ChemistrySelect

Research Article
doi.org/10.1002/slct.202400417

Chemistry Europe
European Chemical Societies Publishing

www.chemistryselect.org

Synthesis and Anti-Hyperalgesic Efficacy of MP-103, a Non-Racemic Enantiomeric Mixture of a New 1,4-Diazabicyclo[4.3.0]nonan-9-one

Laura Micheli,*[a] Alessandra Toti,[a] Lorenzo Di Cesare Mannelli,[a] Carla Ghelardini,[a] Letizia Crocetti,[b] Carlo Farina,[c] and Michael Scherz[c]

With the aim to identify novel and improved drug candidates for the non-opioid management of neuropathic pain, a few chiral fluorobenzenesulfonylamide derivatives of 1,4-diazabicyclo[4.3.0]nonan-9-one, a rigid bicyclic analogue of piracetam, were prepared and characterized in animal models of chemotherapy-induced neuropathic pain. The *R*-enantiomers of these novel compounds are generally more potent than their corresponding *S*-enantiomers. An oral dose of *R*-2-fluorophenyl derivative **8a** is better tolerated when compared to the *R*-3-fluorophenyl derivative **9a**, (mouse Rota-Rod test). Consequently, the enantiomeric 2-fluorophenyl derivatives (**8a** and **8b**) are thoroughly investigated in an enlarged panel of inflammatory and neuropathic pain models, including several models of chemotherapy-induced neuropathic pain. The *R*-enantiomer (**8a**) is consistently more potent in its anti-hypersensitivity profile than the *S*-enantiomer (**8b**). Surprisingly, the non-racemic enantiomeric mixture consisting of a 2-to-1, or better still, a 3-to-1 mixture of the *R*-enantiomer (**8a**) over the *S*-enantiomer (**8b**) is more potent than the *R*-enantiomer (**8a**) alone or than their racemic mixture. These results are reminiscent of our previous report on MP-101, a non-racemic mixture of dimiracetam enantiomers. Although further investigations will be required to rationalize these findings at the pharmacokinetic or molecular level, racetam derivatives appear to be promising candidates for the management of persistent pain.

1. Introduction

Neuropathic pain is among the most difficult-to-treat types of pain and there remains yet today a significant unmet medical need for novel non-opioid treatment options.[1] The current recommendations for the pharmacological treatment of neuropathic pain were published by Neuropathic Pain Special Interest Group (NeuPSIG) of the International Association for the Study of Pain (IASP) and, although the guidelines suggest multiple therapeutic interventions, first-line drugs still today provide unsatisfactory pain relief in the clinic,[2] thus leading to the quest for new therapeutic approaches.

The role of glutamate receptors, especially the ionotropic subtype of glutamate receptors, in pathological conditions such as chronic pain or neuropathic pain has been recently reviewed.[3] Pharmacological, electrophysiological, and behavioral evidence supports the notion that ionotropic glutamate receptors play a crucial role in signaling pathways of neuropathic and inflammatory pain, and that their modulation may be useful in the treatment of these pathologies.[4]

Nootropic drugs, also called racetams, comprise a series of derivatives of γ-aminobutyric acid whose prototype, piracetam, was identified by Giurgea.[5,6] Historically they have been used to improve learning and memory, or in the treatment of epilepsy. Recent studies have shown that certain nootropic drugs such as dimiracetam,[7,8] nefiracetam[9] or levetiracetam,[10–12] may be useful to relieve neuropathic pain both in animal models and in patients.[13] Although their molecular mechanism of action is still poorly defined and their pharmacology appears to change significantly with minor chemical modifications,[13] it appears that racetams' interactions with ionotropic glutamate receptors may have a significant impact on their ability to modulate spinal and brain pre- and post-synaptic neurotransmission via α-amino-3-hydroxy-5-methyl-4-isoxazole-propionate (AMPA) and/or N-methyl-D-aspartate (NMDA) receptors.

We have previously described a surprising synergistic interaction of the individual enantiomers of dimiracetam to counteract NMDA + glycine-triggered release of pre-loaded [^{3}H]-D-aspartate from spinal cord synaptosomal preparations, possibly by negatively modulating specific subtypes of the NMDA receptor.[7] We found that certain ratios of enantiomers were more potent than either the racemic mixture or the individual enantiomers alone. This pattern was also observed in multiple in vivo models of pain, including chemotherapy-induced neuro-

---

[a] L. Micheli, A. Toti, L. Di Cesare Mannelli, C. Ghelardini
Department of Neuroscience, Psychology, Drug Research and Child Health, NEUROFARBA-Pharmacology and Toxicology Section, University of Florence, Viale G. Pieraccini 6, Florence 50139, Italy
E-mail: laura.micheli@unifi.it

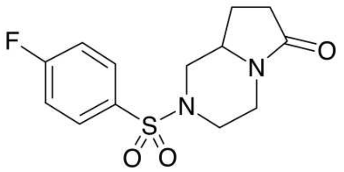
[b] L. Crocetti
Department of Neuroscience, Psychology, Drug Research and Child Health, NEUROFARBA-Pharmaceutical and Nutraceutical Section, University of Florence, Via Ugo Schiff 6, Sesto Fiorentino, Florence 50019, Italy

[c] C. Farina, M. Scherz
Metys Pharmaceuticals c/o Novaremed AG, Thiersteinerallee 17, Basel 4053, Switzerland

pathic pain. We have given the name MP-101 to the 3:1 mixture of dimiracetam enantiomers, and we have reasoned that MP-101's reversal of excitatory transmission-sustained wind-up may be responsible for breaking the reinforcing pain perception in several neuropathic conditions.[8,14]

Looking for structurally similar and less extensively explored compounds, we were attracted by a novel and potentially interesting series of 1,4-diazabicyclo[4.3.0]nonan-9-one derivatives which displayed a very potent nootropic activity in a scopolamine-induced passive avoidance test in mice,[15,16] and whose effects were proposed to be exerted, at least in part, through the NMDA receptor.

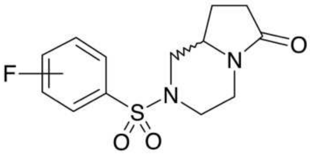
In particular, DM-232 (**1**) (see the chemical structure below) was particularly attractive owing to either its very high potency as a cognition enhancer, with a minimally effective dose of 1–10 μg kg^{-1} s.c. in the mouse passive avoidance test, and to its close relationship with piracetam nootropics, as it possessed a 2-pyrrolidinone ring fused into the 1,4-diazabicyclo[4.3.0]-nonane system.[17]



DM-232 (**1**)

As the data available in the literature only concerned the racemic derivative DM-232, we decided to investigate whether the separated *R*- and *S*-enantiomers of **1**, and of its 2- and 3-fluorophenyl isomeric analogues (Table 1) were endowed with anti-neuropathic properties like those we had previously characterized with dimiracetam and MP-101.[7]

Table 1.
Physicochemical data.



$[\alpha]_D$ c = 1, CH_2Cl_2	m.p., °C	Chirality	R	No
+36.34	136–137	*R*	2-F	**8a**
−40.3	142–144	*S*	2-F	**8b**
+43.5	179–180	*R*	3-F	**9a**
−45.10	177–179	*S*	3-F	**9b**
+41.40	158–159	*R*	4-F	**10a**
−47.20	159–160	*S*	4-F	**10b**

2. Results and Discussion

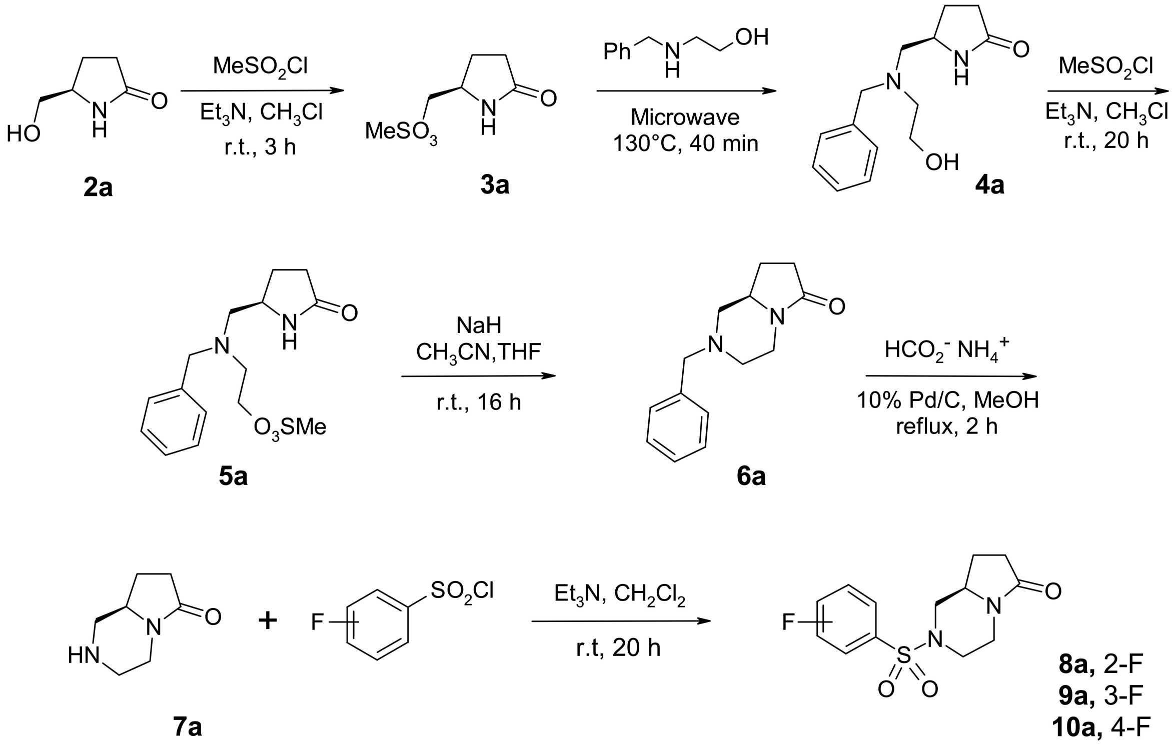
The stereospecific synthetic procedure for the preparation of several (*R*)- or (*S*)-2-(aryl or heteroary/sulfonyl) hexahydropyrrolo[1,2-alpyrazin-6(7H)-one that had been previously reported[18] was used to prepare the *R*- and *S*-enantiomers of the three isomeric 2-, 3- or 4-fluorobenzylsulfonamide derivatives (Scheme 1). The synthesis started from the commercially available 5-hydroxymethylpyrrolidin-2-ones **2a** (*R*-enantiomer) and **2b** (*S*-enantiomer). These were mesylated to **3a** or **3b**, respectively, and reacted with N-benzylethanolamine under microwave irradiation[19] at 130 °C to provide the ethanolamine derivatives **4a** and **4b**. They were readily cyclized to the desired 2-benzylhexahydropyrrolo[1,2-a]pyrazin-6(2H)-ones (**6a** or **6b**, respectively) by mesylation of the hydroxyl group followed by treatment with sodium hydride in acetonitrile.

Removal of the benzyl group with ammonium formate in the presence of 10% palladium on charcoal catalyst afforded the two enantiomeric intermediates **7a** and **7b**, respectively. Final reaction with 2-fluoro-, 3-fluoro- or 4-fluorophenylsulfonyl chloride provided the desired compounds **8a**, **8b**, **9a**, **9b**, **10a**, and **10b**, respectively (see spectra, Figures S3–S8, in Supporting Information). Their main physicochemical parameters, i.e. melting point and optical rotatory power, are compared in Table 1.

The single-dose pharmacological profile of the six compounds was initially investigated in a paclitaxel-induced neuropathic pain model, comparing the activity of the three enantiomeric couples at a standard dose of 3 mg kg^{-1} i.v. (Supplementary Figure S1).

The rat model chosen has been designed to mimic the pain threshold alterations seen in the human condition following repeated paclitaxel treatments.[20] Repeated paclitaxel injections alone severely affected rats' pain threshold, reducing the weight tolerated on the posterior paw in comparison to control rats (vehicle + vehicle group) when a mechanical noxious stimulus was applied. In rats with established paclitaxel-induced hyperalgesia, treatment with 2-fluorophenyl derivative **8a** resulted in a transient restoration of the paw-withdrawal threshold, with a maximal effect (from 32.5 ± 3.1 g at baseline up to 53.3 ± 2.7 g) 30 min after injection. Its *S* enantiomer, compound **8b**, was inactive at the administered dose (Supplementary Figure S1a). A similar profile was observed with 3-fluorophenyl derivatives **9a** and **9b**, with the difference that the *S*-enantiomer was not completely inactive (Supplementary Figure S1b). The 4-fluorophenyl enantiomers **10a** and **10b** were equally active, but their maximal treatment effect at this dose was lower when compared to the 2-fluorophenyl (**8a**) and 3-fluorophenyl (**9a**) derivatives (Supplementary Figure S1c).

Following oral administration of the 2-fluorophenyl or 3-fluorophenyl derivatives **8a** and **9a** at doses between 100 mg kg^{-1} and 1000 mg kg^{-1} in the mouse Rota Rod test, compound **8a** evoked a lower number of falls of the mice from the rotating rod in comparison to compound **9a** (Supplementary Figure S2). The *R*-enantiomer **8a** was therefore chosen for more extensive assessment of its anti-hyperalgesic and anti-allodynic effects in an enlarged panel of in vivo experiments entailing the determination of oral ED_{50} in rat models of



Scheme 1. Synthetic scheme for the preparation of the *R*-enantiomers **8a**, **9a** and **10a** from (*R*)-(-)-5-(hydroxymethyl)pyrrolidin-2-one (**2a**); the *S*-enantiomers **8b**, **9b** and **10b** were prepared in a similar way starting from (*S*)-(-)-5-(hydroxymethyl)pyrrolidin-2-one (**2b**)

neuropathic pain evoked by trauma (CCI model), by chemotherapeutics drugs (taxol, oxaliplatin and vincristine), by streptozotocin-induced metabolic disease (STZ model) and by monoiodoacetate-induced osteoarthritis (MIA model).

Figure 1 shows the anti-allodynic effect of increasing single oral doses of **8a** in the CCI and the STZ models. The trauma caused by the loose ligation of the sciatic nerve significantly reduced the weight borne by the animals on the ipsilateral paw in comparison to the vehicle + vehicle group (Figure 1a; mechanical von Frey test). Treatment with **8a** at the doses of 3 and 10 $mg\,kg^{-1}$ exerted a pain-relieving effect, reducing the hypersensitivity from 30 to 90 minutes after treatment (Figure 1a), without affecting the paw-withdrawal threshold of the contralateral paw (data not shown) unlike what happens when an opioid analgesic is used. The lower dose of 1 $mg\,kg^{-1}$ was still active albeit for a shorter period of time. A long-lasting anti-

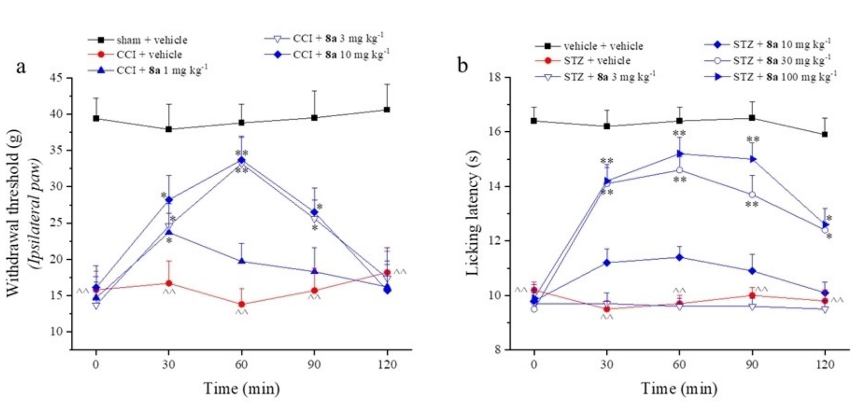


Figure 1. Anti-allodynic and anti-hyperalgesic profile of **8a** in a trauma and metabolic model of neuropathic pain. a) Chronic Constriction Injury (CCI) was used as a rat model of trauma-induced neuropathic pain. Fourteen days after damage, **8a** (1–10 $mg\,kg^{-1}$) was suspended in 1 % CMC and administered by oral gavage. Mechanical allodynia was assessed by the von Frey test before and 30, 60, 90 and 120 minutes after treatments. Each value represents the mean ± S.E.M. of 8 rats. Statistical analysis was one-way ANOVA followed by Bonferroni's post-hoc comparison. ^^$P < 0.01$ vs sham + vehicle; *$P < 0.05$ and **$P < 0.01$ vs CCI + vehicle. b) Streptozotocin (STZ, 100 $mg\,kg^{-1}$, i.v.) was used as a mouse model of diabetes-induced neuropathic pain. Twenty-one days after STZ injection, **8a** (3–100 $mg\,kg^{-1}$) was suspended in 1 % CMC and administered by oral gavage. Thermal hyperalgesia was assessed by the Hot plate test before and 30, 60, 90 and 120 minutes after treatments. Each value represents the mean ± S.E.M. of 8–12 mice. Statistical analysis was one-way ANOVA followed by Bonferroni's post-hoc comparison. ^^$P < 0.01$ vs vehicle + vehicle; *$P < 0.05$ and **$P < 0.01$ vs STZ + vehicle.

hypersensitive effect of **8a** was also recorded in the STZ model: the highest doses tested (30 mg kg^{-1} and 100 mg kg^{-1}) increased the licking latency of the animals in the Hot plate test up to 120 minutes after treatment with a peak of efficacy recorded between 60 and 90 minutes after oral administration. However, the doses of 3 and 10 mg kg^{-1} were ineffective (Figure 1b).

To continue the pharmacological characterization of the *R*-enantiomer **8a**, its efficacy was evaluated in two additional rat models of chemotherapy-induced neuropathy that occurs as a collateral effect after treatment with chemotherapeutic drugs. Both oxaliplatin and vincristine significantly decreased the pain threshold of the rats, strongly affecting their performance when challenged with a noxious mechanical stimulus in the Paw pressure test (Figure 2). In both models, the acute oral treatment with compound **8a** significantly increased the weight borne by the animals on the posterior paw in a dose-dependent manner, counteracting mechanical hyperalgesia with a peak of efficacy 30 minutes after treatment. In the oxaliplatin pain model, compound **8a** at 10 mg kg^{-1} fully counteracted mechanical hyperalgesia with an effect that lasted up to 90 minutes. The doses of 3 and 1 mg kg^{-1} showed a lesser but still significant anti-hyperalgesic activity while the lower dose of 0.3 mg kg^{-1} was inactive (Figure 2a). A similar trend was observed in vincristine-induced neuropathic pain model, where compound **8a** at the dose of 10 mg kg^{-1} possessed the best and long-lasting anti-hyperalgesic profile, whilst the lower doses of 1 and 3 mg kg^{-1} were still active although with a shorter and lower effect (Figure 2b).

The results obtained in these experiments are in line with previous results obtained with the nootropic drug candidates, dimiracetam and MP-101, the corresponding 3-to-1 *R*-to-*S* non-racemic mixture of its enantiomers. Both racemic dimiracetam and MP-101 displayed a broad spectrum of anti-hyperalgesic and anti-allodynic effects with a higher efficacy than the reference drug pregabalin[8] in different models of neuropathic pain induced by trauma,[21] antiretroviral[8] or chemotherapeutic drugs.[22,23] While the mechanism of action of racetams is not fully understood, it has been shown that they can interact with ionotropic glutamate receptors modulating neurotransmitters release from brain and spinal cord synaptosomes.[8] We can thus hypothesize also for this compound a pain relieving effect due to the modulation of NMDA receptors with a consequent reduction of glutamate release. What has been highlighted by a work of Bonifacino and colleagues is that the non-racemic enantiomeric mixture composed by 3:1 part of *R* and *S* dimiracetam respectively, was more potent in comparison to the racemic against neuropathic pain and its comorbidities.[7] We then evaluated in the model of paclitaxel-induced neuropathic pain whether the 1:1, 1:3, 2:1 and 3:1 *R*:*S* mixtures of the two enantiomers **8a** and **8b** behave similarly to that of dimiracetam and MP-101.

Acute intravenous administration of *R*-enantiomer **8a** at 1 mg kg^{-1} increased the paw withdrawal threshold of rats in response to a noxious mechanical stimulus (Paw pressure test). The treatment effect started within 15 minutes and up to 45 minutes after treatment; the highest effect was recorded at 30 minutes, reaching the value of 53.6 ± 1.3 g. The *S*-enantiomer **8b** was ineffective, as in the prior experiment (Supplementary Figure S1a). A statistically significant increase of the paw withdrawal threshold was also observed in rats treated with 1:1, 2:1 and 3:1 *R*:*S* enantiomeric mixtures. In particular 1 mg kg^{-1} of the 3:1 mixture of **8a** + **8b** equaled the anti-hyperalgesic effect of the *R*-enantiomer alone (Figure 3a) showing the same pain relieving profile in the paw pressure test. In the von Frey test, however, the anti-allodynic effect of the 3:1 *R*:*S* mixture was significantly greater than that of the *R*-enantiomer **8a** alone (Figure 3b). In the von Frey paradigm also the 2:1 *R*:*S* mixture was better than the *R*-enantiomer alone. In both settings, the 1:3 *R*:*S* mixture was ineffective (Figures 3a and b).

Finally, pure **8a** and the 1:1, 2:1 and 3:1 enantiomeric *R*:*S* mixtures were compared in a rat model of hyperalgesia induced

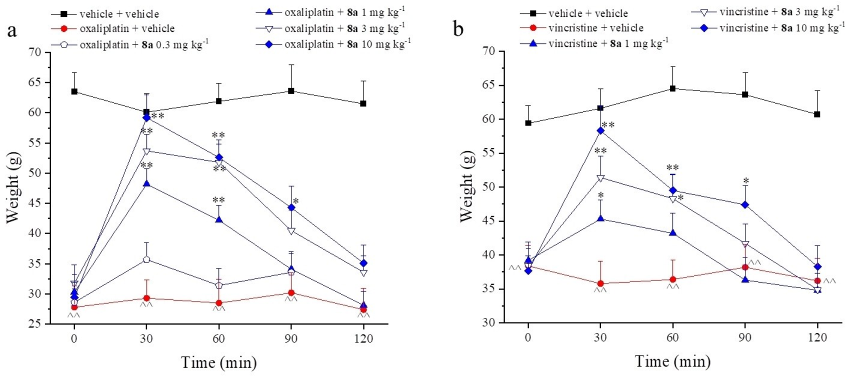


Figure 2. Anti-hyperalgesic profile of **8a** in a rat model of chemotherapy-induced neuropathic pain. a) Oxaliplatin (2.4 mg kg^{-1}, i.p.) was dissolved in 5% glucose solution and injected on days 1–3; 6–10; 13–15 for a total of 11 injections. On day 21, when neuropathy was well established, **8a** (0.3–10 mg kg^{-1}) was suspended in 1% CMC and *per os* administered. Mechanical hyperalgesia was assessed by the Paw pressure test before and 30, 60, 90 and 120 minutes after treatments. Each value represents the mean ± S.E.M. of 8 rats. Statistical analysis was one-way ANOVA followed by Bonferroni's post-hoc comparison. ^^$P < 0.01$ vs vehicle + vehicle; *$P < 0.05$ and **$P < 0.01$ vs oxaliplatin + vehicle. b) Vincristine (150 μg kg^{-1}, i.v.) was dissolved in saline and injected on five non-consecutive days (1, 3, 5, 8, 10). On day 11, **8a** (1–10 mg kg^{-1}) was suspended in 1% CMC and *per os* administered. Mechanical hyperalgesia was assessed by the Paw pressure test before and 30, 60, 90 and 120 minutes after treatments. Each value represents the mean ± S.E.M. of 8 rats. Statistical analysis was one-way ANOVA followed by Bonferroni's post-hoc comparison. ^^$P < 0.01$ vs vehicle + vehicle; *$P < 0.05$ and **$P < 0.01$ vs vincristine + vehicle.

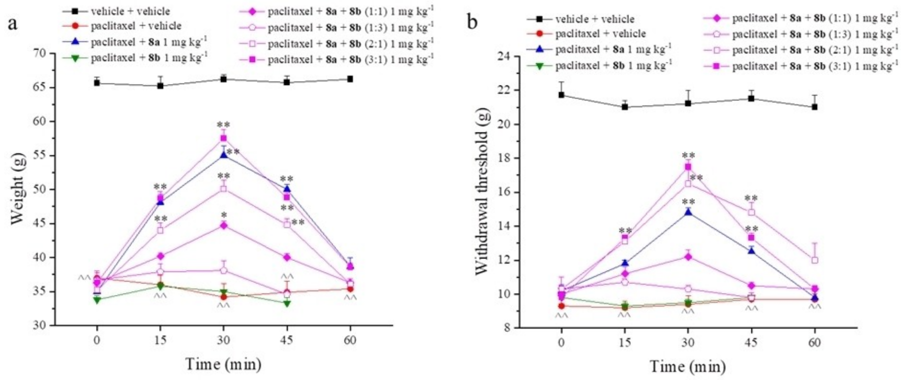


Figure 3. Anti-hyperalgesic effect of the *R* and *S* enantiomeric mixtures in a rat model of paclitaxel-induced neuropathic pain. Paclitaxel (2 mg kg^{-1}, i.p.) was dissolved in a mixture of 10% Cremophor EL in saline solution and injected on four alternate days (days 1, 3, 5 and 8). On day 14, when neuropathy was well established, **8a** and **8b** (1 mg kg^{-1}) and the **8a** + **8b** enantiomeric mixtures 1:1, 1:3, 2:1 and 3:1 at the final dose of 1 mg kg^{-1} were dissolved in saline/DMSO 2% and intravenously (i.v.) injected. a) Paw pressure test and b) von Frey test were used to assess mechanical hyperalgesia and allodynia, respectively, before and 15, 30, 45 and 60 minutes after treatments. Each value represents the mean ± S.E.M. of 6 rats. Statistical analysis was one-way ANOVA followed by Bonferroni's post-hoc comparison. ^^P < 0.01 vs vehicle + vehicle; *P < 0.05 and **P < 0.01 vs paclitaxel + vehicle.

by the intra-articular injection of MIA (Figure 4), which is intended to mimic the effects of human osteoarthritis. Prior to the treatment with the test compounds, the pain threshold was reduced in animals with monolateral pain (MIA + vehicle group) in comparison to the control animals (vehicle + vehicle). The *R*-enantiomer **8a** was able to revert this hyperalgesia in a statistically significant manner between 15 and 30 minutes after administration. The corresponding effect exhibited by the 3:1 *R*:*S* enantiomeric mixture of **8a** + **8b** was significantly higher than the effect of the *R*-enantiomer **8a** alone from 15 to 45 minutes after treatment. Treatment with **8a** + **8b** 2:1 mixture also induced a significantly greater anti-hyperalgesic effects, as compared to the single enantiomer administration. Again, the effects of the 2:1 *R*:*S* mixture were lower when compared to those recorded for the corresponding 3:1 mixture (Figure 4).

Also in the case of the von Frey test, the *R*-enantiomer **8a** induced a significant reduction of allodynia 15–30 minutes after administration. Again, administration of 3:1 and 2:1 *R*:*S* enantiomeric mixtures of **8a** + **8b** were able to increase the pain relief in a statistically significant way in comparison with the effects observed in animals treated with the *R*-enantiomer **8a** alone, at 15–45 minutes after the administration.

In this experimental paradigm the treatment with the racemic mixture (**8a** + **8b**, 1:1) led to statistically significant reversal of hyperalgesia and reduction of allodynia, as compared to the control group. However, in the Paw pressure test the mean values, determined at 15–45 minutes after administration of the racemate, were slightly lower than those recorded after the administration of *R*-enantiomer alone. A similar effect was observed for the racemic mixture at 15–30 minutes after treatment in the von Frey test.

Our results indicate that enantiomeric mixtures of **8a** and **8b** containing a 2:1 or a 3:1 ratio of the *R*-enantiomer as compared with *S*-enantiomer are more potent than either the racemic mixture or the pure *R*-enantiomer alone. We have given the name MP-103 to the 3:1 **8a**:**8b** mixture. The superior

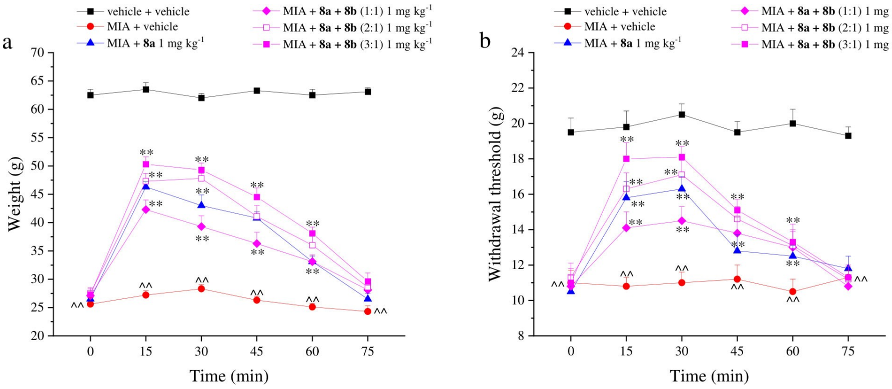


Figure 4. Anti-hyperalgesic effect of the *R* and *S* enantiomeric mixtures in a model of MIA-induced osteoarthritis. MIA (2 mg/25 μl) was dissolved in saline solution and intra-articularly injected on day 1. On day 14, when neuropathy was well established, **8a** (1 mg kg^{-1}) and the **8a** + **8b** enantiomeric mixtures 1:1, 2:1 and 3:1 at the final dose of 1 mg kg^{-1} were dissolved in saline/DMSO 2% and intravenously (i.v.) injected. a) Paw pressure test and b) von Frey test were used to assess mechanical hyperalgesia and allodynia, respectively, before and 15, 30, 45 and 60 minutes after treatments. Each value represents the mean ± S.E.M. of 6 rats. Statistical analysis was one-way ANOVA followed by Bonferroni's post-hoc comparison. ^^P < 0.01 vs vehicle + vehicle; **P < 0.01 vs MIA + vehicle.

potency of MP-103 appears to be more pronounced in the von Frey test, in both paclitaxel and MIA models.

This unexpected, potentiated effect between the enantiomers of compound **8** is similar to the effects observed with non-racemic mixtures of the dimiracetam enantiomers.[7] With dimiracetam, the 3:1 *R*:*S* mixture (MP-101) is significantly more potent than either of the individual enantiomers, and more potent than the racemic (1:1 *R*:*S*) mixture of dimiracetam. This was true in *in vitro* assays of $[^3H]$-Asp release from spinal synaptosomes induced by NMDA plus glycine, and in in vivo assays of pain, cognition and depression.[7] Although a full *in vitro* profile of these novel fluorosulfonyldiazabicyclo[4.3.0]-nonan-9-one derivatives is not yet available, previously published *in vitro* data show that DM-232 (**1**), affects excitatory neurotransmission by reversing the kynurenic acid antagonism of NMDA in hippocampal slices pre-loaded with $[^3H]$NE,[24] and without affecting the release of $[^3H]$NE induced by depolarizing stimuli other than NMDA receptor activation.[17] Also, as the DM-232 reversal of the kynurenic acid antagonism was prevented/reversed by NBQX, a possible involvement of AMPA receptors in the effects of DM-232 was suggested,[25,26] at least as far as the pro-cognitive activity was concerned. Whether the relative potencies of MP-103 and its individual enantiomers exhibit the same pattern in these *in vitro* systems as we report here in multiple in vivo settings, remains to be investigated.

3. Conclusion

Looking for novel lead compounds suitable for developing novel, more effective and safer anti-neuropathic agents, three novel chiral couples of fluorobenzenesulfonyl derivatives of 1,4-diazabicyclo[4.3.0]nonan-9-one have been synthesized and preliminarily characterized in a model of paclitaxel-induced mechanical hyperalgesia and allodynia in rodents. Based on these preliminary results and on their preliminary tolerability profile in the Rota rod and Irwin tests, the *R*-enantiomer of the 2-fluorophenyl derivative **8a** was extensively investigated and its anti-hyperalgesic and anti-allodynic activity was confirmed in different animal models of neuropathic pain (CCI, streptozotocin MIA oxaliplatin- and vincristine-induced models of persistent pain).

Most remarkably, we were also able to confirm that in two different paradigms, paclitaxel and MIA-persistent pain models, whilst racemic mixtures (**8a**+**8b** in a 1:1 ratio) were generally less effective than the *R*-enantiomer (**8a**) alone, enantiomeric mixtures of **8a**+**8b** in a *R*:*S* 2:1 or, better, 3:1 ratios were more effective than the *R*-enantiomer alone, especially when mechanical allodynia was used as an anti-neuropathic endpoint.

These results appear to confirm and strengthen our previous results obtained with MP-101, a similar enantiomeric mixture of three parts of *R*-dimiracetam and one part of *S*-dimiracetam, possibly suggesting that several and complex molecular interactions are involved in the mechanism of action of bicyclic nootropic (or racetam) compounds, at least as far as their anti-neuropathic effect is concerned.

This is a suggestive hypothesis that will need to be investigated and confirmed by further and extensive pharmacological investigations.

Experimental Section/Methods

Chemistry

Reagents obtained from commercial sources were used without further purification. In order to monitor the progress of the reaction, thin layer chromatography (TLC) was performed using Merck silica gel 60 F254 precoated plates. Flash chromatography was performed using Merck silica gel 60, 230–400 mesh. The reported yields are optimized. Melting points were determined on a Buchi melting point B545 apparatus and are uncorrected. ^{1}H-NMR and ^{13}C-NMR spectra were recorded on a Bruker AC 300 or an Avance 400 instrument (Bruker Biospin Version 002 with SGU, Bruker Inc., Billerica, MA, USA). Chemical shifts (δ) are in parts per million (ppm) approximated by the nearest 0.01 ppm, using the solvent as internal standard. Coupling constants (J) are in Hz; they were calculated by Top Spin 3.1 and approximated by 0.1 Hz. Data are reported as follows: chemical shift, multiplicity (exch, exchange; br, broad; s, singlet; d, doublet; t, triplet; q, quartet; m, multiplet; or a combination of those, e.g.: dd), integral, assignments, and coupling constant. Mass spectra were recorded by HPLC Waters Alliance e2695 with Photodiode Detector 2998, Column Oven and Mass Spectrometer ZQ. For compound **9a**, the mass spectrometry analysis was performed by analyzing the compound by a Thermo LTQ (Linear Ion Trap, LIT), coupled with a HPLC Dionex Ultimate 3000 through an ElectroSpray (ESI) source (Waltham, MA). The ion trap worked in scan mode recording a range of m/z from 150 to 750 in positive polarity. Samples were prepared at $5\ \mu g\ mL^{-1}$ of concentration in methanol while the injection volume was 5 μL.

Synthesis of 1,4-diazabicyclo[4.3.0]nonan-9-one Derivatives

(*R*)-5-(Methanesulfonyloxymethyl)pyrrolidin-2-one (**3a**)

To a solution of (*R*)-(-)-5-(hydroxymethyl)pyrrolidin-2-one (**2a**, 3 g, 26.04 mol) in ethanol-free $CHCl_3$ (90 mL), triethylamine (3.95 g, 39.06 mmol) and methanesulfonyl chloride (3.57 g, 31.26 mmol) were added dropwise at 0 °C and the mixture was stirred at room temperature for 3 hours, with dichloromethane (DCM, 90 mL) and washed with a saturated aqueous solution of $NaHCO_3$, (3x45 mL). After drying (Na_2SO_4) the solvent was removed under reduced pressure and the residue was purified by flash chromatography (DCM/MeOH 9/1) to afford the title compound as a pale yellow solid (3.45 g, 68% yield), m.p. 72–74 °C. $[\alpha]_D^{25} = -19.1$ (c=1, 96% EtOH); ^{1}H-NMR (300 MHz, $CDCl_3$) 6.21 (br.s., 1 H), 4.27 (dd, 1 H), 4.09 (dd, 1 H), 3.95–4.05 (m, 1 H), 3.07 (s, 3 H), 2.24–2.47 (m, 3 H), 1.80–1.94 (m, 1 H).

(*S*)-5-(Methanesulfonyloxymethyl)pyrrolidin-2-one (**3b**) was similarly obtained in 65% yield starting from (*S*)-(+)-5-(hydroxymethyl)-pyrrolidin-2-one (**2b**). $[\alpha]_D^{25} = +18.5$ (c=1, 96% EtOH), ^{1}H-NMR spectrum identical to that of **3a**.

(*R*)-5-((Benzyl(2-hydroxyethyl)amino)methyl)pyrrolidin-2-one (**4a**)

A mixture of **3a** (1 g, 5.18 mmol) and 2-(benzylamino)ethanol (3.12 g, 20.72 mmol) was heated at 130 °C in a microwave oven (Personal Chemistry Emrys Optimizer) for 40 minutes. The residue was partitioned between water and dichloromethane, the organic phase was washed with brine, dried (Na_2SO_4) and evaporated under vacuum. The crude residue was purified by flash chromatography (DCM/MeOH from 95:5 to 90:1) to afford the title compound as a yellow oil (1.26 g, 98% yield). $[\alpha]_D^{25} = -40$ (c = 1, 96% EtOH). ^{1}H-NMR (300 MHz, $CDCl_3$) δ 7.76 (br. s., 1H), 7.19–7.43 (m, 5H), 3.62–3.88 (m, 3H), 3.37–3.62 (m, 3H), 2.73–2.85 (m, 1H), 2.47–2.63 (m, 3H), 2.21–2.42 (m, 2H), 2.02–2.21 (m, 1H), 1.48–1.66 (m, 1H).

(*S*)-5-((Benzyl(2-hydroxyethyl)amino)methyl)pyrrolidin-2-one (**4b**) was similarly obtained in 96% yield starting from compound **3b**. $[\alpha]_D^{25} = +38.5$ (c = 1, 96% EtOH), ^{1}H-NMR spectrum identical to that of **4a**.

(*R*)-5-((Benzyl(2-(methanesulfonyloxy)ethyl)amino)methyl)pyrrolidin-2-one (**5a**)

To a solution of compound **4a** (3.5 g, 14.1 mmol) in ethanol-free $CHCl_3$ (55 mL), triethylamine (2.85 g, 28.22 mmol) and a solution of methanesulfonyl chloride (3.23 g, 28.22 mol) in 10 mL of $CHCl_3$ were added at 0 °C. The mixture was allowed to warm to room temperature and stirring was continued for 20 hours. The reaction mixture was diluted with dichloromethane (60 mL) and washed with a saturated solution of $NaHCO_3$ (3×50 mL). After drying (Na_2SO_4) and removal of the solvent under vacuum, the crude was purified by flash chromatography (using a mixture of DCM-EtOH-petroleum ether- 33% NH_4OH-Et_2O 300:180:900:9.9:360) to afford the title compound (3.3 g, 73% yield). UPLC/MS: 327.1 (MH^+)

(*S*)-5-((Benzyl(2-(methanesulfonyloxy)ethyl)amino)methyl)pyrrolidin-2-one (**5b**) was similarly obtained in 63% yield starting from compound **4b**.

(*R*)-2-Benzylhexahydropyrrolo[1,2-a]pyrazin-6(2H)-one (**6a**)

Compound **5a** (2.9 g, 8.89 mmol) was dissolved in a mixture of CH_3CN/THF (1:1, 40 mL) and then 60% NaH (462 mg, 11.56 mmol) was added portion-wise at room temperature, under a nitrogen atmosphere. After stirring for 16 hours, the solvent was evaporated under vacuum and the residue was taken up with water and extracted with DCM. The organic phase was washed with brine, dried (Na_2SO_4) and evaporated under vacuum. The residue was purified by flash chromatography (using a mixture of DCM-EtOH-petroleum ether - 33% NH_4OH-Et_2O 300:180:900:9.9:360) to afford the title compound as a yellow oil (1.75 g, 85% yield). $[\alpha]_D^{25} = +55.54$ (c = 1, 96% EtOH). ^{1}H-NMR (300 MHz, $CDCl_3$) δ 7.26–7.40 (m, 5H), 3.98–4.07 (m, 1H), 3.60–3.73 (m, 1H), 3.61 (d, 1H), 3.52 (d, 1H), 2.80–3.02 (m, 3H), 2.34–2.45 (m, 2H), 1.94–2.22 (m, 2H), 1.75 (dd, 1H), 1.50–1.66 (m, 1H). UPLC/MS: 231.1 (MH^+).

(*S*)-2-Benzylhexahydropyrrolo[1,2-a]pyrazin-6(2H)-one (**6b**) was similarly obtained in 97% yield starting from compound 5b. $[\alpha]_D^{25} = -55.55$ (c = 1, 96% EtOH), ^{1}H-NMR spectrum identical to that of **6a**.

(*R*)-Hexahydropyrrolo[1,2-a]pyrazin-6(2H)-one (**7a**)

A mixture of **6a** (2 g, 8.69 mmol), ammonium formate (3.83 g, 60.8 mmol) and 10% Pd/C (500 mg) in MeOH (90 mL) was refluxed for 2 hours. After cooling to room temperature, the catalyst was filtered off and the solvent was evaporated under vacuum. The residue was purified by flash chromatography (DCM-MeOH-32% NH_4OH 70:30:3) to afford the title compound as a colorless oil (1.1 g, 90% yield). $[\alpha]_D^{25} = +28.04$ (c = 1, 96% EtOH), ^{1}H-NMR (300 MHz, $CDCl_3$) δ 4.02 (td, 1H), 3.45–3.63 (m, 1H), 3.17 (dd, 1H), 2.98–3.07 (m, 1H), 2.74–2.89 (m, 1H), 2.61 (td, 1H), 2.28–2.47 (m, 3H), 2.08–2.25 (m, 1H), 1.48–1.68 (m, 1H).

(*S*)-Hexahydropyrrolo[1,2-a]pyrazin-6(2H)-one (**7b**) was similarly obtained in 82% yield starting from compound **5b**. $[\alpha]_D^{25} = -31.46$ (c = 1, 96% EtOH), ^{1}H-NMR spectrum identical to that of **7a**.

(*R*)-(-2-(2-Fluorophenylsulfonyl)hexahydropyrrolo[1,2-a]pyrazin-6(2H)-one (**8a**)

To a solution of compound **7a** (2 g, 14.4 mmol) and TEA (1.72 g, 17.2 mmol) in di-chloromethane (60 mL), a solution of 2-fluorophenylsulfonyl chloride (14.4 mmol, in 40 mL of CH_3CN) was added dropwise at 0 °C. After stirring the solution at room temperature for 20 hours, the solvent was removed and the residue was treated with water and extracted with ethyl acetate. The organic phase was washed with brine, dried (Na_2SO_4) and evaporated under vacuum. The crude residue was triturated with isopropyl ether and collected on a Buchner filter to provide the desired compound as a white powder (3.04 g, 71% yield), m.p. 136–137 °C. $[\alpha]_D^{25} = +36.34$ (c = 1, CH_2Cl_2). MS (ESI Pos, 3.2 KV, 25 V, 350 °C): 299.24 (MH^+). ^{1}H-NMR (400 MHz, $CDCl_3$) δ 7.84 (t, 1H, Ar, $J = 6.8$ Hz), 7.60–7.55 (m, 1H, Ar), 7.31–7.19 (m, 2H, Ar), 4.09–4.00 (m, 2H, -CH_2-), 3.84 (d, 1H, -CH-, $J = 12.4$ Hz), 3.71–3.68 (m, 1H, -CH-), 2.93 (td, 1H, -CH-, $J_1 = 12.6$ Hz, $J_2 = 3.2$ Hz), 2.53–2.17 (m, 5H, 2 x CH_2 and -CH-), 1.60–1.50 (m, 1H, -CH-). ^{13}C-NMR (100 MHz, $CDCl_3$) δ 173.35, 159.94, 157.59, 135.45, 131.10, 125.71, 124.70, 117.40, 55.34, 51.80, 44.74, 39.48, 30.06, 21.73.

(*S*)-(-2-(2-Fluorophenylsulfonyl)hexahydropyrrolo[1,2-a]pyrazin-6(2H)-one (**8b**) was similarly obtained in 66% yield starting from compound **7b**. M.p. = 142–144 °C, $[\alpha]_D^{25} = -40.30$ (c = 1, CH_2Cl_2). MS (ESI Pos, 3.2 KV, 25 V, 350 °C): 299.24 (MH^+). ^{1}H-NMR (400 MHz, $CDCl_3$) δ 7.83 (t, 1H, Ar, $J = 6.8$ Hz), 7.60–7.55 (m, 1H, Ar), 7.30–7.19 (m, 2H, Ar), 4.09–3.99 (m, 2H, -CH_2-), 3.84 (d, 1H, -CH-, $J = 10.8$ Hz), 3.71–3.67 (m, 1H, -CH-), 2.93 (td, 1H, -CH-, $J_1 = 12.6$ Hz, $J_2 = 3.2$ Hz), 2.52–2.20 (m, 5H, 2 x CH_2 and -CH-), 1.60–1.50 (m, 1H, -CH-). ^{13}C-NMR (100 MHz, $CDCl_3$) δ 173.29, 160.25, 157.83, 135.45, 131.10, 125.68, 124.72, 117.41, 55.35, 52.00, 45.18, 39.57, 29.86, 21.83.

(*R*)-(-2-(3-Fluorophenylsulfonyl)hexahydropyrrolo[1,2-a]pyrazin-6(2H)-one (**9a**) was obtained in 79% yield by reacting compound **7a** with 3-fluorophenylsulfonyl chloride, as described for **8a**. M.p. = 179–180 °C. $[\alpha]_D^{25} = +43.50$ (c = 1, CH_2Cl_2). ITMS (ESI) m/z: 299.31 $[M+H]^+$. ^{1}H-NMR (400 MHz, DMSO-d_6) δ 7.71–7.67 (m, 1H, Ar), 7.62–7.57 (m, 3H, Ar), 3.86–3.77 (m, 2H, -CH_2-), 3.67 (d, 1H, -CH-, $J = 11.6$ Hz), 3.62–3.57 (m, 1H, -CH-), 2.81 (td, 1H, -CH-, $J_1 = 12.6$ Hz, $J_2 = 3.6$ Hz), 2.18–2.05 (m, 5H, 2 x CH_2 and -CH-), 1.55–1.50 (m, 1H, -CH-). ^{13}C-NMR (100 MHz, DMSO-d_6) δ 172.83, 163.59, 161.32, 138.14, 132.40, 124.15, 121.01, 114.93, 54.82, 51.65, 45.29, 39.08, 29.69, 21.51.

(*S*)-(-2-(3-Fluorophenylsulfonyl)hexahydropyrrolo[1,2-a]pyrazin-6(2H)-one (**9b**) was obtained in 71% yield by reacting compound **7b** with 3-fluorophenylsulfonyl chloride, as described for **8a**. M.p. = 177–179 °C. $[\alpha]_D^{25} = -45.10$ (c = 1, CH_2Cl_2). MS (ESI Pos, 3.2 KV, 25 V, 350 °C): 299.24 (MH^+). ^{1}H-NMR (300 MHz, DMSO-d_6) δ 7.76–7.69 (m, 1H, Ar), 7.64–7.58 (m, 3H, Ar), 3.88–3.78 (m, 2H, -CH_2-), 3.70–3.60 (m, 2H, -CH_2-), 2.84 (td, 1H, -CH-, $J_1 = 12.6$ Hz, $J_2 = 3.6$ Hz), 2.23–2.06 (m, 5H, 2 x CH_2 and -CH-), 1.55–1.50 (m, 1H, -CH-). ^{13}C-NMR (100 MHz, DMSO-d_6) δ 173.25, 163.75, 161.34, 138.27, 132.38, 124.12, 121.01, 114.95, 54.82, 51.87, 45.69, 39.20, 29.85, 21.41.

(*R*)-(-2-(4-Fluorophenylsulfonyl)hexahydropyrrolo[1,2-a]pyrazin-6(2H)-one (**10a**) was obtained in 69% yield by reacting compound **7a** with 4-fluorophenylsulfonyl chloride, as described for **8a**. M.p. = 158–159 °C. $[\alpha]_D^{25} = +41.40$ (c = 1, CH_2Cl_2). MS (ESI Pos, 3.2 KV, 25 V, 350 °C): 299.00 (MH^+). ^{1}H-NMR (400 MHz, DMSO-d_6) δ 7.36–7.79 (m, 2H, Ar), 7.46 (t, 2H, Ar, $J = 8.8$ Hz), 3.84 (dd, 1H, -CH-, $J_1 = 13.2$ Hz, $J_2 = 2.4$ Hz), 3.76 (dd, 1H, -CH-, $J_1 = 11.2$ Hz, $J_2 = 2.4$ Hz), 3.66–3.58 (m, 2H, -CH_2-), 2.81 (td, 1H, -CH-, $J_1 = 12.6$ Hz, $J_2 = 3.2$ Hz), 2.24–1.99 (m, 5H, 2 x CH_2 and -CH-), 1.54–1.44 (m, 1H, -CH-). ^{13}C-NMR (100 MHz, DMSO-d_6) δ 173.13, 166.62, 164.05, 132.42, 131.03, 130.94, 117.28, 117.06, 54.78, 51.76, 45.70, 39.04, 29.96, 21.64.

(*S*)-(-2-(4-Fluorophenylsulfonyl)hexahydropyrrolo[1,2-a]pyrazin-6(2H)-one (**10b**) was obtained in 67% yield by reacting compound **7b** with 4-fluorophenylsulfonyl chloride, as described for **8a**. M.p. = 159–160 °C. $[\alpha]_D^{25} = -47.20$ (c = 1, CH_2Cl_2). MS (ESI Pos, 3.2 KV, 25 V, 350 °C): 299.00 (MH$^+$). ^{1}H-NMR (400 MHz, DMSO-d$_6$) δ 7.83–7.79 (m, 2H, Ar), 7.47 (t, 2H, Ar, $J = 8.8$ Hz), 3.84 (dd, 1H, -CH-, $J_1 = 13.2$ Hz, $J_2 = 2.4$ Hz), 3.76 (dd, 1H, -CH-, $J_1 = 11.4$ Hz, $J_2 = 2.2$ Hz), 3.66–3.57 (m, 2H, -CH$_2$-), 2.81 (td, 1H, -CH-, $J_1 = 12.6$ Hz, $J_2 = 3.4$ Hz), 2.22–1.99 (m, 5H, 2 x CH$_2$ and -CH-), 1.54–1.46 (m, 1H, -CH-). ^{13}C-NMR (100 MHz, DMSO-d$_6$) δ 173.15, 166.47, 164.04, 132.44, 131.04, 130.95, 117.31, 117.08, 54.78, 51.94, 45.56, 39.03, 29.77, 21.41.

Pharmacological Studies

Animals

Adult male Sprague Dawley rats or adult male CD-1 mice (Envigo, Varese, Italy) weighing at arrival approximately 200–250 g and 25–30 g respectively were used. Four rats or ten mice were housed per cage and were fed a standard laboratory diet. Food and water were freely available. Room temperature and relative humidity were constantly kept at 22 ± 2 °C and 55 ± 15 %, respectively, and a 12-hour light and 12-hour darkness cycle maintained. All animal manipulations were carried out according to the Directive 2010/63/EU of the European parliament and of the European Union council (22 September 2010) on the protection of animals used for scientific purposes. The ethical policy of the University of Florence complies with the Guide for the Care and Use of Laboratory Animals of the US National Institutes of Health (NIH Publication No. 85-23, revised 1996; University of Florence assurance number: A5278-01). Formal approval to conduct the experiments described was obtained from the Italian Ministry of Health (No. 498/2022-PR) and from the Animal Subjects Review Board of the University of Florence. Experiments involving animals have been reported according to ARRIVE guidelines.[27] All efforts were made to minimize animal suffering and to reduce the number of animals used.

Preparation of Compounds for *In Vivo* Treatments

R and *S* enantiomers for oral administration were dissolved in 1 % CMC (carboxymethylcellulose sodium salt) and given in a volume of 10 ml kg^{-1} body weight. For endovenous treatment, enantiomers or their mixtures were dissolved in saline/DMSO 2 % and administered in a volume of 1 ml kg^{-1}. Control animals received an equal volume of vehicle (saline/DMSO 2 % or CMC 1 %).

Animal Models

Chronic Constriction Injury (CCI)-Induced Neuropathic Pain And Treatments

Neuropathy was induced according to the procedure described by Bennett and colleagues.[28] Briefly, rats were anaesthetized with 2 % isoflurane. Under aseptic conditions, the right (ipsilateral) common sciatic nerve was exposed by blunt dissection at the level of the mid-thigh. Proximal to the trifurcation, the nerve was carefully freed of the adhering tissue from the surrounding connective tissue, and 4 chromic catgut ligatures (3-0, Ethicon, Norderstedt, Germany) were tied loosely around the nerve with about 1-mm spacing between each other. After haemostasis was confirmed, the incision was closed in layers. The animals were allowed to recover from surgery and then housed one per cage with free access to water and standard laboratory chow. Another group of rats was subjected to sham surgery in which the sciatic nerve was only exposed but not ligated. *R* enantiomer **8a** was orally administered on day 14, when neuropathy was well established, in a range of doses between 1 mg kg^{-1} to 10 mg kg^{-1}.

Sodium Monoiodoacetate-Induced Osteoarthritis and Treatments

Unilateral osteoarthritis was induced by injection of sodium monoiodoacetate (MIA; Sigma-Aldrich, Milan, Italy) into the tibiotarsal joint.[29,30] On day 1, rats were slightly anaesthetized by 2 % isoflurane, the left leg skin was sterilized with 75 % ethyl alcohol and the lateral malleolus located by palpation; then, a 28-gauge needle was inserted vertically to penetrate the skin and turned distally for insertion into the articular cavity at the gap between the tibiofibular and tarsal bone until a distinct loss of resistance was felt. Two milligrams of MIA in 25 μl saline was delivered into the left articular cavity. Control rats were treated with an equal volume of saline. Behavioral measurements were performed starting from day 14, when osteoarthritic-like focal lesions in articular cartilage and subchondral bone thickening were exerted by MIA. *R* enantiomer **8a** (1–3 mg kg^{-1}) or the enantiomeric mixtures **8a** + **8b** 1:1; 2:1 and 3:1 were intravenously injected. Control animals received an equivalent volume of vehicle.

Oxaliplatin-Induced Neuropathic Pain Model and Treatments

Oxaliplatin (2.4 mg kg^{-1}; Carbosynth, Compton, UK) was dissolved in 5 % glucose solution and injected intraperitoneally (i.p.) on days 1–3; 6–10; 13–15 for a total of 11 injections according to Cavaletti and colleagues[31] with minor modifications. Oxaliplatin was dissolved in a 5 % glucose solution. *R* enantiomer **8a** was acutely *per os* administered on day 21, when neuropathy was well established, in a range of doses between 0.3 mg kg^{-1} to 10 mg kg^{-1}. Control animals received an equivalent volume of vehicles.

Paclitaxel-Induced Neuropathic Pain Model and Treatments

Paclitaxel (2.0 mg kg^{-1}) (Carbosynth, Compton, UK) were injected intraperitoneally (i.p.) on four alternate days (days 1, 3, 5 and 8) according to.[20] Paclitaxel was dissolved in a mixture of saline solution and 10 % Cremophor EL (vehicle), a derivative of castor oil and ethylene oxide that is used clinically for paclitaxel injections. Compounds were administered on day 14, when neuropathy was well established. Three mg/ml of enantiomers **8a**, **8b**, **9a**, **9b**, **10a** and **10b** were dissolved in saline/DMSO 2% and intravenously (i.v.) injected (1 ml kg^{-1}) for the experiment reported in the Supplementary Figure S1. Subsequently, in the same animal models, 1 mg kg^{-1} of **8a**, **8b** or the enantiomeric mixtures **8a** + **8b** 1:1; 1:3, 2:1 and 3:1 were acutely i.v. administered. For both experiments, control animals received an equal volume of vehicle (saline/DMSO 2 %).

Vincristine-Induced Neuropathic Pain and Treatments

Vincristine, at 150 μg kg^{-1} was dissolved in saline solution and intravenously (i.v.) injected on five non-consecutive days (1, 3, 5, 8, 10) for a cumulative dose of 750 mg kg^{-1} i.v.[32] **8a** (1–10 mg kg^{-1}) were acutely *per os* administered starting on day 11, when neuropathy was well established. Control animals received an equal volume of vehicle only.

Streptozotocin (STZ)-Induced Neuropathic Pain and Treatments

Mice were intravenously (i.v.) administered with STZ, (Sigma Aldrich, Milan, Italy) 100 mg kg^{-1}[33] (with modification). Since STZ has stability problems, the solution was prepared immediately before the injection in freshly prepared citrate buffer. To maintain cleanliness and avoid the development of any infection due to excessive urination, animal bedding was changed frequently. **8a** (3–

100 mg kg^{-1}) was acutely *per os* administered once when neuropathy was established (day 21). Control animals received an equal volume of vehicles.

Assessment of Mechanical Hyperalgesia (Paw Pressure Test)

The nociceptive threshold of rats was determined with an analgesimeter (Ugo Basile, Varese, Italy), according to the method previously described. Briefly, a constantly increasing pressure was applied to a small area of the dorsal surface of the hind paw using a blunt conical probe by a mechanical device. Mechanical pressure was increased until vocalization or a withdrawal reflex occurred while rats were lightly restrained. Vocalization or withdrawal reflex thresholds were expressed in grams. A cut-off was set at 150 g.[34,35]

Assessment of Mechanical Allodynia (von Frey test)

The mechanical allodynia was measured with an electronic von Frey hair unit (Ugo Basile, Varese, Italy) as described previously. Briefly, animals were placed in 20 cm×20 cm Plexiglas boxes equipped with a metallic mesh floor, 20 cm above the bench. Animals were allowed to get used to the environment for 15 minutes before the test. The withdrawal threshold was evaluated by applying forces ranging from 0 to 50 g with a 0.2 g accuracy. Punctuate stimulus was delivered to the mid-plantar area of each anterior paw from below the mesh floor through a plastic tip and the withdrawal threshold was automatically displayed on the screen. The paw sensitivity threshold was defined as the minimum force required to obtain a robust and immediate withdrawal reflex of the paw. Measurements were performed on the posterior paw. Voluntary movements associated with locomotion were not considered as a withdrawal response. Stimuli were applied to each posterior paw at 5 s intervals. Measurements were repeated 5 times and the final value was obtained by averaging the 5 measurements.[36]

Assessment of Thermal Hyperalgesia (Hot-Plate Test)

Thermal hyperalgesia was assessed using the hot-plate apparatus (Ugo Basile, Varese, Italy). With minimal animal-handler interaction, mice were taken from home-cages, and placed onto the hot surface maintained at a constant temperature of 49 ± 1 °C. Ambulation was restricted by a cylindrical Plexiglas chamber (diameter: 10 cm, height: 15 cm), with open top. Pain-related behavior (paw lifting or licking) was observed, and the time (s) of the first sign was recorded. The cut-off time latency was set at 30 s.[37,38]

Assessment of Motor Coordination (Rota Rod Test)

The apparatus consisted of a base platform and a rotating rod with a diameter of 3 cm and a non-slippery surface. The rod was placed at a height of 15 cm from the base. The rod, 30 cm in length, was divided into 5 equal sections by 6 disks. Thus, up to five mice were tested simultaneously on the apparatus, with a rod-rotating speed of 16 revolutions per minute. The integrity of motor coordination was assessed on the basis of the number of falls from the rod in 30 s.[39] The performance time was measured before and 15, 30, 45 and 60 minutes after treatment with **8a** and **8b**.

Statistical Analysis

In vivo assessments were made by researchers blinded to animal treatments. Data are expressed as mean ± standard error of the mean (S.E.M.). One-way ANOVA followed by Bonferroni post hoc comparison test was applied. P-values <0.05 were considered significant. Statistical analysis and curves and graphs were carried out using OriginPro 9 software (OriginLab, Northampton, MA, USA).

Supporting Information Summary

Supporting Information is available from the Wiley Online Library or from the author.

Funding Sources

This work was supported by the Italian Ministry of Instruction, University and Research (MIUR) by the University of Florence and by the NeuroDeRisk project funded from the Innovative Medicines Initiative 2 Joint Undertaking under grant agreement No 821528. This Joint Undertaking receives support from the European Union's Horizon 2020 research and innovation program and EFPIA.

Acknowledgements

The authors would like to extend their sincere appreciation to Carlo Parini, Marisa Martinelli, Silvano Ronzoni and Stefania Gagliardi (NiKem Research Srl), Tina Pazzi (Chimete Srl) for the synthesis of compounds. Open Access publishing facilitated by Università degli Studi di Firenze, as part of the Wiley - CRUI-CARE agreement.

Conflict of Interests

C.F. and M.S. were employees of Metys Pharmaceuticals AG during the conduct of this work. All other authors declare that there are no conflicts of interest regarding the publication of this article.

Keywords: Hyperalgesia · Allodynia · Chemotherapy-induced neuropathic pain · Non-racemic enantiomeric mixtures · Pyrrolidinone derivatives · Racetams

[1] L. Colloca, T. Ludman, D. Bouhassira, R. Baron, A. H. Dickenson, D. Yarnitsky, R. Freeman, A. Truini, N. Attal, N. B. Finnerup, C. Eccleston, E. Kalso, D. L. Bennett, R. H. Dworkin, S. N. Raja, *Nat. Rev. Dis. Prim.* **2017**, *3*, 17002.

[2] N. B. Finnerup, N. Attal, S. Haroutounian, E. McNicol, R. Baron, R. H. Dworkin, I. Gilron, M. Haanpää, P. Hansson, T. S. Jensen, P. R. Kamerman, K. Lund, A. Moore, S. N. Raja, A. S. C. Rice, M. Rowbotham, E. Sena, P. Siddall, B. H. Smith, M. Wallace, *Lancet Neurol.* **2015**, *14*, 162–173.

[3] M. Zhuo, *Neuropharmacology* **2017**, *112*, 228–234.

[4] D. Bleakman, A. Alt, E. S. Nisenbaum, *Semin. Cell Dev. Biol.* **2006**, *17*, 592–604.

[5] C. E. Giurgea, *Fundamentals to a Pharmacology of the Mind*, Thomas **1981**.

[6] C. E. Giurgea, F. E. Moeyersoons, A. C. Evraerd, *Arch. Int. Pharmacodyn. Ther.* **1967**, *166*, 238–251.

[7] T. Bonifacino, L. Micheli, C. Torazza, C. Ghelardini, C. Farina, G. Bonanno, M. Milanese, L. Di Cesare Mannelli, M. W. Scherz, *Cells* **2022**, *11*, 4027.

[8] R. G. Fariello, C. Ghelardini, L. Di Cesare Mannelli, G. Bonanno, A. Pittaluga, M. Milanese, P. Misiano, C. Farina, *Neuropharmacology* **2014**, *81*, 85–94.

[9] M. H. Rashid, H. Ueda, *J. Pharmacol. Exp. Ther.* **2002**, *303*, 226–231.

[10] J. V. Holbech, M. Otto, F. W. Bach, T. S. Jensen, S. H. Sindrup, *Eur. J. Pain* **2011**, *15*, 608–614.

[11] B. A. Lynch, N. Lambeng, K. Nocka, P. Kensel-Hammes, S. M. Bajjalieh, A. Matagne, B. Fuks, *Proc. Natl. Acad. Sci. U. S. A.* **2004**, *101*, 9861–9866.

[12] P.-H. Yen, Y.-C. Kuan, K.-W. Tam, C.-C. Chung, C.-T. Hong, Y.-H. Huang, *J. Formos. Med. Assoc.* **2021**, *120*, 755–764.

[13] A. G. Malykh, M. R. Sadaie, *Drugs* **2010**, *70*, 287–312.

[14] J. F. Herrero, J. M. Laird, J. A. López-García, *Prog. Neurobiol.* **2000**, *61*, 169–203.

[15] S. Scapecchi, E. Martini, D. Manetti, C. Ghelardini, C. Martelli, S. Dei, N. Galeotti, L. Guandalini, M. Novella Romanelli, E. Teodori, *Bioorg. Med. Chem.* **2004**, *12*, 71–85.

[16] D. Manetti, C. Ghelardini, A. Bartolini, C. Bellucci, S. Dei, N. Galeotti, F. Gualtieri, M. N. Romanelli, S. Scapecchi, E. Teodori, *J. Med. Chem.* **2000**, *43*, 1969–1974.

[17] M. N. Romanelli, N. Galeotti, C. Ghelardini, D. Manetti, E. Martini, F. Gualtieri, *CNS Drug Rev.* **2006**, *12*, 39–52.

[18] C. Ghelardini, M. Martinelli, S. R. Carlo Parini (**2009**) Nitrogen-containing bycyclic compounds active on chronic pain conditions. WO 2009/103176 A1, issued 2009.

[19] M. Asif, S. S. Alvi, T. Azaz, A. R. Khan, B. Tiwari, B. B. Hafeez, M. Nasibullah, *Int. J. Mol. Sci.* **2023**, *24*, 7336.

[20] R. C. Polomano, A. J. Mannes, U. S. Clark, G. J. Bennett, *Pain* **2001**, *94*, 293–304.

[21] C. Farina, S. Gagliardi, C. Ghelardini, M. Martinelli, M. Norcini, C. Parini, P. Petrillo, S. Ronzoni, *Bioorg. Med. Chem.* **2008**, *16*, 3224–3232.

[22] L. Di Cesare Mannelli, M. Maresca, L. Micheli, C. Farina, M. W. Scherz, C. Ghelardini, *Cancer Chemother. Pharmacol.* **2017**, *80*, 1091–1103.

[23] L. Di Cesare Mannelli, M. Maresca, C. Farina, M. W. Scherz, C. Ghelardini, *Neurotoxicology* **2015**, *50*, 101–107.

[24] A. Pittaluga, A. Bonfanti, D. Arvigo, M. Raiteri, *Naunyn. Schmiedebergs. Arch. Pharmacol.* **1999**, *359*, 272–279.

[25] C.-C. Tan, J.-T. Yu, H.-F. Wang, M.-S. Tan, X.-F. Meng, C. Wang, T. Jiang, X.-C. Zhu, L. Tan, *J. Alzheimers. Dis.* **2014**, *41*, 615–631.

[26] N. Galeotti, C. Ghelardini, A. Pittaluga, A. M. Pugliese, A. Bartolini, D. Manetti, M. N. Romanelli, F. Gualtieri, *Naunyn. Schmiedebergs. Arch. Pharmacol.* **2003**, *368*, 538–545.

[27] J. C. McGrath, E. Lilley, *Br. J. Pharmacol.* **2015**, *172*, 3189–3193.

[28] G. J. Bennett, Y.-K. Xie, *Pain* **1988**, *33*, 87–107.

[29] M. Maresca, L. Micheli, L. Cinci, A. R. Bilia, C. Ghelardini, L. Di Cesare Mannelli, *J. Pharm. Pharmacol.* **2017**, *69*, 1858–1870.

[30] L. Di Cesare Mannelli, L. Micheli, M. Zanardelli, C. Ghelardini, *BMC Musculoskelet. Disord.* **2013**, *14*, 228.

[31] G. Cavaletti, G. Tredici, M. G. Petruccioli, E. Dondè, P. Tredici, P. Marmiroli, C. Minoia, A. Ronchi, M. Bayssas, G. G. Etienne, *Eur. J. Cancer* **2001**, *37*, 2457–2463.

[32] F. Marchand, A. Alloui, T. Pelissier, A. Hernández, N. Authier, P. Alvarez, A. Eschalier, D. Ardid, *Brain Res.* **2003**, *980*, 117–120.

[33] E. Lucarini, E. Pagnotta, L. Micheli, C. Parisio, L. Testai, A. Martelli, V. Calderone, R. Matteo, L. Lazzeri, L. Di Cesare Mannelli, C. Ghelardini, *Molecules* **2019**, *24*, 3006.

[34] G. E. Leighton, R. E. Rodriguez, R. G. Hill, J. Hughes, *Br. J. Pharmacol.* **1988**, *93*, 553–560.

[35] L. Micheli, M. Bozdag, O. Akgul, F. Carta, C. Guccione, M. C. Bergonzi, A. R. Bilia, L. Cinci, E. Lucarini, C. Parisio, C. T. Supuran, C. Ghelardini, L. Di Cesare, Mannelli, *Int. J. Mol. Sci.* **2019**, *20*, 1923.

[36] L. Micheli, L. Di Cesare Mannelli, L. Mattoli, S. Tamimi, E. Flamini, S. Garetto, J. Lucci, E. Giovagnoni, L. Cinci, M. D'Ambrosio, C. Luceri, C. Ghelardini, *Nutrients* **2020**, *12*, 1618.

[37] L. Micheli, L. Di Cesare Mannelli, E. Lucarini, C. Parisio, A. Toti, B. Fiorentino, M. A. Rigamonti, L. Calosi, C. Ghelardini, *Front. Pharmacol.* **2020**, *11*, 576624.

[38] L. Micheli, M. Vasarri, E. Barletta, E. Lucarini, C. Ghelardini, D. Degl'Innocenti, L. Di Cesare Mannelli, *Mar. Drugs* **2021**, *19*, 48.

[39] L. Micheli, E. Lucarini, E. Trallori, C. Avagliano, C. Caro, R. Russo, A. Calignano, C. Ghelardini, A. Pacini, L. Di Cesare Mannelli, *Nutrients* **2019**, *11*, 1778.