chaparro, S. Hidalgo-Figueroa, S. Estrada-Soto, E. Hernández-Núñez, H. Torres-Gómez, D. Schepmann, B. Wünsch, Discovery of 2-(3,4-dichlorophenoxy)-N-(2-morpholin-4-ylethyl)acetamide: A selective σ1 receptor ligand with antinociceptive effect. Biomed Pharmacother. 79 (2016) 284-293.

[21] D.S. Goodsell, A.J. Olson, Automated docking of substrates to proteins by simulated annealing. Proteins 8 (3) (1990) 195-202.

[22] Molecular Operating Environment (MOE), 2018.01; Chemical Computing group ULC, 1010 Sherbooke St. West, Site #910, Montreal, QC, Canada, H3A 2R7, (2019) http://www.chemcomp.com

[23] H. R. Schmidt, S. Zheng, E. Gurpinar, A. Koehl, A. Manglik, A.C. Kruse, Crystal structure of the human sigma 1 receptor. Nature, 532 (2016), 527-530.

[24] F. Cheng, W. Li, Y. Zhou, J. Shen, Z. Wu, G. Liu, P.W. Lee, Y. Tang, AdmetSAR: a comprehensive source and free tool for assessment of chemical ADMET properties J. Chem. Inf. Model. 52 (2012) 3099-3105.

[25] B. Colín-Lozano, S. Estrada-Soto, F. Chávez-Silva, A. Gutiérrez-Hernández, L. Cerón-Romero, A. Giacoman-Martínez, J. C. Almanza-Pérez, E. Hernández-Núñez, Z. Wang, X. Xie, M. Cappiello, F. Balestri, U. Mura, G. Navarrete-Vazquez, Design, Synthesis and in Combo Antidiabetic Bioevaluation of Multitarget Phenylpropanoic Acids. Molecules 23 (2018) 340.

[26] M. De la O-Arciniega, M.I. Díaz-Reval, A.R. Cortés-Arroyo, A.M. Domínguez-Ramírez, F.J. López-Muñoz, Anti-nociceptive synergism of morphine and gabapentin in neuropathic pain induced by chronic constriction injury. Pharmacol Biochem Behav. 92 (2009) 457-464.

[27] Y. Wan Involvement of hyperpolarization-activated, cyclic nucleotide-gated cation channels in dorsal root ganglion in neuropathic pain. Sheng Li Xue Bao 60 (2008) 579-580.

[28] Q.Y. Li, H.Y. Xu, H.J. Yang, Effect of proinflammatory factors TNF-α,IL-1β, IL-6 on neuropathic pain. Zhongguo Zhong Yao Za Zhi. 42 (2017) 3709-3712.

[29] L. Martinez-Caro, J.M. Laird, Allodynia and hyperalgesia evoked by sciatic mononeuropathy in NKI receptor knockout mice. Neuroreport. 11 (2000) 1213-1217.

[30] Y. Chen, V.A. Derkach, P.A. Smith, Loss of Ca(2+)-permeable AMPA receptors in synapses of tonic firing substantia gelatinosa neurons in the chronic constriction injury model of neuropathic pain. Exp Neurol. 279 (2016) 168-177.

[31] T. Schläger, D. Schepmann, K. Lehmkuhl, J. Holenz, J. M. Vela, H. Buschmann, B. Wünsch, Combination of two pharmacophoric systems: synthesis and pharmacological evaluation of spirocyclic pyranopyrazoles with high σ_1 receptor affinity. J Med Chem. 54 (2011) 6704-6713.

[32] C. Parenti, A. Marrazzo, G. Aricò, R. Parenti, L. Pasquinucci, S. Ronsisvalle, G. Ronsisvalle, G.M. Scoto, The antagonistic effect of the sigma 1 receptor ligan d (+)-MR200 on persistent pain induced by inflammation. Inflamm Res. 63 (2014) 231-237.

[33] C. Bourgeois, E. Werfel, F. Galla, K. Lehmkuhl, H. Torres-Gómez, D. Schepmann, B. Kögel, T. Christoph, W. Straßburger, W. Englberger, M. Soeberdt, S. Hüwel, H.J. Galla, B. Wünsch, Synthesis and pharmacological evaluation of 5-pyrrolidinylquinoxalines as a novel class of peripherally restricted κ-opioid receptor agonists. J Med Chem. 57 (2014) 6845-6860.

[34] J. V. Espinosa-Juárez, O. A. Jaramillo-Morales, G. Navarrete-Vázquez, L. A. Melo-Hernández, M. Déciga-Campos, F. J. López-Muñoz, N-(2-morpholin-4-yl-ethyl)-2-(1-naphthyloxy) acetamide inhibits the chronic constriction injury-generated hyperalgesia via the antagonism of sigma-1 receptors. Eur J Pharmacol. 812 (2017) 1-8.

[35] B.A. García-Martínez, O.A. Jaramillo-Morales, J.V. Espinosa-Juárez, G. Navarrete-Vázquez, L.A. Melo-Hernández, J. R. Medina-López, A. M. Domínguez-Ramírez, D. Schepmann, B. Wünsch, F.J. López-Muñoz, Antinociceptive effects of a new sigma-1 receptor antagonist (N-(2-morpholin-4-yl-ethyl)-2-(1-naphthyloxy)acetamide) in two types of nociception. Eur J Pharmacol. 771 (2016) 10-17.

[36] J.F. Horga de la Parte, A. Horga, Pregabalin: new therapeutic contributions of calcium channel alpha2delta protein ligands on epilepsy and neuropathic pain. Rev Neurol. 42 (2006) 223-237.