DUAL SIGMA-1 RECEPTOR ANTAGONISTS AND HYDROGEN SULFIDE-RELEASING COMPOUNDS FOR PAIN TREATMENT: DESIGN, SYNTHESIS, AND PHARMACOLOGICAL EVALUATION

Maria Dichiara,[a] Antonia Artacho-Cordón,[b] Rita Turnaturi,[a] Miriam Santos-Caballero,[b] Rafael González-Cano,[b] Lorella Pasquinucci,[a] Carla Barbaraci,[a] Isabel Rodríguez-Gómez,[c] Manuel Gómez-Guzmán,[d] Agostino Marrazzo,[a] Enrique J. Cobos[b,*] and Emanuele Amata[a,*]

[a]Department of Drug and Health Sciences, Medicinal Chemistry Section, Università degli Studi di Catania, Viale Andrea Doria 6, 95125 Catania, Italy

[b]Department of Pharmacology, Faculty of Medicine and Biomedical Research Center (Neurosciences Institute), University of Granada and Biosanitary Research Institute ibs.Granada, 18016 Granada, Spain

[c]Department of Physiology, Faculty of Medicine, University of Granada, 18016, Granada, Spain.

[d]Department of Pharmacology, Faculty of Pharmacy and Biomedical Research Center, University of Granada and Biosanitary Research Institute ibs.Granada, 18016, Granada, Spain.

Corresponding authors:

*Emanuele Amata: phone, (+39) 0957384102; email, eamata@unict.it

*Enrique J. Cobos: phone, (+34) 958249302; email, ejcobos@ugr.es

Preprint version. Please cite original version:

Dichiara M, Artacho-Cordón A, Turnaturi R, Santos-Caballero M, González-Cano R, Pasquinucci L, Barbaraci C, Rodríguez-Gómez I, Gómez-Guzmán M, Marrazzo A, Cobos EJ, Amata E. Dual Sigma-1 receptor antagonists and hydrogen sulfide-releasing compounds for pain treatment: Design, synthesis, and pharmacological evaluation. Eur J Med Chem. 2022 Feb 15;230:114091. doi: 10.1016/j.ejmech.2021.114091.

Abstract

The development of σ_1 receptor antagonists hybridized with a H_2S-donor is here reported. We aimed to obtain improved analgesic effects when compared to σ_1 receptor antagonists or H_2S-donors alone. In an *in vivo* model of sensory hypersensitivity, thioamide **1a** induced analgesia which was synergistically enhanced when associated with the σ_1 receptor antagonist BD-1063. The selective σ_1 receptor agonist PRE-084 completely reversed this effect. Four thioamide H_2S-σ_1 receptor hybrids (**5a**–**8a**) and their amide derivatives (**5b**–**8b**) were synthesized. Compound **7a** (AD164) robustly released H_2S and showed selectivity for σ_1 receptor over σ_2 and opioid receptors. This compound induced marked analgesia that was reversed by PRE-084. The amide analogue **7b** (AD163) showed only minimal analgesia. Further studies showed that **7a** exhibited negligible acute toxicity, together with a favorable pharmacokinetic profile. To the best of our knowledge, compound **7a** is the first dual-acting ligand with simultaneous H_2S-release and σ_1 antagonistic activities.

Keywords: Sigma-1 Receptor, Antagonist, Hydrogen Sulfide Donor, Analgesia, Dual Ligands

Abbreviations: σ, sigma; KO, knockout; H_2S, hydrogen sulfide; LPS, lipopolysaccharide; K_{ATP}, ATP-sensitive potassium; [(±)-HP-mII, (±)-haloperidol metabolite II; MOR, μ-opioid receptor; DOR, δ-opioid receptor; KOR, κ-opioid receptor; WSP-1, Washington State Probe-1; PBS, phosphate buffered solution; s.c., subcutaneous; hERG, human ether-a-go-go related gene; MABP, mean arterial blood pressure; BBB, blood-brain barrier.

1. Introduction

Pain is a global health burden, with millions of people suffering from chronic pain, and an estimate of 18% of individuals in developed countries with chronic pain conditions. The current cost of chronic pain to the healthcare system is significant, and arguably unsustainable considering the rapid ageing of the population [1]. Current analgesics show limited efficacy in many pain conditions or a number of side effects which limit their use and therefore, there is an urgent need of novel analgesics [2].

Sigma (σ) receptors are involved in several biological processes and pathological conditions [3]. Two subtypes are currently known, denoted as sigma-1 (σ_1) and sigma-2 (σ_2) receptors, having different structure, biological function, and pharmacological profile [3, 4]. σ_1 receptor is a Ca^{2+}-sensing chaperone which acts as a regulatory subunit of several ion channels and G-protein coupled receptors, with an important role on neurotransmission [5]. Several evidences support the modulatory role of σ_1 receptor in nociception, mainly based on the pain-attenuated phenotype in σ_1 receptor knockout (KO) mice and on the antinociceptive effect exerted by σ_1 receptor antagonists on pain of different types including inflammatory pain, osteoarthritis, neuropathic pain induced by either mechanical injury or antineoplastics, and visceral pain [5].

Hydrogen sulfide (H_2S) is an endogenous gasotransmitter involved in the modulation of the daily cellular activities including inflammation, nociception and the regulation of the structure and function of blood vessels such that the downregulation of H_2S pathways is involved in the pathogenesis of a variety of vascular diseases, such as hypertension and atherosclerosis [6, 7]. Under physiological conditions, cells produce small but significant amounts of H_2S that contribute to enhance the neutrophil/endothelium adhesion process, leading to neutrophil migration toward the inflammatory site and thus inflammatory hypernociception [8, 9]. In contrast to the pro-nociceptive role of endogenous H_2S, the systemic pretreatment of mice with

an exogenous H_2S-donor (NaHS) inhibited nociceptive stimuli both in a lipopolysaccharide (LPS)-induced inflammatory and in a zymosan-induced articular hypernociception model by opening ATP-sensitive potassium (K_{ATP}) channels [10]. The antinociceptive effect of the exogenous H_2S-administration has been found to be effective also in rodent models of visceral pain and peripheral neuropathic pain induced by either traumatic nerve injury or anticancer drugs [11-13].

The gathered preclinical evidence for the role of σ_1 receptor and H_2S donors in pain has led to the recent development of the selective σ_1 antagonist E-52862 (S1RA), and the H_2S-antiinflammatory compound ATB-346 (Figure 1). These compounds are currently undergoing phase II clinical trials for pain treatment with excellent safety profiles [14, 15].

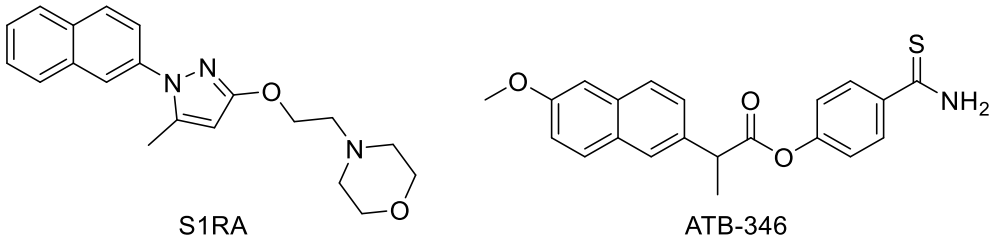


Figure 1. Chemical structures of S1RA and ATB-346.

However, treatments based on a single mechanism of action often lack of efficacy and targeting multiple concurrent mechanisms of nociceptive transmission, by combination pharmacotherapy, is routinely used to alleviate chronic pain as well in reducing side effects [16, 17]. Although combination drug therapy represents the most simple and immediate way to combine drugs with different mechanisms of action, this may have some disadvantages. Indeed, the pharmacokinetics and pharmacodynamics of the two compounds should be compatible regarding latency for the effect, time for maximum effect and time between doses, and also the compounds should be compatible in terms of potential drug-drug interactions [18]. For this reason, polypharmacology

that relies on the use of a single multi-target pharmaceutical ingredient could have inherent advantages over combination therapies, overcoming all these issues related with the combination therapy. As an example, tramadol and tapentadol are well known analgesics with dual opioid agonist and neurotransmitter reuptake-blocker mechanism of action. Moreover, different multitarget drugs are being developed including some opioid-sigma dual compounds [19, 20]. With the aim to identify novel analgesics endowed with multiple mechanisms of action for their use thereof, here we aimed to find whether a H_2S-donor combined with a σ_1 receptor antagonist moiety might induce a synergistically enhanced analgesic effect when compared to these mechanisms acting alone. Here we report for the first time the development of hybrid ligands able to bind σ receptors and to release H_2S useful for pain treatment. Finally, we also performed an initial assessment of the toxicological properties of the most promising hybrid compound.

2. Results and discussion

2.1. Rational design

The choice of an adequate H_2S donor has been done in order to ensure a high and effective H_2S release. Among the different classes of H_2S-donors developed to date, thioamides seem to be preferable having demonstrated a sufficient or higher release than other donors [21]. 4-Carbamothioylbenzoic acid **1a** (Figure 2) was thus selected having a thioamide function bound to the *para* position of benzoic acid. Generally, electron-withdrawing functional groups on the phenyl ring led to faster H_2S generation, while electron-donating groups led to slower H_2S release [22]. Our purposes were to produce chemical compounds able to give a fast-onset analgesic effect although with a fairly stability before administration in order to maximize the real concentration of H_2S released to biological systems.

The new chemical entities (**5**–**8a**) were designed as analogs of the conventional antipsychotic haloperidol (**2a**, Figure 2), a compound that shows a high affinity for σ receptors, showing also a σ_1 receptor antagonist functional profile with antinociceptive and anti-allodynic effects [23, 24]. Different from **2a**, (±)-haloperidol metabolite II [(±)-HP-mII (**2b**, Figure 2)] displays a preferential affinity on σ receptors compared to dopamine receptors acting also as σ_1 receptor antagonist and producing an analgesic effect comparable to that of **2a** [23-25]. Starting from **2b**, we conjugated the secondary hydroxy group with the 4-carbamothioylbenzoic acid as in compound AD95 (**5a**), while the removal of the *para*-F-phenyl group produced compound AD127 (**6a**).

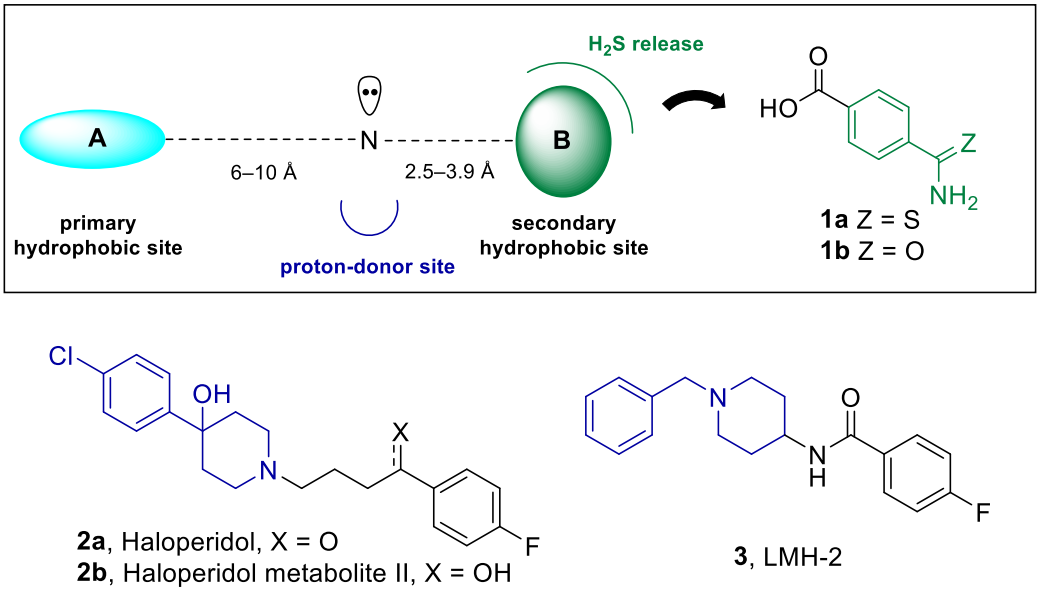


Figure 2. Design strategy and chemical structure of reference compounds.

Recently, LMH-2 (**3**, Figure 2) has been reported as a novel analog of compound **2a** exhibiting high σ_1 receptor affinity and good selectivity over σ_2 receptor [26]. In an *in vivo* model of neuropathic pain, **3** produced dose-dependent antiallodynic effects with more potency than gabapentin. Based on these findings, we replaced the fluorine atom of **3** with the thioamide to

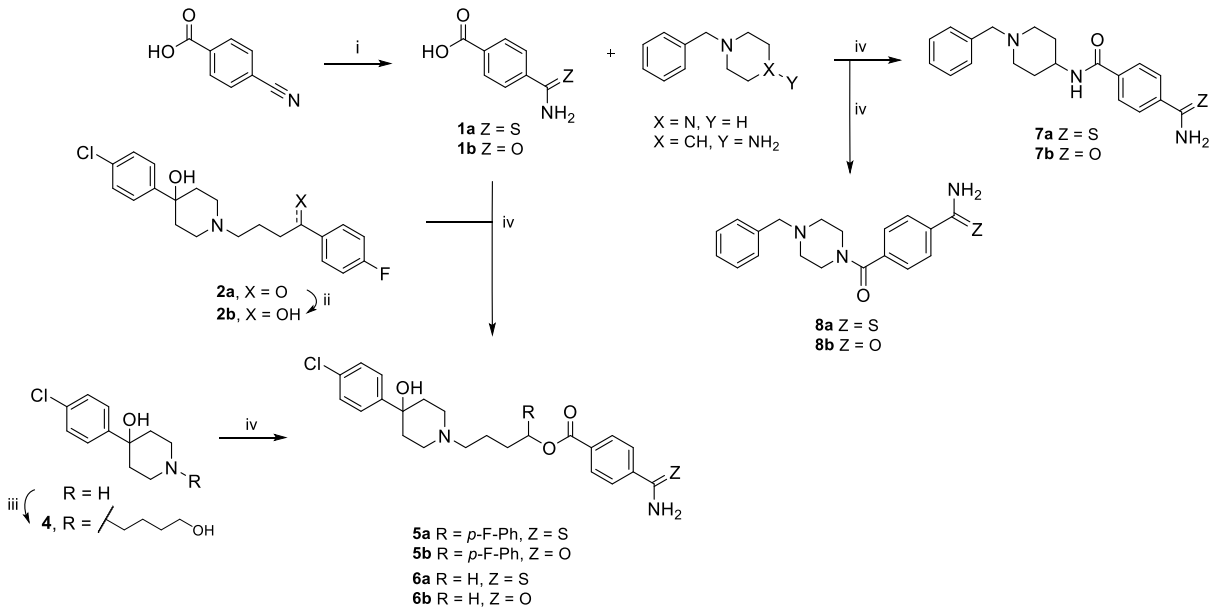
give compound AD164 (**7a**). While compound AD119 (**8a**) was designed as conformationally constrained homologue of compound **7a** where the amide nitrogen was embedded in a cycle and thus tertiary. To better dig into the precise mechanism of the synthetized compounds, derivatives AD162 (**5b**), AD160 (**6b**), AD163 (**7b**) and AD120 (**8b**) bearing an amide instead to the thioamide function have also been prepared as negative controls.

The characterization of the synthesized ligands included an *in vitro* evaluation of (i) the binding affinity against σ and opioid receptors, (ii) assessment of H_2S release ability, and (iii) *in vivo* studies in a model of sensory hypersensitivity. Moreover, we evaluated key aspects of safety pharmacology for the most promising compound, as well as some relevant aspects of its pharmacokinetic profile.

2.2. Synthesis

The H_2S-donor thioamide **1a** and compounds **5**−**8** were synthesized according to the steps illustrated in Scheme 1.

Scheme 1. Synthetic Strategy for the Preparation of Target Compounds.



Reagents and conditions: (i) P_4S_{10}, EtOH, 100 °C, 5 h; (ii) $NaBH_4$, EtOH, rt, 12 h; (iii) 4-chloro-1-butanol, $KHCO_3$, ACN, reflux, 4 h; (iv) EDC, HOBT, DMF, rt, 6 h.

The H_2S-donor scaffold **1a** was prepared starting from commercially available 4-cyanobenzoic acid by thionation with P_4S_{10} and then conjugated with opportune alcohol or amine by coupling reactions to give the final compounds **5a**–**8a**. The reaction of intermediate **2b**, obtained by reduction of **2a** with $NaBH_4$, and **1a** gave ester derivative **5a**. Intermediate **4**, attained by *N*-alkylation with 4-chloro-1-butanol, has been conjugated with **1a** to give ester **6a**. As regard the synthesis of amides **7a** and **8a**, 1-benzylpiperidin-4-amine and 1-benzylpiperazine have been used, respectively. Negative controls **5b**–**8b** were obtained through condensation of the same alcohols or amines with commercial 4-carbamoylbenzoic acid.

2.3. Structure-affinity relationship studies

Compounds were evaluated for affinity at σ receptors and for μ, δ and ƙ opioid receptors (MOR, DOR and KOR, respectively) through radioligand binding assays (Table 1). We also included **2a**,

DTG (**9**), (+)-pentazocine (**10**), BD-1063 (**11**), DAMGO (**13**), naltrindole (**14**) and (–)-U50,488 (**15**) as internal controls that were tested with the same membrane homogenates of the hybrid compounds under investigation (Figure S1). All the reference compounds showed K_i values for σ_1 and σ_2 receptors and the three opioid receptors comparable to those reported in previous studies [27-32]. It is worth pointing out that compounds **1a**,**b** were devoid of affinity for either σ receptors or the three opioid receptors examined.

The esterification of the secondary hydroxy group of compound **2b** with the thioamide **1a** as in compound **5a**, resulted in a substantial decrease in the affinity at both σ receptors. Indeed, as reported in Table 1, compound **2b** has a $K_i\sigma_1$ of 2.7 nM and $K_i\sigma_2$ of 2.4 nM while **5a** esterified with 4-carbamothioylbenzoic acid has a $K_i\sigma_1$ of 156 nM and $K_i\sigma_2$ of 311 nM. The elimination of the *p*-F-phenyl group as in **6a** increased the σ_1 receptor affinity with respect to **5a**, together with an improved selectivity over σ_2 receptor. Indeed, compound **6a** had a $K_i\sigma_1$ of 58 nM *vs* $K_i\sigma_2$ of 266 nM. The sulfur atom seems to promote the affinity at both σ receptors with derivatives **5b** and **6b** bearing the amide function showing lower affinity over both receptor subtypes with $K_i\sigma_1$ of 173 nM *vs* $K_i\sigma_2$ of 618 nM, and $K_i\sigma_1$ of 126 nM *vs* $K_i\sigma_2$ of 933 nM, respectively.

The replacement of the *para*-F-phenyl group of **3** with the thioamide scaffold as in compound **7a**, determined a lowering of affinity towards both σ receptors when compared to the cognate derivative. As reported in Table 1, $K_i\sigma_1$ values of 6.0 nM and $K_i\sigma_2$ of 190 nM were determined for **3**, whereas compound **7a** showed $K_i\sigma_1$ of 94 nM and $K_i\sigma_2$ of 1,125 nM.

The conformationally constrained homologue of **7a**, compound **8a** having the amide nitrogen embedded in a cycle, shows low affinity for σ_1 receptor ($K_i\sigma_1$ 668 nM) and it completely loses the capacity to bind σ_2 receptor ($K_i\sigma_2$ >10,000). Differently from compounds **5a**,**b** and **6a**,**b**, in

compounds **7a** and **8a** the replacement of the thioamide with the amide function as in negative controls **7b** and **8b** determine a lower affinity for both σ receptor subtypes.

Overall, the introduction of the thioamide or amide functions lead to a worsening of the affinity over σ receptors, although remaining, at least for the σ_1 receptor, in the medium or low nM range in most cases.

The interaction with MOR, DOR and KOR was measured in order to exclude additional analgesic effects related with these three opioid receptors (Table 1). The 4-carbamothioylphenyl σ_1 receptor derivatives **5a–8a** show low affinity toward MOR, and negligible for KOR and DOR indicating selectivity for σ_1 receptor over these opioid receptors. Similarly, amide derivatives **5b–8b** have provided to displace the radioligands only at high concentrations, resulting in a low affinity profile for the three receptors.

Table 1. σ and opioid receptors binding assays for compounds **1a**,**b** and **5**−**8**.

	K_i (nM) ± SD[a]				
Cpd	**σ_1**	**σ_2**	**MOR**	**DOR**	**KOR**
1a	>10,000	>10,000	>10,000	>10,000	>10,000
1b	>10,000	>10,000	>10,000	>10,000	>10,000
5a	156 ± 41	311 ± 75	1,458 ± 35	>10,000	>10,000
5b	173 ± 24	618 ± 146	817 ± 30	>10,000	>10,000
6a	58 ± 7.0	266 ± 73	>10,000	>10,000	>10,000
6b	126 ± 15	933 ± 161	>10,000	>10,000	>10,000
7a	94 ± 29	1,125 ± 348	>10,000	>10,000	>10,000
7b	837 ± 125	>10,000	>10,000	>10,000	>10,000
8a	668 ± 137	>10,000	>10,000	>10,000	>10,000
8b	>5,000	>10,000	2,551 ± 88	>10,000	>10,000

Haloperidol (**2a**)	2.6 ± 0.4	77 ± 18			
(±)-HP-mII (**2b**)[b]	2.7 ± 0.8	2.4 ± 0.5			
LMH-2 (**3**)[c]	6.0	190			
DTG (**9**)	124 ± 19	18 ± 1			
(+)-Pentazocine (**10**)	4.3 ± 0.5	1,465 ± 224			
BD-1063 (**11**)	14 ± 2.7	204 ± 31			
DAMGO (**13**)			1.49 ± 0.49		
Naltrindole (**14**)				2.53 ± 0.51	
(–)-U50,488 (**15**)					1.10 ± 0.21

[a]Each value is the mean ± SD of at least two experiments performed in duplicate. [b]Reference 25. [c]Reference 26.

2.4. Hydrogen sulfide release

H_2S release was assessed using a spectrofluorimetric assay based upon the reaction of H_2S with Washington State Probe-1 (WSP-1) to generate benzodithiolone and a fluorophore with excitation and emission maxima of 465 and 515 nm, respectively. The results of H_2S release in phosphate buffered solution (PBS) at regular intervals of 15 min are reported in Figure 3.

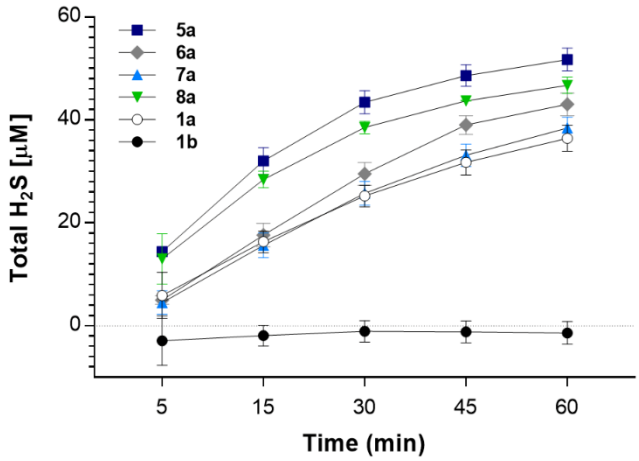


Figure 3. H_2S release measurement at regular incubation intervals in PBS buffer. Data are presented as mean ± SD of at least two experiments in triplicate.

First, we evaluated compound **1a** and its amide derivative **1b** as negative control for their ability to release H_2S. Thioamide **1a** at 100 μM concentration was able to release H_2S in a significant and cumulative fashion over time. The amount of H_2S released by H_2S-donor scaffold **1a** after 1 h incubation was 34.4 μM. No H_2S release was detected for the analogue **1b** (100 μM) bearing an amide function used as negative control. These results exemplify the specificity of the technique used.

Our data show that the aptitude to release H_2S is fully preserved after the H_2S-donor scaffold is covalently conjugated in our compounds, since **5**–**8a** showed a good ability in releasing a significant amount of H_2S in the μM range. In fact, all the synthesized compounds have shown an equal or higher ability in releasing H_2S than the prototypic compound **1a** with the maximum release recorded after 60 min of incubation that correspond with the latest recorder time point. Compounds **5a** and **8a** released 51.7 and 46.7 μM of H_2S respectively, after 1 h of incubation, whereas compounds **6a** and **7a** released H_2S at a slower pace, yielding values of 41.8 and 38.4 μM, respectively.

2.5. Effects of compounds on capsaicin-induced mechanical hypersensitivity

We tested the effects of compounds **1a**,**b** and BD-1063 on sensory hypersensitivity in mice. We used capsaicin-induced mechanical hypersensitivity (allodynia) as a pain model, as it is well known that the increase in sensitivity to pain in the area surrounding capsaicin injection results from central sensitization, which is a key process in chronic pain development and maintenance [33]. In addition, changes in capsaicin-induced mechanical hypersensitivity have been used to

study the behavioral consequences of drug treatment in central sensitization in both humans and rodents [24, 34, 35]. Importantly, capsaicin-induced mechanical hypersensitivity has been used repeatedly to determine the σ_1 agonistic/antagonistic properties of new compounds (including clinical candidates) [36-38], as σ_1 antagonists are able to decrease sensory hypersensitivity while σ_1 agonists reverse the effects of the former [35].

The subcutaneous (s.c.) administration of either BD-1063 or **1a** resulted in a marked dose-dependent reduction of mechanical hypersensitivity (Figure 4A), although **1a** exhibited a higher potency than BD-1063, with ED_{50} values of 6.12 ± 0.34 and 10.31 ± 0.49 mg/kg for **1a** and BD-1063, respectively. The administration of the amide analogue **1b** showed only limited effects at the higher doses tested (Figure 4A). The administration of PRE-084 (**12**, Figure S1) was able to reverse the effect of BD-1063 but not the effect of **1a** (Figure 4B), indicating that σ_1 receptor agonism exclusively decreases the effect of σ_1 antagonism on sensory hypersensitivity without affecting the effect induced by the H_2S release.

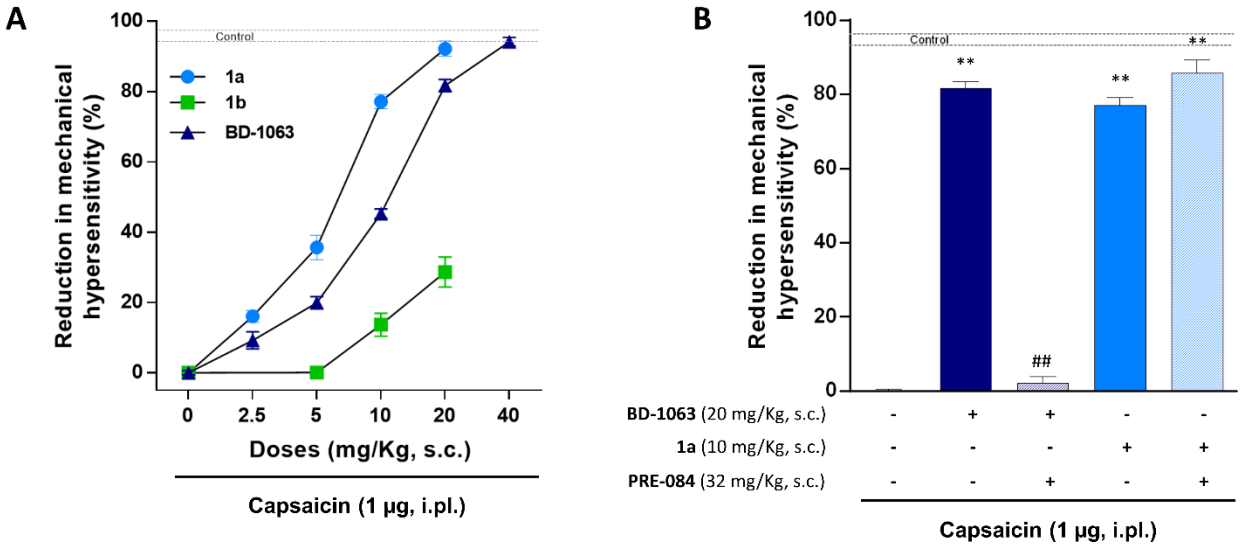


Figure 4. Reduction of capsaicin-induced mechanical hypersensitivity by the systemic administration of **1a**, **1b** and BD-1063 in mice and contribution of σ_1 receptor to their effects. A)

Dose dependency of the antinociceptive effects of the subcutaneous (s.c.) administration of **1a**, **1b** and BD-1063. B) Effects of BD-1063 and compound **1a** alone and in combination with the σ_1 receptor agonist PRE-084. Values are the mean ± SEM obtained from 6–9 animals per group (**$p<0.01$ vs Ctrl, $^{\#\#}p<0.01$ vs BD-1063; one-way ANOVA followed by Student-Newman-Keuls test).

We then evaluated whether the concurrent administration to mice of **1a** and BD-1063 resulted in a supra-additive (synergistic) effect on mechanical hypersensitivity. We tested the association of both compounds in a 1:1 weight ratio and performed isobolographic analyses (see "Experimental Section" for details). The value experimentally determined for the drug association (Zexp) at the ED_{50} level fell on the additivity line and was undistinguishable from the theoretical value predicted for an additive effect (Zadd) (Figure 5A and Table 2). Therefore, the interaction index (ɣ) (which is calculated as Zexp/Zadd) yielded a value close to 1 (Table 2) and this is interpreted as no interaction between treatments [39, 40]. However, when examining the *in vivo* effects of the association between **1a** and BD-1063 at the ED_{75} level we found different results, as the Zexp was below the additivity line and was significantly smaller than the Zadd value, yielding a ɣ value of 0.75 (Figure 5B, and Table 2). These results suggest that there is an apparent synergistic effect of the drug association at the ED_{75} level [39, 40]. Interestingly, the drug combination exhibited an even stronger synergism at the ED_{90} level, as the Zexp was even more distant to the additivity line, and highly significantly different from Zadd, yielding a ɣ value of 0.58 (Figure 5C, and Table 2).

These results indicate that the synergism induced by the administration of both compounds is stronger as the expected antiallodynic effect is more prominent. Therefore, we performed further experiments on the association of **1a** and BD-1063, both at 5 mg/kg, as this combination

produced a robust synergistic decrease of mechanical hypersensitivity (approximately 80%) (Figure 5D). To test for σ_1 receptor involvement on the effects induced by the association of both compounds, mice were treated with the selective σ_1 receptor agonist PRE-084, revealing a marked reversion of the effects. These results indicate that the σ_1 receptor component played an important role on the pronounced effects induced by the association between BD-1063 and **1a**. Notably, the effects of BD-1063 were not enhanced by **1b** (Figure 5D) (i.e. in the absence of H_2S release). Taken together, these results suggest that the σ_1 antagonism and the H_2S release are both needed for the marked effect on the sensory hypersensitivity induced by the association of **1a** with BD-1063, and that we can control for the contribution of both mechanisms by using PRE-084 and testing analog compounds lacking the thioamide function, respectively.

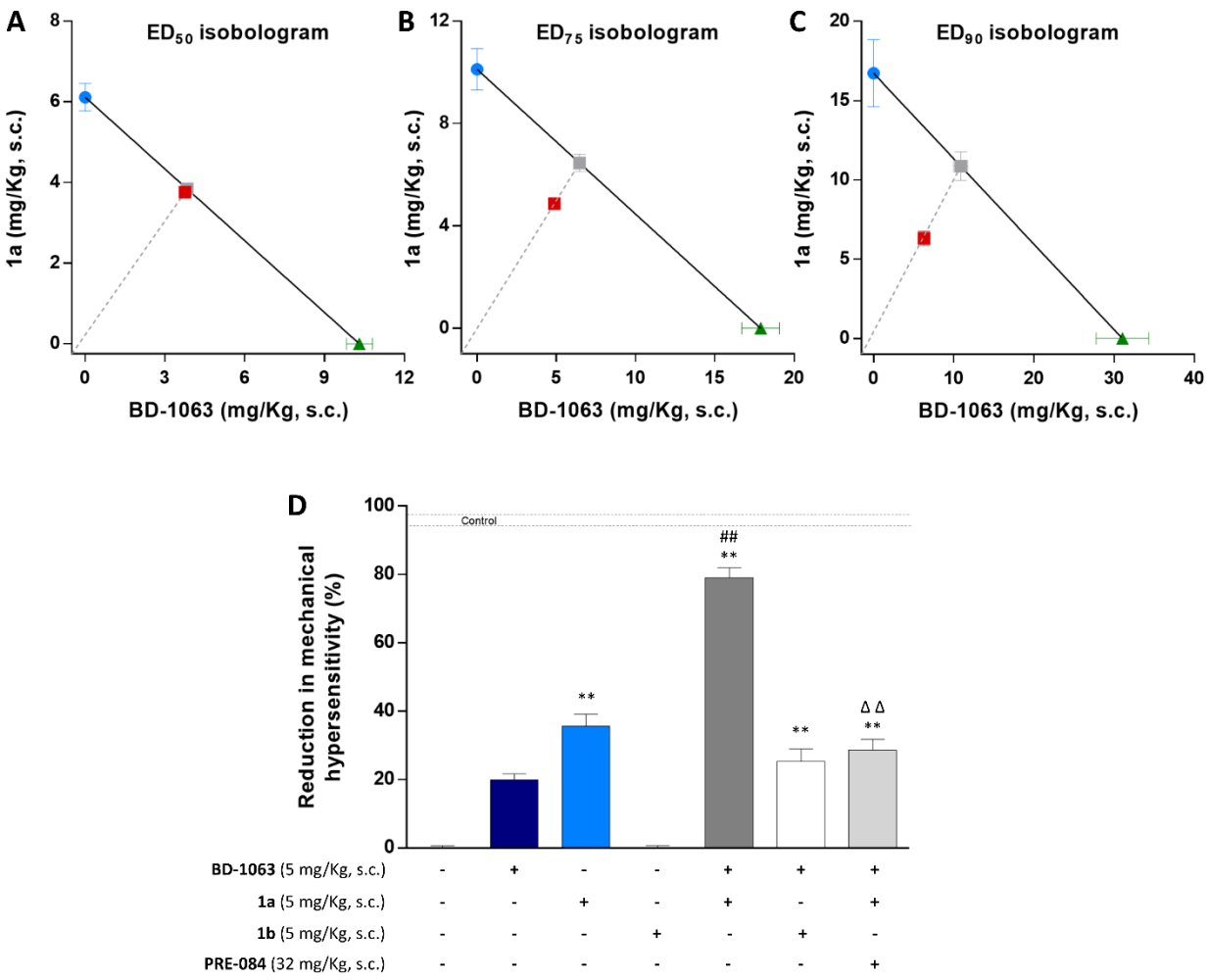


Figure 5. Effects on capsaicin-induced mechanical hypersensitivity of the combination of **1a** and BD-1063. Isobolograms at ED_{50} (A), ED_{75} (B) and ED_{90} (C). The graph shows the individual ED values of each compound (blue circles for **1a** and green triangles for BD-1063) and the line of additivity connecting them, the theoretical calculated ED value for an additive effect (Zadd, grey squares), and the corresponding experimental values (Zexp, red squares). (D) Reduction of capsaicin-induced mechanical hypersensitivity by the s.c. administration of compounds **1a** and **1b** alone and in combination with the selective σ_1 receptor antagonist BD-1063 (all at 5 mg/kg) and the σ_1 receptor agonist PRE-084 (32 mg/kg) in mice. Values are the mean ± SEM obtained from 6–9 animals per group (**$p<0.01$ vs Ctrl, $^{\#\#}p<0.01$ vs **1a** or BD-1063, $^{\Delta\Delta}p<0.01$ vs **1a** + BD-1063; one-way ANOVA followed by Student-Newman-Keuls test).

Table 2. Theoretical (Zadd) and experimental (Zexp) ED_{50}, ED_{75} and ED_{90} values, and interaction index for the 1:1 weight combination of BD-1063 and **1a**.

	Zadd (mg/kg, s.c.)	Zexp (mg/kg, s.c.)	Interaction index
ED_{50}	7.68 ± 0.28	7.52 ± 0.27	0.98
ED_{75}	12.93 ± 0.67	9.75 ± 0.45**	0.75
ED_{90}	21.74 ± 1.79	12.63 ± 0.90**	0.58

Statistically significant differences between Zexp and their respective Zadd: **p <0.01 (Student's t-test).

We thus tested the effects of compounds **5–8** on sensory hypersensitivity in mice. Compound **5a** showed a reversion of capsaicin-induced mechanical hypersensitivity in a dose dependent manner, and with a very high potency. However, the negative control **5b** induced very similar effects when tested at the same doses (Figure S2A). Accordingly, the H_2S release is not participating in the range of doses in which compound **5a** shows the ameliorative effect on sensory hypersensitivity, and that the σ receptors component (or additional activities) are exclusively responsible for the effects observed. The derivative **6a**, lacking the *p*-F-phenyl fragment of **5a**, was not able to completely reverse the sensitizing effect of capsaicin even at high doses (Figure S2A).

Compound **7a** showed a prominent *in vivo* effect, markedly reducing capsaicin-induced mechanical hypersensitivity, whereas the derivative **7b** lacking the thioamide function had minimal effect. This result is a clear evidence of the H_2S contribution to the analgesic effect of **7a** (Figure 6A). The administration of the σ_1 receptor agonist PRE-084 nearly abolished the effect of **7a** at low doses of the later (20 mg/kg), but the effect of σ_1 receptor agonism gradually disappeared when increasing the dose of **7a**, so that at the dose of 40 mg/kg the effect of PRE-084 was not significant (Figure 6B). It is important to note that the dose of 32 mg/kg s.c. of PRE-084 has been previously shown to fully reverse in mice the effects of high doses (64–128 mg/kg)

of S1RA [41, 42], a high affinity and highly selective σ_1 receptor antagonist currently in clinical trials for pain treatment [43]. The findings of these previous studies suggest that this dose of PRE-084 is enough to fully mask the effects of σ_1 receptor antagonism even if the antagonist is administered at high doses. Therefore, our results showing that moderate doses of **7a** are still able to reduce mechanical hypersensitivity in spite of the administration of PRE-084 suggest that additional mechanisms, such as H_2S-mediated actions, participate on the reduction of capsaicin-induced mechanical hypersensitivity induced by this compound. In other words, compound **7a** elicits a qualitatively equivalent antiallodynic effect with respect to the combination of **1a** and BD-1063 (in both cases the effect can be reversed by PRE-084 and it seems dependent on the release of H_2S, as the amide controls were nearly ineffective), suggesting that σ_1 antagonism and H_2S release simultaneously participate on the effects observed in both situations. The use of a single multi-target compound is preferred over the combination of two drugs with a single mechanism of action, since in this latter case, the two compounds must be compatible in terms of pharmacokinetics and potential drug-drug interactions [17]. Therefore, in this sense, **7a** is superior to the combination of a pure σ_1 antagonist and a pure H_2S releasing compound.

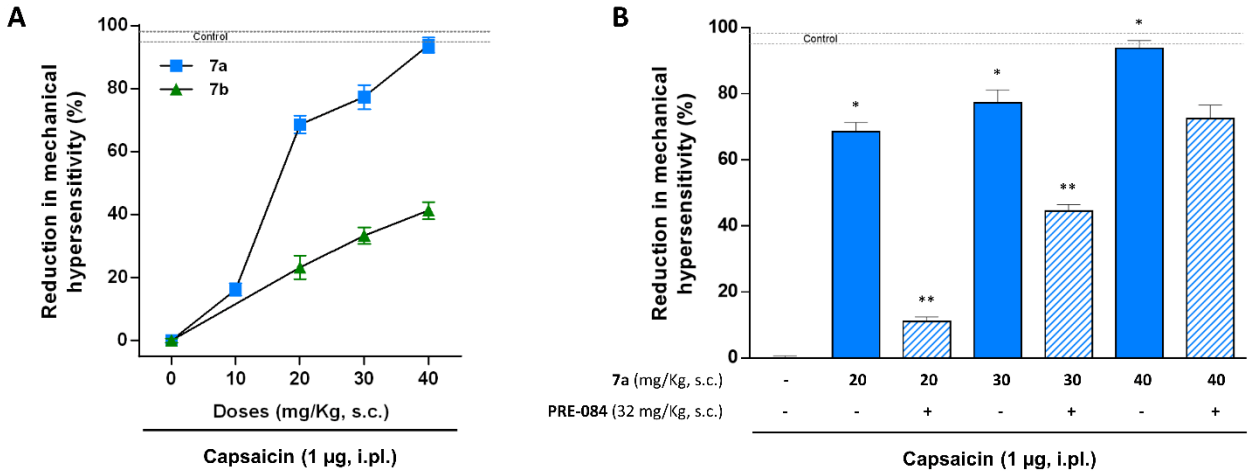


Figure 6. Reduction of capsaicin-induced mechanical hypersensitivity by the systemic administration of **7a** and **7b** in mice, and contribution of σ_1 receptor to their effects. A) Dose dependency of the effects of the subcutaneous (s.c.) administration of **7a** and **7b**. B) Effects of compound **7a** alone and in combination with the σ_1 receptor agonist PRE-084. Values are the mean ± SEM obtained from 6–9 animals per group (*p<0.01 *vs* Ctrl, **p<0.01 *vs* **7a**; one-way ANOVA followed by Student-Newman-Keuls test).

Derivative **8a** was able to decrease mechanical hypersensitivity in a dose dependent manner. When PRE-084 administration was associated to **8a**, at the highest dose tested (40 mg/kg), it resulted in a significant reversion of the effect, indicating the involvement of σ_1 receptor antagonism in the observed effect. However, the amide control **8b** showed similar or even higher analgesic effects when evaluated at the same doses (Figure S2A). It must be noted that compound **8b** had no affinity at σ receptors (Table 1), and in fact its effect was not reversed by PRE-084 (Figure S2B). Thus, the observed *in vivo* effects induced by **8b** are purportedly related to off-target activity. It may be speculated that compound **8a**, although showing a low affinity for σ_1 receptor ($K_i\sigma_1$ 668 nM), might provide a good analgesic *in vivo* activity due to the possible enhancement of the H_2S and σ_1 receptor antagonism combination.

2.6. Safety pharmacology study

Adverse drug reactions are a major problem in drug development [44]. Therefore, once we demonstrated the efficacy of the combination of σ_1 antagonism and H_2S release on an *in vivo* pain model, we tested whether this was accompanied with an apparent toxicity.

The assessment of motor coordination is part of the core battery for CNS safety pharmacology recommended by regulatory agencies [45]. The assessment of drug-induced motor impairment is

particularly relevant for the interpretation of the results from tests for nociception, since animals need to have preserved motor coordination for the performance of the nociceptive responses, which are typically reflex responses. Pharmacological treatment affecting motor functioning might also attenuate nociceptive responses and thereby induce false analgesic-like effects [2]. Therefore, we treated animals with **1a** (5 mg/kg) + BD-1063 (5 mg/kg) or with **7a** (40 mg/kg), at doses able to induce a marked effect on mechanical hypersensitivity, and submitted them to the rotarod test, which is the most standard test for motor coordination in preclinical research. As shown in Figure 7, animals treated with these compounds showed no change in the latency to fall down from the rotating drum in comparison to the baseline value (time 0), at any time-point tested during the 4 h evaluation period. Hence, the results found on capsaicin-induced mechanical hypersensitivity for either the association of the prototypic σ_1 antagonist and H_2S releaser compounds, or our molecule **7a** which combine simultaneously both mechanisms, cannot be attributed to motor impairment. The lack of effect in modifying rotarod latencies of these compounds was not due to any methodological pitfall, because the administration of pregabalin, used as a positive control of a drug known to induce motor deficits [46], induced significantly reduced rotarod latencies (Figure 7).

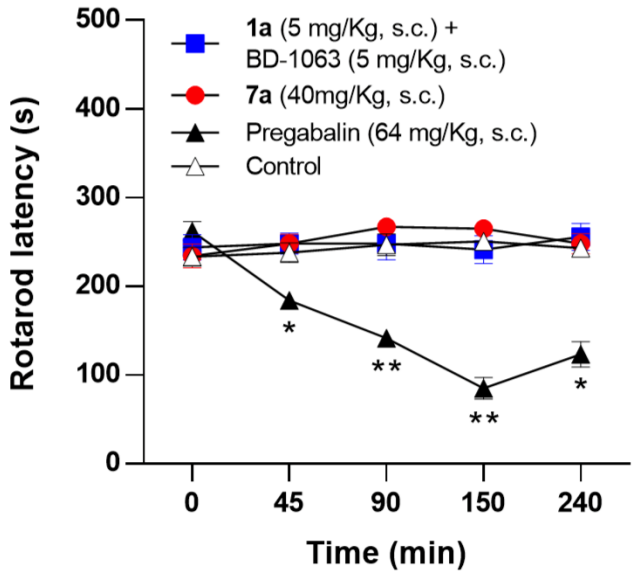


Figure 7. Effect of **1a** + BD-1063, **7a** and pregabalin on motor coordination. The latency to fall-down from the rotarod was recorded in each mouse immediately before (time 0) and at several times after the following subcutaneous (s.c.) treatments: **1a** (5 mg/kg) + BD-1063 (5 mg/kg), **7a** (40 mg/kg), or pregabalin (64 mg/kg). Values are the mean ± SEM from 6–8 animals (significant differences between the values at time 0 and after drug administration: *p<0.05, **p <0.01; 2-way repeated measures ANOVA followed by Student-Newman-Keuls test).

Cardiovascular events are among the most frequent adverse events leading to the failure of drugs in development [47]. We therefore performed electrophysiological assays to test whether compound **7a** blocks the hERG (human ether-a-go-go-related gene) potassium channel activity, which is known to be related to potentially lethal ventricular arrhythmias [47]. Compound **7a** did not block K^+ current up to 1 μM concentration, and induced a 60% inhibition at 10 μM (Table S1) with an estimated IC_{50} of 6.8 μM. This IC_{50} for hERG blockade contrast with the K_i for σ_1 receptor binding, which as commented above is of 94 nM. Therefore, there is a wide window between the affinity of **7a** for one of its main targets and this troublesome off-target effect.

Testing for hERG inhibition is part of the standard core battery of cardiovascular safety pharmacology of regulatory agencies for new compounds [47], together with other important parameters such as blood pressure [45]. This latter measure is particularly relevant for H_2S releasers, as it has been proposed that these compounds might serve to treat hypertension [48]. In the context of a normotensive animal, drug-induced hypotension would be an unwanted side effect. Therefore, we measured the mean arterial blood pressure (MABP) by direct recording in left carotid artery in animals treated with **7a** [49], at doses able to induce a marked effect on mechanical hypersensitivity (30 and 40 mg/kg, s.c.). No differences were found in blood pressure between the groups of animals when receiving vehicle or compound **7a** (Figure 8). Therefore, H_2S released by analgesic doses of **7a** is not enough to alter MABP.

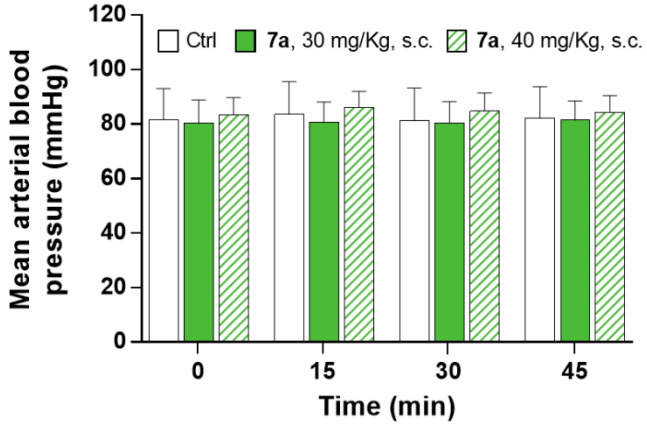


Figure 8. Effect of **7a** on mean arterial blood pressure (MABP). MABP was measured by direct recording in the left carotid artery immediately before (time 0) and at several times after the subcutaneous (s.c.) administration of **7a** (30 or 40 mg/kg) or its solvent. Values are the mean ± SEM obtained from 5–6 animals per group. There were no statistically significant differences between the groups at any time point tested (one-way ANOVA followed by Student-Newman-Keuls test).

2.7. Stability and solubility profile

The water solubility and chemical stability for compound **7a** were experimentally determined, while partition coefficients theoretically calculated (Table 3). Compound **7a** (free base) displayed a water solubility of 1.25 mM (0.44 mg/mL) at rt. The chemical stability was evaluated *in vitro* at 37 °C in an aqueous phosphate buffer solution at pH 7.4 and in saline solution at rt monitored as reduction of thioamide **7a** peak. Compound **7a** showed optimal stability in aqueous phosphate buffer solution at pH 7.4 having a $t_{1/2}$ equal to 93 min allowing for a good time frame for H_2S activity once applied to biological systems. Furthermore, compound **7a** resulted to be stable in saline solution at rt. Indeed, compound **7a** has a low onset for H_2S release in saline solution having a $t_{1/2}$ equal to 250 min. This corresponds to a negligible loss of H_2S due to hydrolysis of the compound and potential H_2S volatilization giving a useful time window for compound handling and optimal *in vivo* administration. Partition coefficients logP and logD were of 2.5 and 1.4, respectively, demonstrating an overall good distribution between hydrophilic and lipophilic phases. Finally, compound **7a** was evaluated in silico for its brain/plasma distribution by a statistical method previously developed by the authors [50]. As can be noted in Table S2, compound **7a** shows four computed descriptors in the BBB^+ scenario (logP, polar surface area, oxygen atom count and ionization state), three in the BBB^+/BBB^- scenario (hydrogen bound acceptor, nitrogen atom count and nitrogen-oxygen count), while two in the BBB^- scenario (logD and hydrogen bound donor) indicating a balanced distribution inside and outside the blood–brain barrier. It is worth mentioning that σ_1 receptors are expressed at highest levels in the peripheral than in the central nervous system, and recent studies show that these peripheral σ_1 receptors play a pivotal role on pain processing [43]. Compound **2a** has been

also included as reference compound (Table S3). It can be noted that compound **2a**, with a logBB of 1.34, has a more BBB^+ oriented probabilistic scenario [50].

Table 3. Water solubility, partition coefficients and chemical stability for compound **7a**.

	Medium	$t_{1/2}$ (min)
chemical stability[a]	Phosphate buffer pH 7.4	93
	Saline solution	250
water solubility (mM)[a]		1.25
LogP[b]		2.5
LogD[b]		1.4

[a]Each value is the mean ± SD of three experiments performed in triplicate. [b]*n*-Octanol/water partition coefficients were theoretically calculated by the ChemAxon program JChem for Excel 19.9.0.467.

3. Conclusion

This study describes the development of novel hybrid compounds able to release H_2S and to bind σ_1 receptor as candidates for pain treatment. We first started to investigate the role of the σ_1 receptor antagonist BD-1063 or H_2S-donor alone (**1a**) and the association of both mechanisms in capsaicin-induced mechanical hypersensitivity. Later, four hybrid ligands have been developed (**5**–**8a**) having a 4-carbamothioylphenyl moiety covalently joined to appropriate σ receptor ligands. To better dig into the precise mechanism, cognate derivatives lacking the thioamide function have been prepared as negative control (**5**–**8b**). All the compounds have been evaluated for affinity at σ and opioid receptors and ability to release H_2S. The analgesic properties of the synthetized ligands have been evaluated, and a pair of compounds (**7a**,**b**) has been identified to reproduce the effect of the combination of **1a** and BD-1063 (i.e. the effect of **7a** can be reversed

by PRE-084 and it was dependent on H_2S release, as the amide control was nearly ineffective). The other pairs of compounds have been excluded for several reasons including imbalanced sigma receptor affinity (**5a**,**b**), no maximal effect reached (**6a**,**b**), and off-target effects (**8a**,**b**). The candidate with the desired *in vivo* pharmacological profile (**7a**) has been further evaluated to assess its safety profile, including the blockade of hERG, an off-target related to cardiac toxicity, changes in mean arterial blood pressure and motor-coordination by rotarod performance. The *in vitro* pharmacokinetic profile has been also evaluated, including water solubility and chemical stability. All these results suggest that the novel developed hybrid σ_1 antagonist/H_2S donor **7a** deserves further investigation for its potential use in the management of pain conditions.

4. Experimental section

4.1. Chemistry

4.1.1. General remarks

Reagent grade chemicals were purchased from Carlo Erba (Milano, Italy), Fluorochem (Hadfield, Derbyshire, England), Merck KGaA (Darmstadt, Germany) and Tokyo Chemical Industry (Tokyo, Japan), and were used without further purification. All reactions involving air-sensitive reagents were performed under N_2 in oven-dried glassware using the syringe-septum cap technique. Flash chromatography purification was performed on a Merck silica gel 60 (40–63 μm; 230–400 mesh) stationary phase. Nuclear magnetic resonance spectra (^{1}H NMR and ^{13}C NMR recorded at 200 and 500 MHz) were obtained on VARIAN INOVA spectrometers using $CDCl_3$ or DMSO-d_6. Trimethylsilane (TMS) was used as internal standard. Chemical shifts (δ) are given in parts per million (ppm) and coupling constants (*J*) in Hertz (Hz). The following abbreviations are used to designate the multiplicities: s = singlet, d = doublet, t = triplet, m =

multiplet, br = broad. The purity of all tested compounds, whether synthesized or purchased, reached at least 95% as determined by microanalysis (C, H, N) that was performed on a Carlo Erba instrument model E1110; all the results agreed within ± 0.4% of the theoretical values. Reactions were monitored by thin-layer chromatography (TLC) performed on 250 μm silica gel Merck 60 F_{254} coated aluminum plates; the spots were visualized by UV light or iodine chamber. Compound nomenclatures were generated with ChemBioDraw Ultra version 16.0.0.82. Analytical UHPLC analysis was performed using a Thermo Scientific UHPLC/PAD-FL Chromatography UltiMate3000 RSLC system. Compound detection utilized photodiode array detector (PAD) set at λ=200, 209, 254 and 290 nm. The column was a Waters Acquity C18 1 mm × 50 mm, 1.8 μm particle size, running H_2O/acetonitrile gradient with a 200 μL/min flow. UHPLC-MS grade solvents were purchased from Carlo Erba (Milano, Italy) and used without further purification. The compound solutions to be analyzed were first filtered with 0.45 μm/13 mm cellulose acetate syringe filters (Carlo Erba, Milano, Italy).

4.1.2. 4-carbamothioylbenzoic acid (**1a**). P_4S_{10} (6.79 mmol, 1.5 g) was added to cold EtOH (10 mL) and the solution was stirred for 1 h at rt. Then, 4-cyanobenzoic acid (3.40 mmol, 0.5 g) was added in one portion and the resulting mixture was heated under reflux for 5 h. Reaction mixture was poured into ultrapure ice-water and the precipitated was filtered off. The obtained solid was dissolved in EtOAc and washed with $NaHCO_3$. The organic layer was removed, and the aqueous was acidified with HCl (pH 1–2) (10 mL) and extracted with EtOAc (25 mL), brine (5 mL), and dried under anhydrous Na_2SO_4. The solvent was removed under vacuum to provide the desired product that was used as it without further purification. Yield: 70 %, yellow solid. ^{1}H NMR (500 MHz, DMSO-d_6) δ 12.99−13.31 (m, 1H), 10.02 (br. s., 1H), 9.63 (br. s., 1H), 7.86−8.00 (m, 4H).

^{13}C NMR (125 MHz, DMSO-d_6) δ 199.4, 166.7, 143.2, 128.8, 127.3. Anal. calcd for $C_8H_7NO_2S$: C, 53.03; H, 3.89; N, 7.73. Found: C, 53.13; H, 3.90; N, 7.74.

4.1.3 4-(4-chlorophenyl)-1-(4-hydroxybutyl)piperidin-4-ol (**2**). To a solution of (4-chlorophenyl)piperidin-4-ol (1.42 mmol, 0.3 g) in ACN (10 mL), 4-chloro-1-butanol (1.42 mmol, 0.13 mL) and $KHCO_3$ (2.84 mmol, 0.28 g) were added and the mixture was refluxed for 4 h. The reaction diluted with EtOAc (10 mL), washed with $NaHCO_3$ saturated solution (2 x 5 mL), and brine (5 mL), and dried under anhydrous Na_2SO_4. The solvent was removed under vacuum and the residue purified via silica gel chromatography with EtOAc and then 5% MeOH in EtOAc. Yield: 80%, white solid. ^{1}H NMR (200 MHz, $CDCl_3$) δ 7.42 (d, *J* = 8.5 Hz, 2H), 7.28 (d, *J* = 8.5 Hz, 2H), 3.39−3.73 (m, 2H), 2.74−2.99 (m, 2H), 2.33−2.66 (m, 4H), 1.93−2.28 (m, 2H), 1.56−1.85 (m, 6H). Anal. calcd for $C_{15}H_{22}ClNO_2$: C, 63.48; H, 7.81; N, 4.94. Found: C, 63.55; H, 7.82; N, 4.95.

4.1.4. 4-(4-chlorophenyl)-1-(4-(4-fluorophenyl)-4-hydroxybutyl)piperidin-4-ol (**4**). To a solution of **2a** (1.33 mmol, 0.5 g) in EtOH (50 mL), $NaBH_4$ (1.33 mmol, 0.051 g) was added at 0 °C. The mixture was stirred at rt for 12 h and quenched with 20 mL ultrapure water. The mixture has been evaporated to remove EtOH and the residue diluted with saturated Na_2CO_3 solution and extracted with CH_2Cl_2 (10 mL). The combined organic layers were dried over anhydrous Na_2SO_4, filtered, and evaporated to dryness. Yield: 97 %, white solid. ^{1}H NMR (200 MHz, $CDCl_3$) δ 7.21−7.57 (m, 6H), 6.93−7.07 (m, 2H), 4.58−4.70 (m, 1H), 3.03 (d, *J* = 11.07 Hz, 1H), 2.75−2.91 (m, 1H), 2.39−2.71 (m, 4H), 2.09−2.33 (m, 2H), 1.58−2.05 (m, 6H). Anal. calcd for $C_{21}H_{25}ClFNO_2$: C, 66.75; H, 6.67; N, 3.71. Found: C, 66.88; H, 6.68; N, 3.72.

4.1.5. General procedure for the synthesis of compounds **5**−**8.** To a solution of 4-carbamothioylbenzoic acid (**1a**) or 4-carbamoylbenzoic acid (1.01 mmol) in DMF (5 mL),

HOBT (1.53 mmol, 0.21 g), and EDC (1.52 mmol, 0.29 g) were added. After stirring for 20 min, the appropriate amine or alcohol (2.02 mmol) has been added and the resulting mixture was stirred at rt for 5 h. After the reaction was complete, it was dissolved in EtOAc (20 mL), washed with H_2O (2 x 10 mL), and brine (5 mL), and dried under anhydrous Na_2SO_4. The solvent was removed under vacuum and the residue purified via silica gel chromatography to provide the desired product.

4.1.5.1. 4-(4-(4-chlorophenyl)-4-hydroxypiperidin-1-yl)-1-(4-fluorophenyl)butyl 4-carbamothioylbenzoate (**5a**, **AD95**). The compound has been prepared using **1a** (1.01 mmol, 0.18 g) and **4** as alcohol (2.02 mmol, 0.76 g). The residue was purified with 5% EtOH in CH_3Cl. Yield: 65%, yellow solid. ^{1}H NMR (500 MHz, $CDCl_3$) δ 8.07 (d, *J* = 8.3 Hz, 2H), 7.90 (d, *J* = 8.3 Hz, 2H), 7.76 (br. s., 1H), 7.37−7.45 (m, 4H), 7.30−7.36 (m, 2H), 7.06 (t, *J* = 8.8 Hz, 1H), 6.00 (t, *J* = 6.8 Hz, 1H), 2.84 (br. s., 2H), 2.52 (d, *J* = 7.8 Hz, 4H), 2.06−2.27 (m, 4H), 1.53−2.05 (m, 4H). ^{13}C NMR (50 MHz, DMSO-d_6) δ 199.2, 164.5, 147.7, 143.8, 142.9, 131.3, 129.0, 128.4, 128.0, 127.6, 126.7, 125.0, 123.3, 118.5, 115.6, 115.1, 110.4, 88.9, 75.5, 68.5, 48.4. Anal. calcd for $C_{29}H_{30}ClFN_2O_3S$: C, 64.37; H, 5.59; N, 5.18. Found: C, 64,53; H, 5,61; N, 5.19.

4.1.5.2. 4-(4-(4-chlorophenyl)-4-hydroxypiperidin-1-yl)-1-(4-fluorophenyl)butyl 4-carbamoylbenzoate (**5b**, **AD162**). The compound has been prepared using 4-carbamoylbenzoic acid (1.01 mmol, 0.17 g) and **4** (2.02 mmol, 0.76 g). The residue was purified with 5% MeOH in CH_2Cl_2 and then 5% of 3% NH_4OH/MeOH solution in CH_2Cl_2. Yield: 60%, white solid. ^{1}H NMR (200 MHz, DMSO-d_6) δ 8.17 (s, 1H), 8.05−8.12 (m, 2H), 7.96−8.04 (m, 2H), 7.61 (s, 1H), 7.42−7.58 (m, 4H), 7.30−7.41 (m, 2H), 7.21 (t, *J* = 8.8 Hz, 2H), 5.91−6.06 (m, 1H), 4.77−5.01 (m, 1H), 2.55 (br. s., 2H), 2.18−2.44 (m, 4H), 1.73−2.15 (m, 4H), 1.38−1.66 (m, 4H). ^{13}C NMR (50 MHz, DMSO-d_6) δ 167.1, 164.6, 149.2, 138.8, 136.8, 132.1, 130.7, 129.2, 128.6, 128.6,

127.9, 127.7, 126.9, 115.5, 115.1, 99.8, 69.6, 49.2. Anal. calcd for $C_{29}H_{30}ClFN_2O_4$: C, 66.34; H, 5.76; N, 5.34. Found: C, 66.47; H, 5.77; N, 5.35.

4.1.5.3. 4-(4-(4-chlorophenyl)-4-hydroxypiperidin-1-yl)butyl 4-carbamothioylbenzoate (**6a, AD127**). The compound has been prepared using **1a** (1.01 mmol, 0.18 g) and **2** as alcohol (2.02 mmol, 0.57 g). The residue was purified with 5% EtOH in CH_3Cl. Yield: 65%, yellow solid. ^{1}H NMR (200 MHz, DMSO-d_6) δ 10.10 (br. s., 1H), 9.69 (br. s., 1H), 7.79−8.28 (m, 4H), 7.15−7.65 (m, 4H), 4.88 (s, 1H), 4.32 (t, *J* = 6.0 Hz, 2H), 2.65 (d, *J* = 10.1 Hz, 3H), 2.18−2.45 (m, 4H), 1.39−2.03 (m, 8H). ^{13}C NMR (50 MHz, DMSO-d_6) δ 200.2, 167.7, 146.6, 131.7, 128.4, 127.9, 127.1, 126.4, 67.5, 66.3, 56.9, 55.4, 33.5, 21.2, 20.5. Anal. calcd for $C_{23}H_{27}ClN_2O_3S$: C, 61.80; H, 6.09; N, 6.27. Found: C, 61.91; H, 6.10; N, 6.28.

4.1.5.4. 4-(4-(4-chlorophenyl)-4-hydroxypiperidin-1-yl)butyl 4-carbamoylbenzoate (**6b**, **AD160**). The compound has been prepared using 4-carbamoylbenzoic acid (1.01 mmol, 0.17 g) and **2** (2.02 mmol, 0.57 g). The residue was purified with 5% MeOH in CH_2Cl_2. Yield: 80%, white solid. ^{1}H NMR (200 MHz, DMSO-d_6) δ 8.17 (s, 1H), 7.90−8.09 (m, 8H), 7.59 (br. s., 1H), 4.23−4.42 (m, 4H), 3.28−3.49 (m, 4H), 1.53−1.91 (m, 4H), 1.27−1.45 (m, 2H), 1.01−1.20 (m, 2H). ^{13}C NMR (50 MHz, DMSO-d_6) δ 167.0, 165.3, 149.2, 138.4, 132.1, 130.7, 129.2, 127.8, 126.7, 69.6, 49.1, 29.0, 23.0. Anal. calcd for $C_{23}H_{27}ClN_2O_4$: C, 64.11; H, 6.32; N, 6.50. Found: C, 64.24; H, 6.33; N, 6.49.

4.1.5.5. *N*-(1-benzylpiperidin-4-yl)-4-carbamothioylbenzamide (**7a**, **AD164**). The compound has been prepared using **1a** (1.01 mmol, 0.18 g) and 1-benzylpiperidin-4-amine (2.02 mmol, 0.38 g). The residue was purified with 4% MeOH in CH_2Cl_2. Yield: 50%, yellow solid. ^{1}H NMR (200 MHz, DMSO-d_6) δ 10.00 (br. s., 1H), 9.61 (br. s., 1H), 8.36 (d, *J* = 7.4 Hz, 1H), 7.76−8.03 (m, 4H), 7.14−7.44 (m, 5H), 3.75 (br. s., 1H), 3.46 (br. s., 2H), 2.82 (d, *J* = 9.8 Hz, 2H), 2.01 (t, *J* =

10.9 Hz, 2H), 1.46−1.88 (m, 4H). ^{13}C NMR (50 MHz, DMSO-d_6) δ 199.3, 164.9, 141.4, 136.7, 128.8, 128.2, 127.1, 126.8, 52.2, 31.5. Anal. calcd for $C_{20}H_{23}N_3OS$: C, 67.96; H, 6.56; N, 11.89. Found: C, 68.09; H, 6.57; N, 11.91.

4.1.5.6. *N*-(1-benzylpiperidin-4-yl)terephthalamide (**7b**, **AD163**). The compound has been prepared using 4-carbamoylbenzoic acid (1.01 mmol, 0.17 g) and 1-benzylpiperidin-4-amine (2.02 mmol, 0.38 g). The product is purified by precipitation in EtOAc. Yield: 63%, white solid. ^{1}H NMR (200 MHz, DMSO-d_6) δ 8.37 (d, *J* = 7.4 Hz, 1H), 8.09 (s, 1H), 7.83−8.01 (m, 4H), 7.50 (s, 1H), 7.18−7.41 (m, 5H), 3.77 (d, *J* = 7.0 Hz, 1H), 3.46 (s, 2H), 2.69−2.91 (m, 2H), 1.89−2.12 (m, 2H), 1.43−1.86 (m, 4H). ^{13}C NMR (50 MHz, DMSO-d_6) δ 167.2, 165.0, 138.6, 137.0, 136.3, 128.7, 128.1, 127.3, 127.1, 126.8, 62.1, 52.2, 31.4. Anal. calcd for $C_{20}H_{23}N_3O_2$: C, 71.19; H, 6.87; N, 12.45. Found: C, 71.33; H, 6.88; N, 12.42.

4.1.5.7. 4-(4-benzylpiperazine-1-carbonyl)benzothioamide (**8a**, **AD119**). The compound has been prepared using **1a** (1.01 mmol, 0.18 g) and 1-benzylpiperazine (2.02 mmol, 0.36 g). The residue was purified with 2−4% EtOH in CH_2Cl_2. Yield: 64%, yellow solid. ^{1}H NMR (200 MHz, DMSO-d_6) δ 10.00 (br. s., 1H), 9.60 (br. s., 1H), 7.90 (d, *J* = 8.2 Hz, 2H), 7.41 (d, *J* = 8.2 Hz, 2H), 7.20−7.36 (m, 5H), 3.62 (br. s., 2H), 3.50 (s, 2H), 3.31 (br. s, 2H), 2.36 (d, *J* = 1.6 Hz, 4H). ^{13}C NMR (50 MHz, DMSO-d_6) δ 199.3, 168.2, 140.2, 138.3, 128.9, 128.2, 127.4, 127.0, 126.5, 61.8, 22.6. Anal. calcd for $C_{19}H_{21}N_3OS$: C, 67.23; H, 6.24; N, 12.38. Found: C, 67.36; H, 6.25; N, 12.40.

4.1.5.8. 4-(4-benzylpiperazine-1-carbonyl)benzamide (**8b**, **AD120**). The compound has been prepared using 4-carbamoylbenzoic acid (1.01 mmol, 0.17 g) and 1-benzylpiperazine (2.02 mmol, 0.36 g). The residue was purified with 2–4% EtOH in CH_2Cl_2. Yield: 71%, white solid.

^{1}H NMR (200 MHz, DMSO-d_6) δ 8.05 (br. s., 1H), 7.85−7.97 (m, 2H), 7.40−7.50 (m, 3H), 7.20−7.37 (m, 5H), 3.62 (br.s., 2H), 3.50 (s, 2H), 3.25−3.41 (m, 4H), 2.37 (d, *J* = 4.3 Hz, 2H). ^{13}C NMR (50 MHz, DMSO-d_6) δ 168.3, 167.2, 138.5, 137.8, 135.0, 128.9, 128.2, 127.6, 126.8, 61.8. Anal. calcd for $C_{19}H_{21}N_3O_2$: C, 70.57; H, 6.55; N, 12.99. Found: C, 70.71; H, 6.56; N, 13.01.

4.2. Receptors radioligand binding assays

4.2.1. Materials

Brain and liver homogenates for σ_1 and σ_2 receptors, MOR, DOR, and KOR binding assays were prepared from male Dunkin-Hartley guinea pigs and Sprague Dawley rats (Italian Minister of Health project code 335/1984F.N.JL.T; ENVIGO RMS S.R.L., Udine, Italy). Animals (200−250 g) were euthanized with CO_2 in a euthanasia chamber and sacrificed by decapitation. [^{3}H](+)-Pentazocine (26.9 Ci/mmol) (Italian Minister of Health permit to import and use SP/051 10/03/2019), [^{3}H]1,3-di-o-tolylguanidine ([^{3}H]DTG, 35.5 Ci/mmol), [^{3}H]-DAMGO (48.4 Ci/mmol), [^{3}H]-(2-D-Ala)-[Tyrosyl-3,5-] DELTORPHIN II (54.7 Ci/mmol) and [^{3}H]-U69,593 (49.3 Ci/mmol) were purchased from PerkinElmer (Zaventem, Belgium). Unlabeled (+)-pentazocine was prepared by alkylation of (+)-normetazocine as reported in Scheme S1 (Italian Minister of Health permit to produce and use SP/072 05/04/2019). (+)-Normetazocine was obtained by separation from the racemic mixture (±)-normetazocine that was gently provided by Fabbrica Italiana Sintetici (Montecchio Maggiore, Italy). Unlabeled naloxone hydrochloride and DAMGO were purchased from Tocris (Cookson, MI, USA). (–)-U50,488 and naltrindole hydrochloride were from Sigma-Aldrich (St. Louis, MO, USA). Ultima Gold MV Scintillation cocktail was from PerkinElmer (Milano, Italy). All the other materials were obtained from Merck KGaA (Darmstadt, Germany). The test compound solutions were prepared by dissolving

approximately 10 µmol of test compound in DMSO so that a 10 mM stock solution was obtained. The required test concentrations for the assay (σ receptors assays from 10^{-5} to 10^{-11} M; opioid receptors assay from 10^{-5} to 10^{-9} M) have been prepared by diluting the DMSO stock solution with the respective assay buffer. All experiments were performed using ultrapure water obtained with a Millipore Milli-Q Reference Ultrapure Water Purification System (Millipore, Burlington, MA, USA). Membrane homogenates have been prepared with a Dounce glass homogenizer (Wheaton, Millville, NJ, USA) with a loose inner tolerance pestle first and a tight inner tolerance pestle later in a cylindrical glass tube of 40 mL volume. Centrifugations have been accomplished using a Beckmann J2-20 centrifuge and a JA-21 rotor with 40 mL volume tubes (Beckman Coulter, Brea, CA, USA). The bound radioactivity has been determined using a Beckman LS 6500 liquid scintillation counter (Beckman Coulter, Brea, CA, USA).

4.2.2. Preparation of membrane homogenates from guinea pig brain for σ_1 receptor binding assay

Fresh guinea pig brain cortices were homogenized ice-cold Tris (50 mM, pH 7.4) containing 0.32 M sucrose. The suspension was centrifuged at 1,030 × *g* for 10 min at 4 °C. The supernatant was separated and centrifuged at 41,200 × g for 20 min at 4 °C. The obtained pellet was suspended in ice-cold Tris (50 mM, pH 7.4), incubated at rt for 15 min and centrifuged at 41,200 × g for 15 min at 4 °C. The final pellet was resuspended with ice-cold Tris buffer, and frozen at -80 °C in ~1 mL portions containing about 5 mg protein/mL.

4.2.3. Preparation of membrane homogenates from guinea pig brain for KOR binding assay

Guinea pig brain were homogenized in ice-cold Tris buffer (50 mM, pH 7.4). The suspension was centrifuged at 40,000 × *g* for 20 min at 4 °C. The pellet was resuspended in ice-cold Tris buffer, incubated at 37 °C for 30 min and centrifuged at 40,000 × *g* for 20 min at 4 °C. The final

pellet was resuspended in ice-cold Tris buffer and frozen at -80 °C in ~1 mL portions containing about 10 mg protein/mL.

4.2.4. Preparation of membrane homogenates from rat liver for σ_2 receptor binding assay

Rat livers were homogenized with cold 0.32 M sucrose. The suspension was centrifuged at 1,030 × *g* for 10 min at 4 °C. The supernatant was separated and centrifuged at 31,100 × *g* for 20 min at 4 °C. The pellet was resuspended in ice-cold Tris buffer (50 mM, pH 8) and incubated at rt for 30 min. Then, the suspension was centrifuged at 31,100 × *g* for 20 min at 4 °C. The final pellet was resuspended in ice-cold Tris buffer and stored at -80 °C in ~1 mL portions containing about 6 mg protein/mL.

4.2.5. Preparation of membrane homogenates from Sprague Dawley rats for MOR and DOR binding assay

Sprague Dawley rat brains were homogenized in ice-cold Tris buffer (50 mM, pH 7.4). The suspension was centrifuged at 40,000 × *g* for 20 min at 4 °C. The pellet was resuspended in ice-cold Tris buffer, incubated at 37 °C for 30 min and centrifuged at 40,000 × *g* for 20 min at 4 °C. The final pellet was resuspended in ice-cold Tris buffer and frozen at -80 °C in ~1 mL portions containing about 10 mg protein/mL.

4.2.6. Protein determination

The protein concentration was determined by the method of Bradford. The Bradford solution was prepared by dissolving 10 mg of Coomassie Brilliant Blue G 250 in 5 mL of 95% ethanol. To this solution, 10 mL of 85% phosphoric acid were added and the mixture was stirred and filled to a total volume of 100 mL with ultrapure water. The calibration curve was built with bovine

serum albumin as standard compound at 7 different concentrations ranging from 60 µg/mL to 210 µg/mL with blank correction. In a 96-well plate, 30 µL of the calibration solution or 30 µL of the membrane receptor preparation were mixed with 240 µL of the Bradford solution, respectively. After 5 min of incubation at rt, the UV absorbance was measured at λ=595 nm using a microplate spectrophotometer reader (Synergy HT, BioTek, Winooski, VT, USA).

4.2.7. σ_1 Receptor Ligand Binding Assays

In vitro σ_1 receptor ligand binding assays were carried out in Tris buffer (50 mM, pH 7.4) for 150 min at 37 °C. The thawed membrane preparation of guinea pig brain cortex (250 µg/sample) was incubated with increasing concentrations of test compounds and [^{3}H](+)-pentazocine (2 nM) in a final volume of 0.5 mL. The K_d value of [^{3}H](+)-pentazocine was 2.9 nM. Unlabeled (+)-pentazocine (10 µM) used to measure non-specific binding. Bound and free radioligand were separated by fast filtration under reduced pressure using a Millipore filter apparatus through Whatman GF/6 glass fiber filters (25 mm diameter), which were presoaked in a 0.5% poly(ethyleneimine) water solution for 120 min. Each filter paper was rinsed three times with 3 mL ice-cold Tris buffer (50 mM, pH 7.4), dried at rt, and incubated overnight with 3 mL scintillation cocktail into 6 mL pony vials. The bound radioactivity has been determined by liquid scintillation counting.

4.2.8. σ_2 Receptor Ligand Binding Assays

In vitro σ_2 receptor ligand binding assays were carried out in Tris buffer (50 mM, pH 8.0) for 120 min at rt. The thawed membrane preparation of rat liver (250 µg/sample) was incubated with increasing concentrations of test compounds and [^{3}H]DTG (2 nM) in the presence of (+)-pentazocine (5 µM) as σ_1 receptor masking agent in a final volume of 0.5 mL. The K_d value of

[^{3}H]DTG was 17.9 nM. Non-specific binding was evaluated with unlabeled DTG (10 μM). Bound and free radioligand were separated by fast filtration under reduced pressure using a Millipore filter apparatus through Whatman GF/6 glass fiber filters (25 mm diameter), which were presoaked in a 0.5% poly(ethyleneimine) water solution for 120 min. Each filter paper was rinsed three times with 3 mL ice-cold Tris buffer (10 mM, pH 8), dried at rt, and incubated overnight with 3 mL scintillation cocktail into 6 mL pony vials. The bound radioactivity has been determined by liquid scintillation counting.

4.2.9. Opioid Receptor Ligand Binding Assays

MOR and DOR binding experiments were carried out by incubating 400 μg/sample and 500 μg/sample of rat brain membranes, respectively for 45 min at 35 °C either with 1 nM [^{3}H]-DAMGO or 2 nM [^{3}H]-(2-D-Ala)-[Tyrosyl-3,5-]DELTORPHIN II in 50 mM Tris-HCl (pH 7.4). For KOR binding assays, guinea pig brain membranes (400 μg/sample) were incubated for 30 min at 30 °C with 1 nM [^{3}H]-U69,593. Test compounds were added in a final volume of 1 mL. The K_d values of [^{3}H]DAMGO, [^{3}H]-(2-D-Ala)-[Tyrosyl-3,5-]DELTORPHIN II and [^{3}H]-U69,593 were 1.0, 1.5 and 2.3 nM, respectively. Nonspecific binding was assessed in the presence of 10 μM of unlabeled naloxone. The reaction was terminated by filtering the solution under reduced pressure using a Millipore filter apparatus through Whatman glass fiber filters GF/C for MOR and DOR, GF/B for KOR, presoaked for 1h in a 0.5% poly(ethyleneimine) solution. Filters were washed with 50 mM ice-cold Tris-HCl buffer (2×4 mL), dried at rt, soaked overnight in 4 mL of scintillation cocktail into 6 mL pony vials and counted on a liquid scintillation counter.

4.2.10. Data analysis

The K_i-values were calculated with the program GraphPad Prism® 7.0 (GraphPad Software, San Diego, CA, USA). The K_i-values are given as mean value ± SD from at least two independent experiments performed in duplicate.

4.3. Fluorescence analysis for H_2S determination

H_2S generation was measured using WSP-1 at a final concentration of 100 µM [51]. A 10 mM solution of the appropriate compound in DMSO was diluted with PBS (10 mM, pH 7.4) containing 1 mM of surfactant cetrimonium bromide (CTAB) to give the desired final concentration of 100 µM. The calibration was carried out with NaHS as standard compound at 7 different concentrations ranging from 20 µM to 80 µM with blank correction. In a 96-well plate, 70 µL of the calibration solution or 70 µL of the tested compound were mixed with 140 µl of WSP1 stock solution and diluted at a final volume of 280 µL with the same buffer. The fluorescence signal was recorded at λ=476 nm in a microplate spectrophotometer reader (Synergy HT, BioTek) for 5 different time periods incubating in the dark at rt. The H_2S releasing curves were obtained by plotting H_2S concentration versus time.

4.4. *In vivo* pharmacology

4.4.1. Experimental animals

Experiments were performed in female WT-CD1 (Charles River, Barcelona, Spain) mice weighing 25–30 g. Mice were acclimated in our animal facilities for at least 1 week before testing and were housed in a room under controlled environmental conditions: 12/12 h day/night cycle, constant temperature (22 ± 2 °C), air replacement every 20 min, and they were fed a standard laboratory diet (Harlan Teklad Research Diet, Madison, WI, USA) and tap water ad libitum until the beginning of the experiments. Behavioral test was conducted during the light

phase (from 9.00 h to 15.00 h), and randomly throughout the oestrous cycle. Animal care was in accordance with institutional (Research Ethics Committee of the University of Granada, Spain), regional (Junta de Andalucía, Spain) and international standards (European Communities Council Directive 2010/63).

4.4.2. Drugs and drug administration

The experimental compounds were dissolved in 5% DMSO (Merck KGaA, Darmstadt, Germany) in physiological sterile saline (0.9% NaCl). As selective σ_1 receptor drugs, we used the σ_1 receptor antagonist BD-1063 (1–[2–(3,4–dichlorophenyl)ethyl]–4–methylpiperazine dihydrochloride) and the σ_1 receptor agonist PRE-084 (2-(4-morpholinethyl)1]- phenyl cyclohexane carboxylate hydrochloride) [52] (both provided by Tocris Cookson, Bristol, UK). Both σ_1 receptor drugs were dissolved in physiological sterile saline. Drug solutions were prepared immediately before the start of the experiments and injected s.c. in a volume of 5 mL/kg into the interscapular area. H_2S donors or their controls were injected 30 min before the administration of capsaicin, used as the chemical algogen. When we studied the effects of the association of BD-1063 with **1a** or **1b**, BD-1063 solution was administered immediately before the other drug. To test for the effects of PRE-084 on the antiallodynia induced by the other drugs, it was administered 5 min before the later. When the effect of the association of several drugs was assessed, each injection was performed in different areas of the interscapular zone to avoid mixture of the drug solutions and any physicochemical interaction between them.

Capsaicin (Sigma-Aldrich Química S.A.) was dissolved in 1% DMSO in physiological sterile saline to a concentration of 0.05 μg/μL (i.e., 1 μg per mouse). Capsaicin solution was injected intraplantarly (i.pl.) into the right hind paw proximate to the heel, in a volume of 20 μL using a

1710 TLL Hamilton microsyringe (Teknokroma, Barcelona, Spain) with a $30^{1/2}$-gauge needle. Control animals were injected with the same volume of the vehicle of capsaicin.

4.4.3. Evaluation of capsaicin-induced secondary mechanical hypersensitivity

Animals were placed for 2 h in individual black-walled test compartments. The test compartments were situated on an elevated mesh-bottomed platform with a 0.5-cm^2 grid to provide access to the ventral surface of the hind paws. In all experiments, punctate mechanical stimulation was applied with a Dynamic Plantar Aesthesiometer (Ugo Basile, Varese, Italy) 15 min after the administration of capsaicin or saline (i.e., 45 min after the injection of the experimental drug). Briefly, a nonflexible filament (0.5 mm diameter) was electronically driven into the ventral side of the right hind paw (which was previously injected with capsaicin or vehicle) at least 5 mm away from the site of the injection towards the fingers. The intensity of the stimulation was fixed at 0.5 g force, as described previously [35]. When a paw withdrawal response occurred, the stimulus was automatically terminated, and the response latency was automatically recorded. The filament was applied three times, separated by intervals of 0.5 min, and the mean value of the three trials was considered the withdrawal latency time of the animal. The degree of effect of drugs on mechanical hypersensitivity induced by capsaicin was calculated as: % reduction in mechanical hypersensitivity = [(LTD - LTS)/(CT - LTS)] x 100, where LTD is latency time in drug-treated animals, LTS is latency time in solvent-treated animals, and CT is the cut-off time (50 s).

4.4.4. Isobolographic analysis of drug effects

Theoretical additive doses (Zadd) for the combination of **1a** + BD-1063, in a 1:1 weight ratio, were computed from the equi-effective doses (ED_{50}, ED_{75}, ED_{90}) of the individual compounds,

according to the method described by Tallarida [39]. The EDs were obtained by non-linear regression of the dose-response data. The respective experimental values (Zexp) were determined from the non-linear regression of the dose-response data of the drug combination, also in a 1:1 weight ratio (3, 5 and 7 mg/kg for each component). The Zadds and Zexps were statistically compared with the use of Student's t-test.[39, 40] We also calculated the interaction index (ɣ) as a measure of the magnitude of the interaction between **1a** and BD-1063 at the three levels of effect tested (ED_{50}, ED_{75}, ED_{90}). According to the method previously described, ɣ= Zexp/Zadd [40]. Therefore, ɣ= 1 means no interaction and ɣ< 1 indicates a synergistic interaction.

4.4.5. Rotarod test

Motor coordination was assessed with an accelerating rotarod (Cibertec, Madrid, Spain), as previously described [46]. Briefly, mice were required to walk against the motion of an elevated rotating drum at increasing speed (4 to 40 rpm over 5 min), and the latency to fall down was recorded with a cut-off time of 300 s. Mice were given three training sessions 24 h before drug testing. On the day of the drug test, rotarod latencies were measured immediately before the drug or saline was administered (time 0) and several times (45, 90, 150, and 240 min) after the s.c. injection. As a comparison drug we used pregabalin, which has been reported to impair rotarod performance [46].

4.4.6. MABP determinations

Mice were randomly divided into control and drug-treated groups. To ensure the homogeneity of the groups, arterial pressures were previously determined in conscious pre-warmed restrained mice by tail-cuff plethysmography with a LE 5001 digital pressure meter (Letica, Barcelona,

Spain). The potential hypotensive effect of compound **6a** was evaluated using a direct method of recording blood pressure measurement by cannulation in the carotid artery. To carry out the experiment, mice were anesthetized by the intraperitoneal injection of equitensin (2.5 mL/kg), and a polyethylene catheter containing 100U heparin in isotonic, sterile saline solution was inserted in the left carotid artery to monitor intra-arterial blood pressure in conscious, unrestrained conditions. Direct blood pressure was recorded continuously with MacLab (AD Instruments, Hastings, UK). Blood pressure was measured before the drug or saline was administered (time 0) and several times (15, 30 and 45 minutes) after the s.c. injection. MABP values obtained during these times were averaged for intergroup comparisons [53].

4.5. Measure of hERG activity

Electrophysiological experiments were performed in CHO-K1 cells which express human ERG using a Qube APC assay. Compound **7a** has been tested employing six different concentrations ranging from 10^{-10} to 10^{-5} M using serial dilution by Eurofins Panlabs (St Charles, MO, United States) according to their standard assay protocol. Briefly, after whole cell configuration is achieved, the cell is held at -80 mV. The cell is held at this voltage for 50 ms to measure the leaking current, which is subtracted from the tail current on-line. Then the cell is depolarized to +40 mV for 500 ms and then to -80 mV over a 100ms ramp to elicit the hERG tail current. This paradigm is delivered once every 8s to monitor the current amplitude. All data were filtered for seal quality, seal drop, and current amplitude. The peak current amplitude was calculated before and after addition of the test compound, and the amount of block was assessed by dividing the test compound current amplitude by the control current amplitude.

4.6. Evaluation of chemical stability

4.6.1. Stability in 50 mM Phosphate Buffer (pH 7.4)

Before addition of compound **7a**, the medium was preheated at 37 °C. A 10 mM solution in DMSO was added to have a final concentration of 200 μM. The resulting solution was incubated at 37 ± 0.5 °C, and at appropriate time intervals, an amount of 500 μL of the reaction mixture was withdrawn and added to 500 μL of acetonitrile. The samples were vortexed and filtered by 0.45 μm filters and analyzed by UHPLC-PDA. Three individual experiments were run in triplicate. *Stability in physiological saline solution 0.9% (w/w)*. A 10 mM solution in DMSO of compound **7a** was added to the medium to have a final concentration of 200 μM. The resulting solution was stirred at rt, and at appropriate time intervals, an amount of 500 μL of the reaction mixture was withdrawn and added to 500 μL of acetonitrile. The samples were vortexed and filtered by 0.45 μm filters and analyzed by UHPLC-PDA. Three individual experiments were run in triplicate. *Data analysis*. The half-life ($t_{1/2}$) of compound **7a** in each medium was determined by fitting the data with one phase exponential decay equation using Prism software 7.00 (Graph Pad, San Diego, CA, USA).

4.6.2. Water solubility

Aqueous solubility was determined by UHPLC-PDA analysis. First, 5 mg of **7a** (free base) were weighted and added to 1 mL of ultrapure water. The suspension was shaken at rt for 24 h and then centrifuged, and the supernatant filtered by 0.45 μm filters. The supernatant was diluted in methanol before analysis. The compound is quantified against a methanol calibration curve built over 7 dilution concentrations ranging from 50 μM to 200 μM with blank correction.

Declaration of competing interest

The authors declare no competing financial interest.

Acknowledgment

The authors acknowledge the Bio-nanotech Research and Innovation Tower of University of Catania (BRIT-UNICT) for instrumental support of the Thermo Scientific UHPLC/PAD-FL Chromatography UltiMate3000 RSLC system. The authors thank Prof. Giuseppina Immè, Dr. Roberto Catalano and Nunzio Giudice from the Department of Physics and Astronomy, University of Catania, for technical and instrumental support of the Beckman LS6500 liquid scintillation counter. Free academic licenses from ChemAxon for their suites of programs are gratefully acknowledged. Authors thank Dr. Luz Romero for her invaluable help in the isobolographic analysis.

Funding

This work was financially supported by University of Catania, PIA.CE.RI. 2020–2022 Linea di intervento 3 Starting Grant project CARETO (grant 57722172136). This study was partially supported by the Spanish State Research Agency (10.13039/501100011033) under the auspices of MINECO (grant number PID2019-108691RB-I00), the Andalusian Regional Government (grant CTS109), the University of Catania PIA.CE.RI. 2020–2022 Linea di intervento 2 project DETTAGLI (grant 57722172125), and by Italian MUR, PRIN 2017, Code: 201744BN5T.

Appendix A. Supplementary data

Supplementary data to this article can be found online.

References

[1] A. Lazkani, T. Delespierre, B. Bauduceau, F. Pasquier, P. Bertin, G. Berrut, E. Corruble, J. Doucet, B. Falissard, F. Forette, O. Hanon, L. Benattar-Zibi, C. Piedvache, L. Becquemont,

Healthcare costs associated with elderly chronic pain patients in primary care, European journal of clinical pharmacology, 71 (2015) 939-947.
[2] R. González-Cano, Á. Montilla-García, M.C. Ruiz-Cantero, I. Bravo-Caparrós, M. Tejada, F.R. Nieto, E.J. Cobos, The search for translational pain outcomes to refine analgesic development: Where did we come from and where are we going?, Neuroscience and biobehavioral reviews, 113 (2020) 238-261.
[3] N. Ye, W. Qin, S. Tian, Q. Xu, E.A. Wold, J. Zhou, X.C. Zhen, Small Molecules Selectively Targeting Sigma-1 Receptor for the Treatment of Neurological Diseases, Journal of medicinal chemistry, 63 (2020) 15187-15217.
[4] E. Arena, M. Dichiara, G. Floresta, C. Parenti, A. Marrazzo, V. Pittalà, E. Amata, O. Prezzavento, Novel Sigma-1 receptor antagonists: from opioids to small molecules: what is new?, Future medicinal chemistry, 10 (2018) 231-256.
[5] M. Merlos, L. Romero, D. Zamanillo, C. Plata-Salamán, J.M. Vela, Sigma-1 Receptor and Pain, Handbook of experimental pharmacology, 244 (2017) 131-161.
[6] P.K. Moore, M. Bhatia, S. Moochhala, Hydrogen sulfide: from the smell of the past to the mediator of the future?, Trends in pharmacological sciences, 24 (2003) 609-611.
[7] B. Lv, S. Chen, C. Tang, H. Jin, J. Du, Y. Huang, Hydrogen sulfide and vascular regulation - An update, Journal of advanced research, 27 (2021) 85-97.
[8] M. Bhatia, J. Sidhapuriwala, S.M. Moochhala, P.K. Moore, Hydrogen sulphide is a mediator of carrageenan-induced hindpaw oedema in the rat, British journal of pharmacology, 145 (2005) 141-144.
[9] A. Kawabata, T. Ishiki, K. Nagasawa, S. Yoshida, Y. Maeda, T. Takahashi, F. Sekiguchi, T. Wada, S. Ichida, H. Nishikawa, Hydrogen sulfide as a novel nociceptive messenger, Pain, 132 (2007) 74-81.
[10] T.M. Cunha, D. Dal-Secco, W.A. Verri, Jr., A.T. Guerrero, G.R. Souza, S.M. Vieira, C.M. Lotufo, A.F. Neto, S.H. Ferreira, F.Q. Cunha, Dual role of hydrogen sulfide in mechanical inflammatory hypernociception, European journal of pharmacology, 590 (2008) 127-135.
[11] L. Di Cesare Mannelli, E. Lucarini, L. Micheli, I. Mosca, P. Ambrosino, M.V. Soldovieri, A. Martelli, L. Testai, M. Taglialatela, V. Calderone, C. Ghelardini, Effects of natural and synthetic isothiocyanate-based H(2)S-releasers against chemotherapy-induced neuropathic pain: Role of Kv7 potassium channels, Neuropharmacology, 121 (2017) 49-59.
[12] E. Distrutti, S. Cipriani, B. Renga, A. Mencarelli, M. Migliorati, S. Cianetti, S. Fiorucci, Hydrogen sulphide induces micro opioid receptor-dependent analgesia in a rodent model of visceral pain, Molecular pain, 6 (2010) 36.
[13] J.Q. Lin, H.Q. Luo, C.Z. Lin, J.Z. Chen, X.Z. Lin, Sodium hydrosulfide relieves neuropathic pain in chronic constriction injured rats, Evidence-based complementary and alternative medicine : eCAM, 2014 (2014) 514898.
[14] J.L. Wallace, P. Nagy, T.D. Feener, T. Allain, T. Ditrói, D.J. Vaughan, M.N. Muscara, G. de Nucci, A.G. Buret, A proof-of-concept, Phase 2 clinical trial of the gastrointestinal safety of a hydrogen sulfide-releasing anti-inflammatory drug, British journal of pharmacology, 177 (2020) 769-777.
[15] L. Romero, D. Zamanillo, X. Nadal, R. Sánchez-Arroyos, I. Rivera-Arconada, A. Dordal, A. Montero, A. Muro, A. Bura, C. Segalés, M. Laloya, E. Hernández, E. Portillo-Salido, M. Escriche, X. Codony, G. Encina, J. Burgueño, M. Merlos, J.M. Baeyens, J. Giraldo, J.A. López-García, R. Maldonado, C.R. Plata-Salamán, J.M. Vela, Pharmacological properties of S1RA, a

new sigma-1 receptor antagonist that inhibits neuropathic pain and activity-induced spinal sensitization, British journal of pharmacology, 166 (2012) 2289-2306.
[16] J. Mao, M.S. Gold, M.M. Backonja, Combination drug therapy for chronic pain: a call for more clinical studies, The journal of pain, 12 (2011) 157-166.
[17] I. Gilron, T.S. Jensen, A.H. Dickenson, Combination pharmacotherapy for management of chronic pain: from bench to bedside, The Lancet. Neurology, 12 (2013) 1084-1095.
[18] W. Zhang, Y. Bai, Y. Wang, W. Xiao, Polypharmacology in Drug Discovery: A Review from Systems Pharmacology Perspective, Current pharmaceutical design, 22 (2016) 3171-3181.
[19] M. García, M. Virgili, M. Alonso, C. Alegret, J. Farran, B. Fernández, M. Bordas, R. Pascual, J. Burgueño, A. Vidal-Torres, A.R. Fernández de Henestrosa, E. Ayet, M. Merlos, J.M. Vela, C.R. Plata-Salamán, C. Almansa, Discovery of EST73502, a Dual μ-Opioid Receptor Agonist and σ(1) Receptor Antagonist Clinical Candidate for the Treatment of Pain, Journal of medicinal chemistry, 63 (2020) 15508-15526.
[20] M. García, M. Virgili, M. Alonso, C. Alegret, B. Fernández, A. Port, R. Pascual, X. Monroy, A. Vidal-Torres, M.T. Serafini, J.M. Vela, C. Almansa, 4-Aryl-1-oxa-4,9-diazaspiro[5.5]undecane Derivatives as Dual μ-Opioid Receptor Agonists and σ(1) Receptor Antagonists for the Treatment of Pain, Journal of medicinal chemistry, 63 (2020) 2434-2454.
[21] C.R. Powell, K.M. Dillon, J.B. Matson, A review of hydrogen sulfide (H(2)S) donors: Chemistry and potential therapeutic applications, Biochemical pharmacology, 149 (2018) 110-123.
[22] Y. Zhao, S. Bhushan, C. Yang, H. Otsuka, J.D. Stein, A. Pacheco, B. Peng, N.O. Devarie-Baez, H.C. Aguilar, D.J. Lefer, M. Xian, Controllable hydrogen sulfide donors and their activity against myocardial ischemia-reperfusion injury, ACS chemical biology, 8 (2013) 1283-1290.
[23] C.M. Cendán, J.M. Pujalte, E. Portillo-Salido, J.M. Baeyens, Antinociceptive effects of haloperidol and its metabolites in the formalin test in mice, Psychopharmacology, 182 (2005) 485-493.
[24] 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.
[25] M. Olivieri, E. Amata, S. Vinciguerra, J. Fiorito, G. Giurdanella, F. Drago, N. Caporarello, O. Prezzavento, E. Arena, L. Salerno, A. Rescifina, G. Lupo, C.D. Anfuso, A. Marrazzo, Antiangiogenic Effect of (±)-Haloperidol Metabolite II Valproate Ester [(±)-MRJF22] in Human Microvascular Retinal Endothelial Cells, Journal of medicinal chemistry, 59 (2016) 9960-9966.
[26] M. Déciga-Campos, L.A. Melo-Hernández, H. Torres-Gómez, B. Wünsch, D. Schepmann, M.E. González-Trujano, J. Espinosa-Juárez, F.J. López-Muñoz, G. Navarrete-Vázquez, Design and synthesis of N-(benzylpiperidinyl)-4-fluorobenzamide: A haloperidol analog that reduces neuropathic nociception via σ(1) receptor antagonism, Life sciences, 245 (2020) 117348.
[27] E. Amata, M. Dichiara, D. Gentile, A. Marrazzo, R. Turnaturi, E. Arena, A. La Mantia, B.R. Tomasello, R. Acquaviva, C. Di Giacomo, A. Rescifina, O. Prezzavento, Sigma Receptor Ligands Carrying a Nitric Oxide Donor Nitrate Moiety: Synthesis, In Silico, and Biological Evaluation, ACS medicinal chemistry letters, 11 (2020) 889-894.
[28] E. Amata, M. Dichiara, E. Arena, V. Pittalà, V. Pistarà, V. Cardile, A.C.E. Graziano, A. Fraix, A. Marrazzo, S. Sortino, O. Prezzavento, Novel Sigma Receptor Ligand-Nitric Oxide Photodonors: Molecular Hybrids for Double-Targeted Antiproliferative Effect, Journal of medicinal chemistry, 60 (2017) 9531-9544.

[29] L. Pasquinucci, C. Parenti, M.C. Ruiz-Cantero, Z. Georgoussi, P. Pallaki, E.J. Cobos, E. Amata, A. Marrazzo, O. Prezzavento, E. Arena, M. Dichiara, L. Salerno, R. Turnaturi, Novel N-Substituted Benzomorphan-Based Compounds: From MOR-Agonist/DOR-Antagonist to Biased/Unbiased MOR Agonists, ACS medicinal chemistry letters, 11 (2020) 678-685.
[30] R. Turnaturi, L. Pasquinucci, S. Chiechio, M. Grasso, A. Marrazzo, E. Amata, M. Dichiara, O. Prezzavento, C. Parenti, Exploiting the Power of Stereochemistry in Drug Action: 3-[(2S,6S,11S)-8-Hydroxy-6,11-dimethyl-1,4,5,6-tetrahydro-2,6-methano-3-benzazocin-3(2H)-yl]-N-phenylpropanamide as Potent Sigma-1 Receptor Antagonist, ACS chemical neuroscience, 11 (2020) 999-1005.
[31] E. Amata, A. Rescifina, O. Prezzavento, E. Arena, M. Dichiara, V. Pittalà, Á. Montilla-García, F. Punzo, P. Merino, E.J. Cobos, A. Marrazzo, (+)-Methyl (1R,2S)-2-{[4-(4-Chlorophenyl)-4-hydroxypiperidin-1-yl]methyl}-1-phenylcyclopropanecarboxylate [(+)-MR200] Derivatives as Potent and Selective Sigma Receptor Ligands: Stereochemistry and Pharmacological Properties, J Med Chem, 61 (2018) 372-384.
[32] R. Turnaturi, C. Parenti, O. Prezzavento, A. Marrazzo, P. Pallaki, Z. Georgoussi, E. Amata, L. Pasquinucci, Synthesis and Structure-Activity Relationships of LP1 Derivatives: N-Methyl-N-phenylethylamino Analogues as Novel MOR Agonists, Molecules (Basel, Switzerland), 23 (2018).
[33] C.J. Woolf, Central sensitization: implications for the diagnosis and treatment of pain, Pain, 152 (2011) S2-s15.
[34] K.M. Park, M.B. Max, E. Robinovitz, R.H. Gracely, G.J. Bennett, Effects of intravenous ketamine, alfentanil, or placebo on pain, pinprick hyperalgesia, and allodynia produced by intradermal capsaicin in human subjects, Pain, 63 (1995) 163-172.
[35] J.M. Entrena, E.J. Cobos, F.R. Nieto, C.M. Cendán, G. Gris, E. Del Pozo, D. Zamanillo, J.M. Baeyens, Sigma-1 receptors are essential for capsaicin-induced mechanical hypersensitivity: studies with selective sigma-1 ligands and sigma-1 knockout mice, Pain, 143 (2009) 252-261.
[36] J.L. Díaz, F. Cuevas, A.I. Oliva, D. Font, M. Sarmentero, P. Álvarez-Bercedo, J.M. López-Valbuena, M.A. Pericàs, R. Enrech, A. Montero, S. Yeste, A. Vidal-Torres, I. Álvarez, P. Pérez, C.M. Cendán, E.J. Cobos, J.M. Vela, C. Almansa, Tricyclic Triazoles as σ(1) Receptor Antagonists for Treating Pain, Journal of medicinal chemistry, 64 (2021) 5157-5170.
[37] J. Wilke, T. Kawamura, H. Xu, A. Brause, A. Friese, M. Metz, D. Schepmann, B. Wünsch, A. Artacho-Cordón, F.R. Nieto, N. Watanabe, H. Osada, S. Ziegler, H. Waldmann, Discovery of a σ(1) receptor antagonist by combination of unbiased cell painting and thermal proteome profiling, Cell chemical biology, 28 (2021) 848-854.e845.
[38] J.L. Díaz, M. García, A. Torrens, A.M. Caamaño, J. Enjo, C. Sicre, A. Lorente, A. Port, A. Montero, S. Yeste, I. Álvarez, M. Martín, R. Maldonado, B. de la Puente, A. Vidal-Torres, C.M. Cendán, J.M. Vela, C. Almansa, EST64454: a Highly Soluble σ(1) Receptor Antagonist Clinical Candidate for Pain Management, Journal of medicinal chemistry, 63 (2020) 14979-14988.
[39] R.J. Tallarida, Drug synergism: its detection and applications, The Journal of pharmacology and experimental therapeutics, 298 (2001) 865-872.
[40] R.J. Tallarida, The interaction index: a measure of drug synergism, Pain, 98 (2002) 163-168.
[41] C. Sánchez-Fernández, Á. Montilla-García, R. González-Cano, F.R. Nieto, L. Romero, A. Artacho-Cordón, R. Montes, B. Fernández-Pastor, M. Merlos, J.M. Baeyens, J.M. Entrena, E.J. Cobos, Modulation of peripheral μ-opioid analgesia by σ1 receptors, The Journal of pharmacology and experimental therapeutics, 348 (2014) 32-45.

[42] I. Bravo-Caparrós, G. Perazzoli, S. Yeste, D. Cikes, J.M. Baeyens, E.J. Cobos, F.R. Nieto, Sigma-1 Receptor Inhibition Reduces Neuropathic Pain Induced by Partial Sciatic Nerve Transection in Mice by Opioid-Dependent and -Independent Mechanisms, Frontiers in pharmacology, 10 (2019) 613.
[43] M.C. Ruiz-Cantero, R. González-Cano, M. Tejada, M. Santos-Caballero, G. Perazzoli, F.R. Nieto, E.J. Cobos, Sigma-1 receptor: A drug target for the modulation of neuroimmune and neuroglial interactions during chronic pain, Pharmacological research, 163 (2021) 105339.
[44] L. Walker, V. Yip, M. Pirmohamed, Chapter 20 - Adverse Drug Reactions, in: S. Padmanabhan (Ed.) Handbook of Pharmacogenomics and Stratified Medicine, Academic Press, San Diego, 2014, pp. 405-435.
[45] R.D. Porsolt, Overview of safety pharmacology, Current protocols in pharmacology, Chapter 10 (2006) Unit10.11.
[46] F.R. Nieto, C.M. Cendán, C. Sánchez-Fernández, E.J. Cobos, J.M. Entrena, M.A. Tejada, D. Zamanillo, J.M. Vela, J.M. Baeyens, Role of sigma-1 receptors in paclitaxel-induced neuropathic pain in mice, The journal of pain, 13 (2012) 1107-1121.
[47] A. Garrido, A. Lepailleur, S.M. Mignani, P. Dallemagne, C. Rochais, hERG toxicity assessment: Useful guidelines for drug design, European journal of medicinal chemistry, 195 (2020) 112290.
[48] C.N. Hsu, Y.L. Tain, Hydrogen Sulfide in Hypertension and Kidney Disease of Developmental Origins, International journal of molecular sciences, 19 (2018).
[49] K.W. Swan, B.M. Song, A.L. Chen, T.J. Chen, R.A. Chan, B.T. Guidry, P.V.G. Katakam, E.K. Kerut, T.D. Giles, P.J. Kadowitz, Analysis of decreases in systemic arterial pressure and heart rate in response to the hydrogen sulfide donor sodium sulfide, American journal of physiology. Heart and circulatory physiology, 313 (2017) H732-h743.
[50] M. Dichiara, B. Amata, R. Turnaturi, A. Marrazzo, E. Amata, Tuning Properties for Blood-Brain Barrier Permeation: A Statistics-Based Analysis, ACS chemical neuroscience, 11 (2020) 34-44.
[51] C. Liu, J. Pan, S. Li, Y. Zhao, L.Y. Wu, C.E. Berkman, A.R. Whorton, M. Xian, Capture and visualization of hydrogen sulfide by a fluorescent probe, Angewandte Chemie (International ed. in English), 50 (2011) 10327-10329.
[52] E.J. Cobos, J.M. Entrena, F.R. Nieto, C.M. Cendán, E. Del Pozo, Pharmacology and therapeutic potential of sigma(1) receptor ligands, Current neuropharmacology, 6 (2008) 344-366.
[53] M. Gómez-Guzmán, R. Jiménez, M. Romero, M. Sánchez, M.J. Zarzuelo, M. Gómez-Morales, F. O'Valle, A.J. López-Farré, F. Algieri, J. Gálvez, F. Pérez-Vizcaino, J.M. Sabio, J. Duarte, Chronic hydroxychloroquine improves endothelial dysfunction and protects kidney in a mouse model of systemic lupus erythematosus, Hypertension (Dallas, Tex. : 1979), 64 (2014) 330-337.