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Research Article

Discovery of a Highly Selective Sigma-2 Receptor Ligand, 1-(4-(6,7-Dimethoxy-3,4-dihydroisoquinolin-2(1*H*)-yl)butyl)-3-methyl-1*H*-benzo[*d*]imidazol-2(3*H*)-one (CM398), with Drug-Like Properties and Antinociceptive Effects In Vivo

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Abstract. The sigma-2 receptor has been cloned and identified as Tmem97, which is a transmembrane protein involved in intracellular Ca^{2+} regulation and cholesterol homeostasis. Since its discovery, the sigma-2 receptor has been an extremely controversial target, and many efforts have been made to elucidate the functional role of this receptor during physiological and pathological conditions. Recently, this receptor has been proposed as a potential target to treat neuropathic pain due to the ability of sigma-2 receptor agonists to relieve mechanical hyperalgesia in mice model of chronic pain. In the present work, we developed a highly selective sigma-2 receptor ligand (sigma-1/sigma-2 selectivity ratio > 1000), 1-(4-(6,7-dimethoxy-3,4-dihydroisoquinolin-2(1*H*)-yl)butyl)-3-methyl-1*H*-benzo[*d*]imidazol-2(3*H*)-one (CM398), with an encouraging *in vitro* and *in vivo* pharmacological profile in rodents. In particular, radioligand binding studies demonstrated that CM398 had preferential affinity for sigma-2 receptor compared with sigma-1 receptor and at least four other neurotransmitter receptors sites, including the norepinephrine transporter. Following oral administration, CM398 showed rapid absorption and peak plasma concentration (Cmax) occurred within 10 min of dosing. Moreover, the compound showed adequate, absolute oral bioavailability of 29.0%. Finally, CM398 showed promising anti-inflammatory analgesic effects in the formalin model of inflammatory pain in mice. The results collected in this study provide more evidence that selective sigma-2 receptor ligands can be useful tools in the development of novel pain therapeutics and altogether, these data suggest that CM398 is a suitable lead candidate for further evaluation.

KEY WORDS: sigma receptors; sigma-2 receptor; neuropathic pain; formalin assay; pharmacokinetic.

INTRODUCTION

Neuropathic pain is a major clinical problem that results in a drastic reduction to the quality of life in patients who are affected with this chronic condition (1,2). It represents one of the most frequent causes of adult disability and a considerable health-care cost which consists of medical expenses and lost workdays. It has been estimated that approximately 20 million individuals in the USA suffer from some form of peripheral neuropathy (3). The most common reasons of developing peripheral neuropathy include physical injury (trauma), chronic diseases (diabetic neuropathy), and exposure to toxins (3). Despite the fact that neuropathic pain is a common medical problem, there are only a few effective treatment options, each with their own limitations, thus the management of chronic pain is quite complicated and challenging. First-line treatments include antidepressant

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agents such as tricyclic antidepressants (TCAs) and serotonin-norepinephrine reuptake inhibitors (SNRIs) or anticonvulsant drugs such as calcium channel alpha-2-delta ligands (gabapentinoids) (4). Nonsteroidal anti-inflammatory drugs (NSAIDs) may be used for mild pain relief, whereas narcotic agents are generally prescribed for pain that does not respond to the first-line treatments (4). Although most of the prescribed drugs are effective for alleviating pain symptoms, they cause several side effects and possess notable liabilities that include sedation, diplopia, and dizziness in case of antiepileptic drugs (5), or tolerance, respiratory depression, dependence, and addiction in case of opioids (6). Therefore, there is a strong need for new therapeutics with fewer side effects and increased effectiveness for pain management. To this end, several new potential targets to treat neuropathic pain have been proposed, and novel agents have shown efficacy in preclinical models, and some have currently reached investigation in clinical trials (7). Among these approaches, sigma receptor ligands emerged as promising tools for alleviating chronic pain (8). Sigma receptors are transmembrane proteins present throughout the central nervous system as well as in peripheral tissues (e.g., spleen, liver, and kidney) (9). Two different receptor subtypes were reported and identified as sigma-1 and sigma-2. The sigma-1 receptor was cloned over two decades ago, and the human crystal structure has been reported (10,11), whereas purification and cloning of the sigma-2 receptor have only recently been published in 2017 (12). The sigma-2 receptor has been confirmed as Tmem97 (transmembrane protein 97) (12). Several literature reports suggest a role for the sigma-1 receptor in pain modulation, and either selective or non-selective sigma-1 receptor ligands have shown antinociceptive effects in animal models (13–16). In particular, the sigma-1 receptor antagonist S1RA (E-52862) (Fig. 1) has completed successfully phase I clinical trials studies and is currently under phase II investigation (17,18). Another example of sigma-1 receptor antagonist is [^{18}F]FTC-146 (Fig. 1) which has entered into clinical trials as a positron emission tomography and magnetic resonance imaging (PET/MRI) diagnostic agent to pinpoint peripheral nerve injury (19–22). Despite many years of research efforts focused on the validation of the sigma-1 subtype as an effective target to treat neuropathic pain (23), the evaluation of the sigma-2 receptor potential as a therapeutic target for pain target has

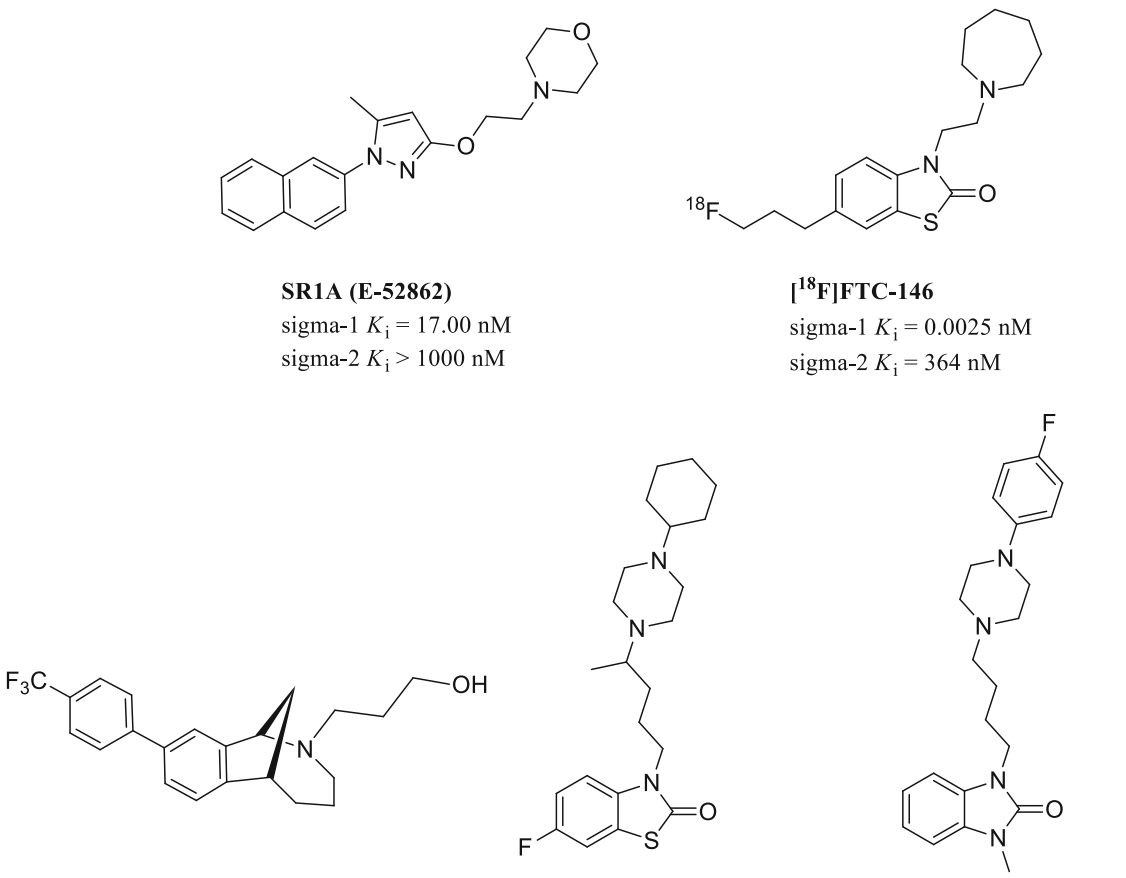

Fig. 1. Chemical structure and affinity values of selective sigma-1 antagonists S1RA (E-52862) and [^{18}F]FTC-146 which are current under clinical studies; selective sigma-2 agonist UKH-1114 and non- selective sigma-1/sigma-2 ligand AZ66 which have showed antineuropathic pain effects in mice; selective sigma-2 benzimidazolone-based analog CM397

also started to gain momentum. Most recently, the phenyl methanobenzazocine derivative (UKH-1114, Fig. 1) as sigma-2/Tmem97 agonist was reported to be able to produce antinociceptive effects when administered IT to spared nerve injury (SNI) mice, suggesting antineuropathic pain effects of sigma-2 ligands (24). Moreover, the non-selective sigma-1/sigma-2 ligand AZ66 (Fig. 1) demonstrated the ability to alleviate multiple modalities of chronic pain with reduced liabilities compared to opioids (16).

Our research group has been working for many years in the development of selective sigma receptor ligands, including benzimidazolone-based selective sigma-2 receptor ligands (25,26). Several of our compounds have been used as effective chemical probes in the elucidation of the putative roles of the sigma receptor subtypes in many diseases such as cancer, drug addiction, and pain (27–31). More recently, our efforts have been focused on the translational research of many of our lead molecules into effective diagnostic or pharmacotherapies through a multi-disciplinary and integrated approach. Herein, we report the synthesis, *in vitro/in vivo* pharmacology evaluation, and preclinical pharmacokinetic studies of CM398, a highly selective sigma-2 receptor ligand (sigma-1/sigma-2 selectivity ratio > 1,000), that demonstrates anti-inflammatory analgesic effects in mice as an initial proof-of-concept.

MATERIALS AND METHODS

Chemistry

Reagents and starting materials were obtained from commercial suppliers and were used without purification. Precoated silica gel GF Uniplates from Analtech were used for thin-layer chromatography (TLC). Column chromatography was performed on silica gel 60 (Sorbent Technologies). ^{1}H and ^{13}C NMR spectra were obtained on a Bruker APX400 (400 and 100 MHz, respectively) in $CDCl_3$ and DMSO-d_6 solution. Chemical shift (δ) values are given in parts per million (ppm) using tetramethylsilane (TMS) and DMSO or $CHCl_3$ as the internal standard; coupling constants (J values) are given in hertz (Hz). For signal multiplicities, the following abbreviations are used as follows: s (singlet), d (doublet), dd (doublets of doublet), ddd (doublet of doublet of doublets), t (triplet), br s (broad singlet), and m (multiplet). The mass spectra (MS) were recorded on a WATERS ACQUITY Ultra Performance LC with ZQ detector in ESI or APCI mode. The high resolution mass spectra (HRMS) were recorded on a Waters Micromass Q-TOF Micro mass spectrometer with a lock spray source. Chemical names were generated using ChemDraw Ultra (CambridgeSoft, version 10.0).

*N-Methyl-2-nitroaniline (**2**).* A solution of 1-fluoro-2-nitro-benzene (2.00 g, 14.17 mmol) in water (5 mL) was added 40% aqueous methylamine (5 mL) at room temperature, and the reaction mixture stirred at room temperature under nitrogen. After 2 h, the reaction mixture was poured into a saturated aqueous solution of sodium chloride (50 mL) and extracted with ethyl acetate (3 × 50 mL). The organic layer was dried over anhydrous sodium sulfate, concentrated *in vacuo*, and the residue was purified by flash column chromatography (hexane/ethyl acetate 9:1) to afford **2** as an orange oil (2.00 g, 93%). ^{1}H NMR (400 MHz, DMSO-d_6) δ 8.14 (d, J = 5.5 Hz, 1H), 8.01 (dd, J = 8.6, 1.5 Hz, 1H), 7.49 (ddd, J = 8.3, 6.9, 1.5 Hz, 1H), 6.91 (dd, J = 8.8, 1.3 Hz, 1H), 6.62 (ddd, J = 8.3, 7.0, 1.2 Hz, 1H), 2.91 (d, J = 5.0 Hz, 3H).^{13}C NMR (101 MHz, DMSO-d_6) δ 146.59, 137.19, 131.48, 126.72, 115.49, 114.80, 30.24. MS (ESI$^+$) *m/z* 153 $[M + H]^+$.

*N^1-Methylbenzene-1,2-diamine (**3**).* A solution of **2** (2.00 g, 13.14 mmol) in methanol (20 mL) was added 10% palladium on carbon (0.10 g) in a portion wise manner. After addition and stirring for an additional 2 h under hydrogenated (H_2) atmosphere at room temperature, the reaction mixture was then filtered through a pad of celite, and the filtrate was concentrated *in vacuo* to afford **3** as brown residue, which was used in the next step without further purification. ^{1}H NMR (400 MHz, DMSO-d_6) δ 6.55 (t, J = 7.3 Hz, 2H), 6.48–6.35 (m, 2H), 4.56 (d, J = 5.9 Hz, 1H), 4.42 (s, 2H), 2.70 (d, J = 3.6 Hz, 3H).^{13}C NMR (101 MHz, DMSO-d_6) δ 137.85, 135.75, 118.40, 117.37, 114.46, 109.78, 30.91. MS (ESI$^+$) *m/z* 123 $[M + H]^+$.

*1-Methyl-1H-benzo[d]imidazol-2(3H)-one (**4**).* A mixture of **3** (2.00 g, 16.40 mmol), 1,1′-carbonyldiimidazole (3.18 g, 19.60 mmol) in anhydrous tetrahydrofuran (25 mL) was stirred at 65°C for 5 h. The mixture was poured onto water and extracted with ethyl acetate (3 × 50 mL). The extract was washed with a saturated aqueous solution of sodium chloride (35 mL) and dried over anhydrous sodium sulfate. The solvent was removed *in vacuo*, and the obtained residue was crystallized from hexane-ethyl acetate (1:1) to afford **4** as white crystals (1.90 g, 79%). ^{1}H NMR (400 MHz, DMSO-d_6) δ 10.82 (s, 1H), 7.06–6.93 (m, 4H), 3.25 (s, 3H).^{13}C NMR (101 MHz, DMSO-d_6) δ 155.07, 131.58, 128.85, 121.47, 121.13, 109.23, 108.21, 27.03. MS (ESI$^+$) *m/z* 149 $[M + H]^+$.

*1-(4-Bromobutyl)-3-methyl-1H-benzo[d]imidazol-2(3H)-one (**5**).* K_2CO_3 (0.56 g, 4.05 mmol) and 1,4-dibromobutane (1.12 mL, 9.45 mmol) were added, under mechanical stirring, to a solution of **4** (0.20 g, 1.35 mmol) in anhydrous DMF (8 mL). The reaction mixture was heated at 60°C for 3 h. After cooling, the mixture was poured into 100 mL of water, extracted with ethyl acetate (3–40 mL), washed with a saturated aqueous solution of sodium chloride, and dried over anhydrous sodium sulfate. The solvent was removed *in vacuo*, and the residue was purified by flash column chromatography (petroleum ether/ethyl acetate 7:3) to afford **5** as a colorless oil (0.27 g, 70%). ^{1}H NMR (400 MHz, $CDCl_3$) d 7.06–7.03 (m, 2H), 6.96–6.91 (m, 2H), 3.88–3.86 (m, 2H), 3.41–3.38 (m, 2H), 3.63 (s, 3H), 1.89–1.85 (m, 4H). ^{13}C NMR (101 MHz, $CDCl_3$) d 154.41, 130.10, 129.17, 121.26, 121.23, 107.53, 107.50, 40.08, 33.13, 29.66, 27.15, 26.98. MS (ESI) *m/z* 305 $[M + Na]^+$ (^{79}Br) and 307 $[M + Na]^+$ (^{81}Br).

*1-(4-(6,7-Dimethoxy-3,4-dihydroisoquinolin-2(1H)-yl)butyl)-3-methyl-1H- benzo[d]imidazol-2(3H)-one Hydrochloride (**CM398**).* K_2CO_3 (0.08 g, 0.62 mmol) and 6,7-dimethoxy-1,2,3,4-tetrahydroisoquinoline hydrochloride (0.05 g, 0.21 mmol) were added, under mechanical stirring, to a solution of **5** (0.05 g, 0.18 mmol) in anhydrous DMF (3 mL). The reaction mixture was heated at 40°C for 1 h.

After cooling, the mixture was poured into 20 mL of water, extracted with ethyl acetate (3–30 mL), washed with a saturated aqueous solution of sodium chloride, and dried over anhydrous sodium sulfate. The solvent was removed *in vacuo*, and the residue was purified by flash column chromatography (methylene chloride/methanol 95:5) to afford **6**, which was converted into the hydrochloride salt by addition of HCl/dioxane and isolated as a white solid (0.026 g, 34%). ^{1}H NMR (400 MHz, DMSO-*d*6) d 11.26 (br s, 1H), 7.24–7.22 (m, 1H), 7.16–7.14 (m, 1H), 7.08–7.06 (m, 2H), 6.79 (s, 1H), 6.77 (s, 1H), 4.35–4.32 (m, 1H), 4.13–4.09 (m, 1H), 3.87 (t, J = 7.2 Hz, 2H), 3.73–3.56 (m, 7H), 3.33–3.19 (m, 7H), 2.89–2.85 (m, 1H), 1.83–1.71 (m, 4H). ^{13}C NMR (101 MHz, DMSO-*d*6) d 153.49, 148.16, 147.51, 129.57, 128.65, 123.23, 120.72, 119.77, 111.40, 109.65, 107.64, 107.60, 55.47, 55.41, 54.22, 50.99, 48.51, 39.65, 26.77, 25.11, 24.14, 20.49. HRMS calcd for $C_{23}H_{30}N_3O_3$ $[M + H]^+$ 396.2287, found 396.2268.

Competition Binding Assays

Radioligand binding studies at sigma and non-sigma receptors were performed using competition binding assays in homogenates of rat brain tissues and following previously reported procedures (26,32). Specific information regarding tissues, radioligands, and drugs used in the assays are reported in Table I.

Metabolic Stability in Rat Liver Microsomes

Phase-I metabolism stability of CM398 was studied in rat liver microsomes. The incubation mixture consisted of liver microsomal protein (1 mg/mL), CM398 (1 μM), phosphate buffer (100 mM, pH 7.4), and reduced nicotinamide adenine dinucleotide phosphate (NADPH, 2 mM). Verapamil (1 μM) was used as a positive control to assess the metabolizing capacity of rat liver microsomes. NADPH deficient microsomal reaction was performed as a negative control to reveal the non-NADPH-dependent degradation, non-specific binding, and chemical instability of the compound in the reaction mixture. In a 96-well plate, 178 μL of buffer, 2 μL of CM398/verapamil stock solution (100 μM), and 10 μL of liver microsomes (protein 20 mg/mL) were added and equilibrated for 5 min in a shaking water bath at 37°C. Total organic content in reaction mixture was 1%. The reaction was started by adding 10 μL of NADPH (40 mM). For the negative control reaction, volume of NADPH was replaced by phosphate buffer. An aliquot (20 μL) from the incubation mixture was quenched at 0, 5, 10, 15, 30, and 45 min with 80 μL of ice cold acetonitrile containing internal standard (IS) to terminate the microsomal reaction. The samples were centrifuged at 4°C for 15 min at 12,000 rpm, and supernatants were analyzed using UPLC/MS-MS for residual CM398 content. The positive and negative controls were also processed similarly. The area under the curve (AUC) was calculated for the analyte and IS in MassLynx, and the ratio of the analyte to IS AUC was used to plot relative concentration *vs*. time and calculate percent degradation of CM398 in the reaction mixture. The elimination half-life ($T_{1/2}$) was calculated as Eq. 1.

$$T_{1/2} = -0.693/k \quad (1)$$

Where k is the slope of the line obtained by plotting natural logarithmic of percentage of CM398 remaining in the reaction mixture *vs*. incubation time. Intrinsic clearance (CL_{int}) and whole liver clearance ($CL_{int,H}$) were calculated from the following equations:

$$CL_{int} = k \times (\text{Incubation volume})/(\text{Microsomal protein}) \quad (2)$$

$$CL_{int,H} = CL_{int} \times \text{MPPGL} \times \text{liver scaling factor} \quad (3)$$

where MPPGL represents the microsomal protein per gram of *Sprague Dawley* rat liver (45 mg/g) (33), and liver scaling factor (40 g/Kg) (34) represents the liver weight per body weight of *Sprague Dawley* rats. *In vivo* hepatic clearance was extrapolated through well-stirred model (Eq. 4) including microsomal protein binding (f_{umic}) (35). The f_{umic} was derived using the equation

Table I. Binding affinities of CM398 for sigma and non-sigma receptors and specific conditions used for the competition binding assays

Target		*Ki* [a](nM)	Tissue	Radioligand	Nonspecific binding
Sigma receptors	Sigma-1	560 ± 8.72	Rat brain	5 nM [^{3}H](+)-pentazocine	10 μM haloperidol
	Sigma-2	0.43 ± 0.015	Rat brain	3 nM [^{3}H]di-o-tolylguanidine	10 μM haloperidol
Monoamine transporters	Dopamine	32.90 ± 1.9	Rat striatum	0.5 nM [^{3}H]WIN 35,428	50 μM cocaine
	Serotonin	244.2 ± 2.4	Rat brainstem	0.2 nM [^{3}H]paroxetine	1.5 μM imipramine
	Norepinephrine	> 1000	Rat cerebral cortex	0.5 nM [^{3}H]nisoxetine	4 μM desipramine
Other neurotransmitter receptors	Dopamine (D_2)	> 1000	Rat brain	5 nM [^{3}H](−)sulpiride	1 μM haloperidol
	Serotonin (5-HT_2)	> 1000	Rat brain	2 nM [^{3}H]ketanserin	1 μM mianserin
	NMDA	> 10,000	Rat brain	5 nM [^{3}H]TCP	10 μM cyclazocine
	Opioid	>1000	Rat brain	1 nM [^{3}H]naloxone	1 μM naloxone

[a] *Affinities (Ki values in nanomolar) were determined in brain tissue homogenates*
The values represent ± S.E.M. from replicate assays. Values of > 10,000 represent less than 30% displacement of the radioligand at that concentration.

(Eq. 5) of Hallifex and Houstan (36). Hepatic extraction ratio was also determined using Eq. 6.

$$\text{Hepatic Clearance } (CL_H) = (Q \times f_u \times CL_{int}/f_{umic})/(Q + (f_u \times CL_{int}/f_{umic})) \quad (4)$$

$$f_{umic} = 1/1 + C \times 10^{(0.072\times(\log P/D)^{\wedge}2+0.067\times \log P/D-1.126)} \quad (5)$$

$$\text{Hepatic extraction ratio} = CL_H/Q \quad (6)$$

where f_u (6.2%) is the unbound fraction of CM398 in rat plasma, logP/D (3.1) is the partition coefficient, *C* is the microsomal protein concentration, and *Q* is the hepatic blood flow in *Sprague Dawley* rats (4.8 L/h/kg) (33).

Plasma Protein Binding Studies

The protein binding of CM398 was evaluated *in vitro* using an ultra-filtration method at concentrations of 1 and 10 μM. The desired concentrations (1 and 10 μM) were obtained by spiking the required volume of stock solutions in rat plasma. Spiked organic content was ≤ 0.5% v/v. The spiked plasma samples were placed in Centrifree® devices (YM-30, Millipore, Bedford, USA) and incubated at 37°C for 30 min. The samples were then centrifuged for 10 min at 1000 g, and ultrafiltrates were collected. Spiked plasma and plasma filtrate samples were processed and analyzed using the UPLC-MS/MS method as described in supporting information. Nonspecific binding was also determined and incorporated in the calculation of plasma protein binding values. The fraction unbound (f_u) and plasma protein binding were calculated as described in Eqs. 7 and 8, respectively (37).

$$f_u = [C_f/(1-NSB)/C_i] \quad (7)$$

$$\text{Protein binding} = (1-f_u) \times 100 \quad (8)$$

Where C_i is the initial concentration, C_f is the concentration in filtrate or free compound, NSB is the nonspecific binding fraction, and f_u is the percent unbound drug concentration.

Pharmacokinetic Studies in Rats

Oral (20 mg/kg) and intravenous (1 mg/kg) pharmacokinetic (PK) studies of CM398 were evaluated in male *Sprague Dawley* rats (*N*=5, each). Right jugular vein cannulated rats (225 ± 25 g) were purchased from Envigo (Indianapolis, USA). Prior to the PK study, the animals were quarantined in the University of Mississippi vivarium for 48 h with a 12-h dark/light cycle and allowed access to standard feed and water *ad libitum*. All animal experiments were performed in accordance with the University of Mississippi Institutional Animal Care and Use Committee (IACUC) pre-approved protocol. Animals were housed in individual Nalgene metabolic cages with mesh floor and receptacles for urine and feces. Rodent feed was removed from the metabolic cages 12–18 h prior to oral (P.O.) dosing, and feed was again provided 4 h post-dose. Animals in the (I.V.) administration study were allowed constant access to standard feed. All animals always had access to water *ad libitum*. Solution formulations of CM398 were prepared in water (5 mg/mL) and normal saline (1 mg/mL) for P.O. and I.V. PK studies, respectively. The formulation for the I.V. study was filtered through 0.2 μm syringe filter. Both formulations were analyzed for CM398 content. The I.V. solutions were administered *via* caudal vein. The P.O. solution was administered *via* oral gavage. Blood samples were collected using the indwelling cannula. An initial blood volume of 0.05 mL was withdrawn to clear the line of heparinized saline. Using a fresh syringe, 0.15 mL of blood was withdrawn and placed in heparin coated micro-centrifuge tube. The cannula was then flushed with 0.20 ml of heparinized saline. For the P.O. study, blood samples were taken pre-dose and at 0.17, 0.33, 0.5, 0.75, 1, 2, 4, 6, 8, 12, and 24 h post-dose. For the I.V. study, blood samples were taken pre- dose and at 0.083, 0.17, 0.33, 0.5, 1, 2, 4, 6, 8, 12, and 24 h post-dose. Rat plasma was separated by centrifugation of blood at 4000*g* for 10 min at 4°C and stored at −80°C refrigerator until analysis. Plasma samples were processed and analyzed using an UPLC-MS/MS method as described in supporting information. Plasma concentration-time data and PK parameters are represented as mean ± standard error of the mean (SEM). Peak plasma concentration (C_{max}) and time to reach C_{max} (T_{max}) were obtained directly from the concentration-time data plot. Concentration *vs.* time data was subjected to noncompartmental analysis using Phoenix WinNonlin (version 6.3; Certara Inc, Missouri, USA). The area under plasma concentration-time curve (AUC) of CM398 was calculated using the linear trapezoidal method. Clearance (CL) was calculated as a ratio of dose and AUC. Absolute oral bioavailability (%F) was calculated using Eq. 9.

$$\%F = [Dose_{intravenous} \times AUC_{(last)oral}/Dose_{(oral)} \times AUC_{(last)intravenous}] \times 100 \quad (9)$$

In Vivo Characterization in a Model of Inflammatory Pain and Statistical Analysis

Thirty-five 10 weeks old male CD-1 mice obtained from the Charles River Laboratories, Wilmington, Massachusetts, USA ,were used in the formalin assay. Mice were housed five to a cage in a temperature and humidity-controlled room at the University of Florida vivarium on a 12:12-h light/dark cycle (lights off at 19:00 h) with free access to food and water except during experimental sessions. All procedures were pre-approved and carried out in accordance with the UF Institutional Animal Care and Use Committee as specified by the 2011 National Institutes of Health Guide for the Care and Use of Laboratory Animals. Animal studies are reported in compliance with the ARRIVE guidelines (38,39). Sample sizes (i.e., number of animals) were predetermined by power analysis, and animals were assigned to groups randomly. Drug

treatment experiments were conducted in a blinded fashion. No animals were excluded from statistical analysis.

After seven mice each received a 10-min pre-treatment with vehicle (saline, 0.9% as a control group), morphine (23.2 μmol/kg, i.p. as a positive control), or CM398 (0.23, 2.32, or 23.2 μmol/kg, i.p.), formalin (10 μL of a 10% solution) was injected into the hind paw as described previously (40,41). Paw-licking duration (in s) during 5 min intervals was recorded for 70 min. The summated response during the final 60 min was analyzed, consistent with nociception attributed to Phase II inflammation (41,42).

All paw-licking data for formalin testing are reported as summed paw licking as area under the curve (AUC) by each animal across the 60-min measured response, ± SEM. Morphine data *vs*. saline was analyzed by Student's *t* test. CM398 data was analyzed by a one-way ANOVA (factor:dose) with significant results between groups further analyzed with Tukey's multiple comparisons *post hoc* test. The data and statistical analysis comply with the recommendations on experimental design and analysis in pharmacology (43). All data are presented as mean ± SEM, with a significance set at $P<0.05$, denoted by the asterisk (*).

RESULT

Chemistry

The straightforward synthetic scheme for CM398 is outlined in Fig. 2. Treatment of 1-fluoro-2-nitro-benzene (**1**) with 40% aqueous methylamine at room temperature gave *N*-methyl-2-nitroaniline (**2**), which was subjected to hydrogenation (H_2) in presence of 10% palladium on activated charcoal to afford N^1-methylbenzene-1,2-diamine (**3**) (25,44,45). The later intermediate was subjected to ring closure using 1,1′-carbonyldiimidazole (CDI) in anhydrous tetrahydrofuran (THF) and heated to 65°C to obtain 1-methyl-2-benzimidazolinone (**4**). Treatment of compound **4** with 1,4-dibromobutane in the presence of anhydrous potassium carbonate in anhydrous dimethylformamide (DMF) gave **5**. The bromo derivative (**5**) was then coupled with 6,7-dimethoxy-1,2,3,4-tetrahydroisoquinoline in the presence of potassium carbonate in DMF to afford the target compound (CM398). Spectral analysis and high-resolution mass spectra of CM398 were consistent with its assigned structure.

Competition Binding Assays

The binding affinity of CM398 for sigma-1 and sigma-2 receptor subtypes is summarized in Table I. CM398 possess subnanomolar affinities for the sigma-2 receptor and over 1000-fold selectivity for the sigma-1 receptor. Moreover, binding affinities *versus* other off-target proteins, including monoamine transporter and other neurotransmitter receptors, were also reported (Table I). The receptor binding profile of CM398 is indicative of its highly preference for the sigma-2 compared with the sigma-1 and to the other non-sigma proteins (selectivity ratio > 1000). Furthermore, CM398 resulted highly selective also for the norepinephrine transporter receptor ($Ki > 1{,}000$ nM) and for the serotonin transporter receptor (500-fold); on the other hand, CM398 displayed significant affinity for the dopamine transporter (Ki = 32.90 nM). However, because dopamine transmission is not strictly related to nociception but rather it may reinforce the noradrenergic effects to inhibit certain type of pain (46), CM398 represented a suitable probe for the scope of this study.

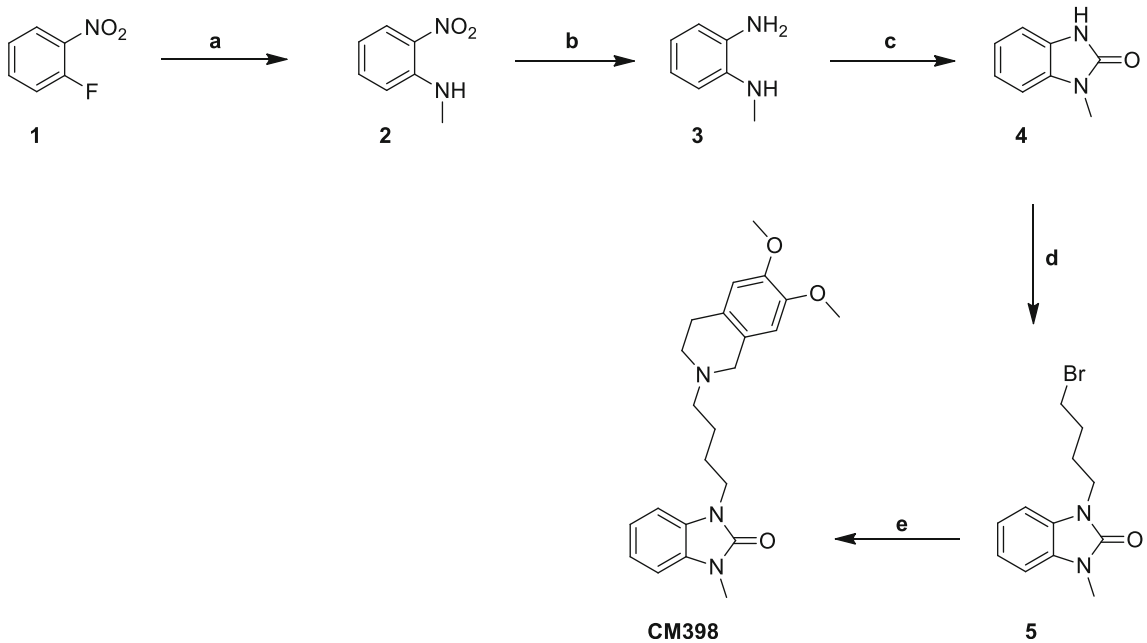

Fig. 2. Synthesis of CM398. Reagents and conditions: **a** CH_3NH_2, H_2O, 2 h, r.t. **b** 10% Pd/C, H_2 (1 atm), MeOH, 2 h. **c** CDI, THF, 65°C, 18 h. **d** 1,4-dibromobutane, K_2CO_3, DMF, 60°C, 2 h. **e** 6,7-dimethoxy-1,2,3,4-tetrahydroisoquinoline, K_2CO_3, DMF, 60°C, 1 h

Metabolic Stability in Rat Liver Microsomes

It is important to understand the fate of a discovery compound undergoing hepatic metabolism; therefore, metabolic stability of CM398 was performed in rat liver microsomes. *In vitro* metabolic stability method is able to determine the metabolic depletion of a compound by cytochrome P450 enzymes in a closed system without considering the physiological factors like blood flow or drug binding within the matrix (35). The *in vitro* half-life of CM398, when incubated in rat liver microsomes, was found to be 0.10 ± 0.01 h. The $CL_{int,H}$ derived from *in vitro* half-life and scaling factors is 12.8 ± 0.3 L/h/kg. Calculated value of f_{umic} was 0.63, which also suggests that only 37% of the compound in liver will be available for metabolism by the liver enzymes. *In vitro* $CL_{int,H}$ was also successfully extrapolated to hepatic clearance and extraction ratio. Compound CM398 exhibited both high hepatic clearance (CL_H, 4.6 ± 0.0 L/h/Kg) and extraction ratio (0.96 ± 0.01). The high extraction ratio suggests that only approximately 4% of CM398 can escape unchanged after a single pass through the liver following its phase I metabolism.

Plasma Protein Binding Studies

The reversible binding of a drug to plasma proteins is an important determinant of its PK and pharmacodynamics characteristics. The protein binding of CM398 was evaluated *in vitro* using ultra-filtration method. The concentration-dependent F_u of CM398 was studied in rat plasma. At concentrations of 1 and 10 μM, the F_u values were 6.7 ± 1.1 and 5.6 ± 0.1%, respectively. Average plasma protein binding of CM398 was found to be 93.8 ± 0.9%. Non-specific binding of CM398 to Centrifree® devices was less than 5.0%.

Pharmacokinetic Studies in Rats

The PK properties of CM398 were studied following the P.O. (20 mg/kg) and I.V. (1 mg/kg) administration of the compound to male *Sprague Dawley* rats. After dosing of CM398, close and continuous visual monitoring of the animals revealed that there was no severe acute toxicity response, as none of the animals showed any signs of behavioral or neurological toxicity during the entire study period. The plasma concentration-time profiles are presented in Fig. 3. PK parameters were estimated using non-compartmental analysis with Phoenix WinNonlin and are presented in Table II. CM398 shows a very rapid absorption as C_{max} (2052.8 ± 252.8 ng/mL) occurred at 0.17 ± 0.00 h (T_{max}) post-dose. Oral absorption is so fast, that the absorption phase cannot be captured. Absolute oral bioavailability was calculated to be 29.0%, indicating adequate total exposure after the oral dose. Total body CL (2.1 ± 0.1 L/h/kg) of CM398 was lower than the total hepatic blood flow (4.8 L/h/kg) (33) in rats, indicating negligible extra-hepatic elimination of the compound. The volume of distribution (V_d, 5.3 ± 0.9 L/kg) of CM398 was larger than the total blood volume of rats (0.085 L/kg) (34), showing extra-vascular distribution of the compound. After P.O. and I.V. dosing, the mean half- life ($T_{1/2}$) was found to be 1.9 ± 0.2 and 1.7 ± 0.3 h, respectively.

In Vivo Characterization

The analgesic effects *in vivo* of CM398 were tested using the formalin model of inflammatory pain in mice. Mice ($n = 7$) were pretreated with vehicle (saline, 0.9%), morphine (23.2 μmol/kg), or CM398 (0.23, 2.32, or 23.2 μmol/kg) through the intraperitoneal (i.p.) route and 10 min later, administered formalin (10 μL in the hind paw). Mice pretreated with saline spent an average of 205.1 ± 32.6 s

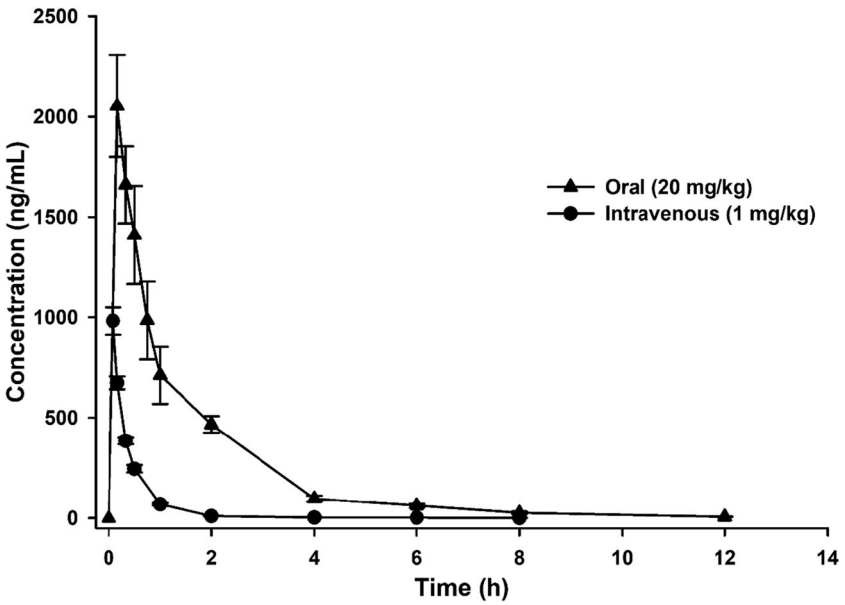

Fig. 3. Mean plasma concentration-time profile of CM398 in male *Sprague Dawley* rats ($N = 5$, each) after a single oral (20 mg/kg) and intravenous (1 mg/kg) administration. Bar represents SEM

Table II. Pharmacokinetic parameters of CM398 after oral dose and intravenous administration in male *Sprague Dawley* rats[a]

Parameters	Oral	Intravenous
C_{max} (ng/mL)	2052.8 ± 252.8	–
T_{max} (h)	0.17 ± 0.00	–
$AUC_{0\rightarrow t}$ (ng h/L)	2733.6 ± 293.4	471.3 ± 23.6
Vd (L/kg)	6.2 ± 0.6	5.3 ± 0.9
T1/2 (h)	1.9 ± 0.2	1.7 ± 0.3
CL (L/h/kg)	2.2 ± 0.2	2.1 ± 0.1
Bioavailability (%)	29.0	–

[a] *Each value represents the average of five rats dosed oral (20 mg/kg) and intravenous (1 mg/kg); values are mean ± SEM.*
AUC area under the plasma concentration-time curve, CL clearance, C_{max} plasma peak concentration, T_{max} time to C_{max}, $T_{1/2}$ elimination half-life, V_d volume of distribution

licking the injected hindpaw. Mice pretreated with morphine spent significantly less time licking their formalin-treated hindpaw (205.1 ± 32.6 s; $p = 0.0002$, Student's t test). Pretreatment with CM398 dose dependently reduced the time spent licking the hind paw (Fig. 4), demonstrating a significant antinociceptive effect doses of 2.32 and 23.2 µmol/kg, i.p. ($F(3, 24) = 7.66$, $p = 0.0009$; one-way ANOVA with Tukey's *post hoc* test).

DISCUSSION

Previous research and preclinical studies have elucidated the role of the sigma-1 receptor in modulating nociception (8,47). In particular, the high expression of sigma-1 receptors in specific areas of the brain such as dorsal spinal cord, thalamus, periaqueductal gray, basolateral amygdala, and rostroventral medulla (48), as well as in peripheral tissues (especially dorsal root ganglia neurons) (49), clearly suggests its involvement in pain modulation.

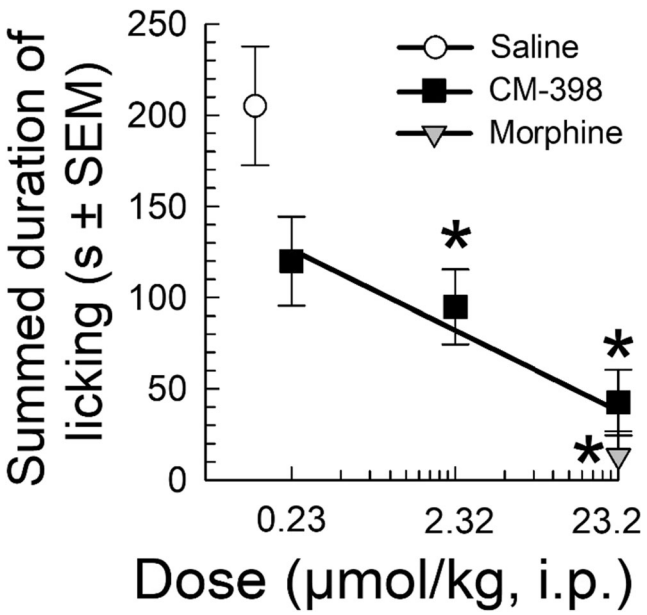

Fig. 4. Antinociceptive effects of CM398 in the formalin test. Values represent summed time licking across 60 min testing period following 5% formalin injection into the mouse hindpaw. $n = 7$ (each); $^*p = 0.02$ *vs.* vehicle response; one-way ANOVA with Tukey's *post hoc* test for CM398, or $p = 0.0002$ *vs.* vehicle response; Student's t test for morphine

From a pharmacological point of view, inhibition of sigma-1 receptor produces a decrease of nociception stimuli through attenuated expression of pain behaviors in several animal models, including mechanical hypersensitivity (capsaicin-induced test), inflammatory pain (formalin assay), and neuropathic pain models (SNI in mice) (50). Moreover, these findings were supported by previous studies which used sigma-1 receptor KO mice (51–53).

In a more recent study, Sahn *et al.* have tested the effect of IT injection of different sigma-1 and sigma-2 receptor ligands in the mouse SNI model (24). As a result, both the sigma-1 and the sigma-2/Tmem97 preferring ligands produced antinociceptive effects, which were significantly different from vehicle, whereas the moderate selective sigma-2 receptor agonist, siramesine, showed only a modest inhibitory effect on mechanical hypersensitivity (24). These results led to further investigations into the putative role of sigma-2 receptors in the modulation of pain. In fact, unlike the results obtained for sigma-1 receptor antagonists, which are consistent with previously reported literature, those obtained by using slightly preferring sigma-2 receptor ligands did not completely clarify if a mixed sigma-1/sigma-2 or a selective sigma-2 compound might be beneficial to treat pain. Unfortunately, as is often the case, the lack of very selective ligands for the protein target adds complexity for elucidating their function. From this perspective, the main goal of this work was the preliminary characterization of a highly selective sigma-2 receptor ligand with drug-like properties, serving as a lead candidate for further preclinical and clinical development.

The radioligand binding assays revealed that CM398 displayed subnanomolar affinities ($Ki = 0.43$ nM) for the sigma-2 receptor and very low affinities ($Ki = 560$ nM) for the sigma-1 receptor, thus, to the best of our knowledge, CM398 represents the most selective sigma-2 receptor ligand reported to date with regard to sigma-1/sigma-2 selectivity ratio (1000-fold) (54). The binding properties of CM398 were consistent with those of a recently reported set of structurally related benzimidazolone-based analogs (25). Structure-affinity relationships (SARs) studies suggested that the presence of both the benzimidazolone as a scaffold and the 6,7-disubstituted tetrahydroisoquinoline as a cyclic amine fragment was optimal in term of sigma binding profile. Specifically, replacement of the 1-(4-fluorophenyl)piperazine (CM397, Fig.1) with the 6,7-dimethoxy-1,2,3,4-tetrahydroisoquinoline moiety significantly increased the affinity for the sigma-2 receptor ($Ki = 2.5$ and 0.43 nM) and the selectivity over the sigma-1 receptor subtype (179-fold and > 1000 fold, respectively).

CM398 also possessed negligible affinities for D_2, 5-HT_2, NMDA, opioid receptors, and norepinephrine transporters (1000–10,000 nM). CM398 did demonstrate notable affinity for dopamine (Ki = 32.90 nM) and serotonin transporters (Ki = 244.2 nM), but these are still 76-fold and > 500-fold over the

affinity for sigma-2 receptors. As mentioned above, the *in vitro* pharmacologic profile of CM398 was well suited to unveil the utility of sigma-2 ligands as potential therapeutic for pain treatment, especially due to the lack of affinity with those target proteins mainly involved in pain relief mechanisms, for instance, opioid receptors, noradrenaline transporters, D_2, and NMDA receptors (55–59).

In regard to the pharmacokinetic evaluation, CM398 demonstrated adequate oral exposure, extravascular distribution, and negligible extra hepatic elimination in rats indicating satisfactory pharmacokinetic properties to support its further development as a pharmacological tool and potential orally active drug candidate.

Finally, we decided to test the analgesic properties of CM398 in a tonic pain stimuli induced by formalin injection rather than allodynia elicited by chronic nerve injuries. The formalin test is a standard animal model of inflammation-induced nociception and frequently included in the battery of behavioral pharmacology tests of pain (60). The 2.32 and 23.2 μmol/kg i.p. doses of CM398 in mice produced a significant reduction of the time spent licking as compared with saline, through attenuation of the localized inflammatory pain produced after injection of formalin in the paw of mice. These results suggest that targeting sigma-2 receptor with a highly selective ligand, like CM398, may be effective in alleviating inflammatory pain. Notably, this initial testing was limited to male mice to be consistent with the previous testing (24). Although sex differences have not been demonstrated in antinociception studies involving sigma-1 receptors (51,52), future studies are needed to examine possible sex effects on sigma-2 receptor-mediated antinociception. Although the main goal of this work was limited to the preliminary evaluation of the pharmacological profile of a potent and highly selective sigma-2 receptor ligand, collectively, this preliminary data offers support for further, extensive characterization.

CONCLUSION

In summary, we synthesized and characterized CM398, a highly selective sigma-2 receptor ligand, with the highest sigma-1/sigma-2 selectivity ratio known to date. Moreover, CM398 acted as a sigma-2 preferring ligand *versus* other non-sigma receptors involved in pain modulation, particularly opioid receptors, NMDA, and norepinephrine transporters. The metabolism studies performed on CM398 showed suitable drug-like properties for the development of an orally active drug. Despite the favorable pharmacological profile of CM398, additional evaluation of analgesic effects in a full battery of behavioral models examining the various modalities of pain is needed. The data provided herein represent further support for the development of sigma-2 receptors ligands as an alternative pain medication.

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