Chiral Aryloxyalkylamines: Selective 5-HT$_{1B/1D}$ Activation and Analgesic Activity

Alessia Carocci, Giovanni Lentini, Alessia Catalano, Maria Maddalena Cavalluzzi, Claudio Bruno, Marilena Muraglia, Nicola Antonio Colabufo, Nicoletta Galeotti, Filomena Corbo, Rosanna Matucci, Carla Ghelardini, and Carlo Franchini

A series of chiral 2,3-dichlorophenoxy and 1-naphthoxy alkylamines were synthesized, and their binding affinities towards 5-HT$_{1D}$ and 5-HT$_{1B}$ receptors were evaluated. In the naphthoxy series, the (R)-prolinol derivative was the most selective 5-HT$_{1D}$ ligand, while (S)-N-methyl-2-(1-naphthoxy)propan-1-amine showed the highest selectivity for 5-HT$_{1B}$. Both compounds performed as 5-HT$_{1D}$ agonists in the isolated guinea pig assay and showed higher analgesic activity than both sumatriptan and the achiral analogue 20b in the mouse hot-plate test. Neither ligand displayed any affinity for nicotinic ACh receptors present in mouse brain membranes, thus indicating that their analgesic activity does not arise through interaction with these receptors.

Introduction

Serotonin (5-HT) receptors are divided into seven classes (5-HT$_1$–7) based upon pharmacological, structural, and signaling properties. 5-HT$_1$ receptors are further divided into several populations that include the 5-HT$_{1A}$ and 5-HT$_{1D}$ subtypes. 5-HT$_{1B}$ receptors are found in rodent brain but not in human brain, whereas the reverse is true for 5-HT$_{1D}$ receptors. Two distinct populations of human 5-HT$_{1D}$ receptors have been identified: 5-HT$_{1D,a}$ and 5-HT$_{1D,b}$ receptors. In an attempt to unify rather confusing nomenclature, these subtypes are now commonly named h5-HT$_{1D}$ and h5-HT$_{1B}$, respectively. The latter is believed to represent the species homologue of the rat 5-HT$_{1B}$ receptor. Receptor mapping studies showed that the h5-HT$_{1B}$ receptor is widely distributed in both neural and vascular tissues of the central nervous system (CNS), whereas the h5-HT$_{1D}$ receptor is restricted to neural tissues including the trigeminal ganglia. Both receptor subtypes are believed to play a pivotal role in both vascular and injured nerve-pain modulation. 5-HT$_{1B/1D}$ receptors are localized on peptidergic nociceptors in the so-called trigeminocephalic complex—a group of cells located in the rostral sections of the spinal cord that are supposed to provide a possible anatomical substrate for migraine pain. Finally, 5-HT$_{1B/1D}$ receptors seem to modulate vascular nociceptive stimuli processed in the thalamus.

5-HT$_{1B/1D}$ receptor subtypes are also involved in the serotonergic descending pain modulating system, and 5-HT$_{1B}$ activation demonstrated to produce a selective blockade of the spinal nociceptive processing. Both subtypes have been implicated in the control of several sensory circuits, and the activation of 5-HT$_{1B}$ and 5-HT$_{1D}$ receptors have opposing actions on some nerves receiving afferent input. Thus, selective h5-HT$_{1D}$ and h5-HT$_{1B}$ agonists could be useful to clarify the role of each subtype in the processing of nociceptive stimuli and might drive the research into new agents for the treatment of migraine and chronic pain. Indeed, it is generally accepted that the clinical efficacy of sumatriptan, the first of a well-known class of therapeutic agents for the treatment of acute migraine headaches, is mediated through action on both h5-HT$_{1D}$ and h5-HT$_{1B}$ receptor subtypes. Activation of the h5-HT$_{1B}$ receptor may cause coronary artery constriction, which precludes the use of sumatriptan in patients with concomitant heart disease. These findings have suggested the development of selective h5-HT$_{1D}$ receptor agonists as potential second-generation antimigraine agents, which may lack the vasoconstrictor action of unselective compounds, such as sumatriptan, thus presenting an improved side-effect profile.

Most h5-HT$_{1D}$ ligands are tryptamine-based and, hence, are likely to suffer from related drawbacks including poor adsorption and low bioavailability. As a result, non-tryptamine agents with selectivity for h5-HT$_{1D}$ over h5-HT$_{1B}$ receptors are...
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attractive targets for development. Studies focused on the identification of novel non-tryptaminergic S-HT\textsubscript{1D} ligands showed that aryloxyalkylamines, such as 2-(2,3-dichlorophenoxy)-N-methylethanolamine (20\textsubscript{a}) and N-methyl-2-(1-naphthyl-oxo)ethanethiol (20\textsubscript{b}), bind to h5-HT\textsubscript{1D} and h5-HT\textsubscript{1B} receptors with high affinity. Indeed, some naphthyl derivatives display approximately tenfold selectivity for h5-HT\textsubscript{1D} over h5-HT\textsubscript{1B} receptors.\textsuperscript{[18,19]} Starting from the observation that both tryptamines\textsuperscript{[20,21]} and non-indole\textsuperscript{[22]} chiral 5-HT\textsubscript{1D}/1B ligands show stereoselectivity, we speculated that both efficacy and selectivity towards h5-HT\textsubscript{1B} and h5-HT\textsubscript{1D} might be modulated by exploiting chirality. Chiral aryloxyalkylamines \textsuperscript{21\textsubscript{a,b}} and \textsuperscript{22\textsubscript{a,b}} were designed by the introduction of a methyl group onto the ethylenic chain of the parent compounds \textsuperscript{20\textsubscript{a,b}}. Two conformationally constrained analogues were designed by including part of the ethylenic chain in a pyrrolidine ring (31\textsubscript{a,b}). Here we report the synthesis of both series of compounds and the preliminary investigation into their pharmacological profiles.

Chemistry

\textit{N}-Methylaryloxyalkylamines \textsuperscript{20\textsubscript{a,b}-22\textsubscript{a,b}} were prepared via the synthetic routes shown in Scheme 1. Compounds \textsuperscript{20\textsubscript{a,b}} were prepared starting from commercially available phthalimido alcohol 4, which was condensed with the appropriate aromatic hydroxyl compound (1\textsubscript{a,b}) under Mitsunobu conditions\textsuperscript{[22]} to give the corresponding aryl ethyl ethers (8\textsubscript{a,b}). N-Methyl-3-aryloxy-2-propylamines 21\textsubscript{a,b} were prepared starting from phthalimido alcohol 7 (prepared as reported in the literature),\textsuperscript{[24]} which was condensed with compounds 1\textsubscript{a,b} under Mitsunobu conditions to give the corresponding aryl alkyl ethers (9\textsubscript{a,b}). N-Methyl-2-aryloxy-1-propylamines 22\textsubscript{a,b} were prepared from compounds 1\textsubscript{a,b}, which were O-alkylated with 3-bromo-2-propanol under Mitsunobu conditions. The resulting bromo derivatives (3\textsubscript{a,b}) underwent a Gabriel reaction with potassium phthalimide to give the corresponding aryl propyl ethers (10\textsubscript{a,b}). Amines 11\textsubscript{a,b-13\textsubscript{a,b}}, obtained by hydrazinolysis\textsuperscript{[25]} of the corresponding phthaloylated intermediates 8\textsubscript{a,b}-10\textsubscript{a,b}, were converted into the corresponding trifluoroacetamido derivatives 14\textsubscript{a,b-16\textsubscript{a,b}} by treatment with trifluoroacetic anhydride in anhydrous THF. N-Alkylation of 14\textsubscript{a,b-16\textsubscript{a,b}} with methyl iodide gave 17\textsubscript{a,b-19\textsubscript{a,b}}, which in turn were hydrolyzed\textsuperscript{[26]} to afford 20\textsubscript{a,b-22\textsubscript{a,b}}. The free amines were converted into the corresponding hydrochlorides (20\textsubscript{a,b-HCl-22\textsubscript{a,b}-HCl}) by treatment with aqueous HCl and azeotropic removal of water.

The homochiral forms of 22\textsubscript{b} were prepared according to Scheme 2, which differs from Scheme 1 only in the first three steps. Thus, 1\textsubscript{b} was condensed with commercially available methyl (R)-(+-)-lactate [(R)-23] and ethyl (S)(--)-lactate [(S)-24] according to Mitsunobu procedure to give the corresponding esters (S)-25 and (R)-26, respectively. (S)- and (R)-27, obtained by reduction of (S)-25 and (R)-26, respectively, were reacted with phthalimide under Mitsunobu conditions to give the phthaloylated intermediates (S)- and (R)-10\textsubscript{b}.

2-(Aryloxymethyl)pyrrolidines 31\textsubscript{a,b} were prepared according to the synthetic route described in Scheme 3. In the first step, the amino groups of the homochiral pyrrolidin-2-ylmethanols (R)- and (S)-28 were Boc protected. The obtained N-Boc aminooacohols (R)- and (S)-29 were reacted with 1\textsubscript{a,b} according to Mitsunobu procedure to give the corresponding aryl alkyl ethers (R)- and (S)-30\textsubscript{a,b}. The removal of the Boc protect-
**Results**

Aryloxyalkylamines 20a,b and their analogues were evaluated for their binding affinity towards 5-HT$_{10}$ and h5-HT$_{18}$ receptors (Table 1); $pK_a$, Log$P$, and Log$D$ values for all of these compounds were experimentally determined in order to evaluate a possible correlation between their physicochemical properties and biological activity. All aryloxyalkylamines presented higher Log$D$ values than sumatriptan. Naphthoxy derivatives (b series) were more basic and showed higher affinity for h5-HT$_{18}$ than the corresponding 2,3-dichlorophenoxy analogues (a series). With the exception of (R)-21 and (S)-31, the same relationships were also observed in 5-HT$_{10}$ affinities. Compounds 21a,b, in which a methyl group has been formally introduced in the 1-position of the ethylamino side chain, were poor 5-HT$_{10}$ and h5-HT$_{18}$ ligands (when compared to reference compounds 20a,b).

The introduction of a methyl group in 2-position of the alkyl chain (22a,b) offers no advantage over the parent compounds (20a,b), if racemates are considered. However, when homochiral forms are concerned, chirality conferred stereochemistry-dependent selectivity in favor of h5-HT$_{18}$. In fact, the addition of a methyl group did not markedly alter h5-HT$_{18}$ affinity regardless of stereochemistry (cf. (R)- and (S)-22b with 20b) but dramatically lowered (S)-22b affinity for 5-HT$_{10}$ (two orders of magnitude). Derivative 22b exhibits high stereo-differentiation in terms of receptor binding, especially for the 5-HT$_{10}$ subtype; the (R)-isomer is the eutomer ($K_i$ distomer/$K_i$ eutomer = 84). Thus, (S)-22b was about ten times more selective for h5-HT$_{18}$ than the reference compound 20b.

A reversal of receptor subtype selectivity was observed when constraining the nitrogen atom into a pyrroline ring to give restricted analogues 31a,b. While h5-HT$_{18}$ affinity was lowered (cf. 20b with (R)- and (S)-31a,b), 5-HT$_{10}$ affinity was slightly improved in (R)-31b, which performed as the most selective 5-HT$_{10}$ ligand of both series (h5-HT$_{18}$ $K_i$/5-HT$_{10}$ $K_i$ = 26). Derivative (S)-31b showed about 20-times lower affinity. When comparing 31a and 31b affinities for 5-HT$_{10}$ receptors, a reversal of the stereoselectivity pattern was observed, with (S)- and (R)-isomer being the eutomers, respectively.

In order to establish their intrinsic activity, the most selective compounds for h5-HT$_{18}$ and 5-HT$_{10}$ (S)-22b and (R)-31b, respectively, together with sumatriptan as a reference compound, were evaluated in the isolated guinea pig ileum assay, where 5-HT$_{10}$ receptor agonists inhibit the substance P evoked contractions in a dose-dependent fashion.$^{29}$ All compounds displayed agonistic activity in the same rank order (EC$_{50}$ values from 12 $\mu$m to 30 $\mu$m) as depicted in Figure 1. In order to establish for each compound whether this effect was 5-HT$_{10}$ mediated, the assay was repeated after desensitizing 5-HT$_{10}$ receptors with sumatriptan at high concentration (Figure 2). The results showed that the sumatriptan-mediated relaxation effect was largely due to 5-HT$_{10}$ contribution. By contrast, the relaxation effects of (S)-22b and (R)-31b are partially 5-HT$_{10}$ mediated (60% and 50%, respectively).

Agnons (S)-22b and (R)-31b, together with sumatriptan and 20b as an in vivo baseline, were evaluated in the hot-plate test (Table 2). Both compounds, administered (i.p.) at a dose of 30 mg kg$^{-1}$, were able to increase the pain threshold in mice in the presence of a thermal stimulus in a statistically significant manner. The analgesic effect induced by (S)-22b and
Clic compounds have been re-
22b and (R)-31b, together with nicotine as a reference compound, were tested for their binding affinities at nAChRs present in mouse brain membranes labeled by [3H]cytisine (Table 3). Only nicotine was able to inhibit the specific binding of [3H]cytisine, showing an IC_{50} value of 6.76 ± 0.16 mM (pIC_{50} = 8.17 ± 0.14), while the two newly synthesized compounds failed to bind to these receptors; (S)-22b and (R)-31b were only partially able to decrease [3H]cytisine-specific binding, with IC_{50} values of 0.692 ± 0.015 mM (pIC_{50} = 3.16 ± 0.07) and 44.9 ± 0.8 μM (pIC_{50} = 4.35 ± 0.08), respectively.

### Table 3. Binding affinities of the tested compounds at nAChR in mouse brain membranes.

<table>
<thead>
<tr>
<th>Compd</th>
<th>[3H]cytisine^{24} pIC_{50}</th>
<th>[3H]cytisine IC_{50} ± SEM [μM]</th>
</tr>
</thead>
<tbody>
<tr>
<td>nicotine</td>
<td>8.17 ± 0.14</td>
<td>0.00676 ± 0.00016</td>
</tr>
<tr>
<td>(S)-22b</td>
<td>3.16 ± 0.07</td>
<td>692 ± 15</td>
</tr>
<tr>
<td>(R)-31b</td>
<td>43.5 ± 0.08</td>
<td>44.9 ± 0.8</td>
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</tbody>
</table>

[a] Values are expressed as pIC_{50} ± SEM of at least three independent experiments.

### Discussion

In agreement with previously reported data, our study confirms that the naphthyl and 2,3-dichlorophenyl rings could be considered as bioisosteric nuclei. Both arylxy moieties may also be considered as bioisosteres of the indole nucleus of tryptaminergic 5-HT_{1B/1D} ligands. Similar observations have been made in the melatoninergic system, where we obtained potent melatonin analogues by replacing the melatonin 5-methoxyindol-3-yl moiety with a m-methoxyphenoxyl group. However, when homochiral bioisosteres 31a and 31b are considered, a reversal of the stereochemistry-dependent 5-HT_{1D} affinity patterns was observed. Thus, it may be argued that the two bioisosteric arylxy moieties do not bind exactly in the same mode to the active site. The naphthylxy derivatives (b series) generally show slightly higher affinities than the 2,3-dichlorophenoxyl analogues (a series), presumably as a consequence of higher basicities. The introduction of a methyl group in the α position to the nitrogen of the ethylamino side chain (compounds 21a,b) was detrimental. The above observations are in agreement with the hypothesis that an aspartic acid residue (Asp118) in the h5-HT_{1D} sequence interacts with the protonated forms of ligands. In fact, while higher pK_a values lead to an increase in the molar ratios of the protonated forms in the biophase, steric hindrance introduced by substitution might hamper the interaction with the anionic binding site and/or prevents the homologues adopting the active conformation. However, these detrimental steric factors did not completely abolish affinity in the pyrrolidine derivatives series (31a,b), probably due to compensating effects of basicity enhancement and/or chain strain imposed by inclusion of the α-substituent in the pyrrolidine ring.

Both (S)-22b and (R)-31b performed as 5-HT_{1D} agonists, showing higher efficacy than sumatriptan in the isolated guinea pig ileum assay, and displayed a more potent analgesic effect than both sumatriptan and the achiral analogue 20b. When evaluated in a nAChR binding assay, compounds (S)-22b and (R)-31b showed no significant affinity towards nAChRs (Table 3), indicating that the analgesic effect of (S)-22b and (R)-31b does not benefit from direct nicotinic mechanisms. Other mechanisms, both centrally- (e.g., noradrenergic modulating system) and peripherally- (e.g., sodium-channel blockade) localized, might contribute and, thus, explain why no clear relationship between h5-HT_{1D}/5-HT_{1D} affinities and in vivo analgesia was found.

### Conclusions

Effective alleviation of pain has still to be achieved. Currently available therapeutic agents are unsatisfactory for about 50% of patients and may be accompanied by several side effects. The last 20 years have witnessed a rise in interest towards the so-called adjuvant analgesic drugs, which have primary indications other than pain but may be analgesics under certain circumstances. In this heterogeneous group of alternative drugs, h5-HT_{1B/1D}-selective agonists acting on the serotonergic pain-modulating system may offer new perspectives. The aryloxyalkylamines reported herein present higher Log D values than sumatriptan. In principal, they should allow pharmacological investigation of parts of the serotonergic pain-modulating system generally unreachable by sumatriptan because of its low Log D values.

The superior analgesic effect of the newly proposed homochiral bioisosteres of sumatriptan, (S)-22b and (R)-31b, compared to that of both the parent compound and the achiral analogue 20b and despite comparable affinity for 5-HT_{1B/1D} receptors, might be ascribed to concurrent unknown mechanisms and/or advantageous pharmacokinetics. Our preliminary results suggest that nicotinic systems should be ruled out. Work has been undertaken to explore the possible participation of nerve sodium-channel blockade.

Since aryloxyalkylamines can be obtained through facile and versatile synthetic routes reported in this paper and elsewhere, the compounds described herein might help to reach the goal described above. The requirements for stereochemistry-specific binding of an arylxyalkylamine to 5-HT_{1D} are generally more stringent than those for h5-HT_{1D} binding. These observations show that it may be possible to design more selective h5-HT_{1D} ligands by modulating the volume of the substituent in the 2-position of 20b alkyl chain in order to reduce 5-HT_{1D} affinity, as observed for (S)-22b. On the other hand, the conformationally constrained analogue (R)-31b may well be considered as the starting point for future SAR studies aimed at improving affinity. Finally, it should be noted that chiral aryloxyalkylamines, despite their relatively low-molecular weight, possess molecular complexity (i.e., relatively high proportion of sp^3-hybridized carbons and chirality) and this should increase the probability that their study will lead to useful drugs.
Experimental Section

Chemistry

Chemicals were purchased from Sigma–Aldrich or Lancaster in the highest quality commercially available. Solvents were RP grade unless otherwise indicated. Compound 7 was prepared as previously described.\(^{24}\) (+)-S)-O-P-Chlorophenylmandelic acid, used as a chiral solvating agent, was prepared in our laboratories.\(^{25}\) Yields refer to purified products and were not optimized. Yields of hydrochlorides refer to the recrystallized products. The structures of the compounds were confirmed by routine spectrometric analyses. Only spectra for compounds not previously described are given. Melting points were determined on a Gallenkamp melting point apparatus in open glass capillary tubes and are uncorrected. The infrared spectra were recorded on a Perkin–Elmer Spectrum One FT spectrophotometer and band positions are given in reciprocal centimeters (\(cm^{-1}\)). \(^1\)H NMR spectra were recorded on either a Varian XL 200 spectrometer (90 MHz) using TMS as an internal standard and CDCl\(_3\) as the solvent or a Varian Mercury-VX spectrometer operating at 300 MHz using CDCl\(_3\) or [D\(_6\)]DMSO (where indicated) as the solvent. Chemical shifts (\(\delta\)) are reported in ppm relative to either TMS or the residual nondeuterated solvent resonance (CDCl\(_3\)); \(\delta = 7.26 \) ppm; [D\(_6\)]DMSO; \(\delta = 2.50 \) ppm; CD\(_3\)OD; \(\delta = 3.30 \) ppm) as internal references. Coupling constants (\(J\)) are given in Hz. \(^1\)C NMR spectra were recorded on a Varian Mercury-VX spectrometer operating at 75 MHz using CD\(_3\)OD as solvent (\(\delta = 47.8\)). Enantiotropic excess (ee) values were determined by chiral \(^1\)H NMR analyses, which were performed on free amines recovered by extraction of a sample of the corresponding hydrochloride salts and dissolved with 1.5 equiv of chiral solvating agent in CDCl\(_3\).\(^{26}\) TLC analyses and/or in a iodine chamber. The purity of the final compounds was confirmed by thin layer chromatography of a sample of the corresponding hydrochloride salts and analyses, which were performed on free amines recovered by extraction of a sample of the corresponding hydrochloride salts and dissolved with 1.5 equiv of chiral solvating agent in CDCl\(_3\).\(^{26}\) Optical rotations were measured on a Perkin–Elmer (Norwalk, CT) 341 spectropolarimeter; concentrations are expressed in 100 \(\mu\)L.\(^{27}\), and the cell length was 1 dm, thus \([\alpha]_{D}^{22}\) values are given in units of \(10^{-1} \text{deg cm}^{-1} \text{g}^{-1}\). Chromatographic separations were performed on silica gel columns by flash chromatography (Kieselgel 60, 0.040–0.063 mm, Merck, Darmstadt, Germany) using the technique described by Still et al.\(^{28}\) TLC analyses were performed on precoated silica gel on aluminium sheets (Kieselgel 60 \(F_254\), Merck). TLC plates were visualized under UV light and/or in a iodine chamber. The purity of the final compounds was determined by elemental analysis.

(R,S)-2-(3-Dichlorophenoy)-N-methylpropan-1-amine Hydrochloride (22a·HCl): The title compound was prepared from 18a using the procedure described for the preparation of 20a·HCl (See Supporting Information).

Compound 22a (72%): MS (70 eV): m/z (%): 234 \([M+1]^+\) (1); 216 \([M]^+\) (1); 215 \([M]^+\) (1); 216 (98), 214 (44). 

\(\text{FTIR (KBr): \(\nu \text{N-H} = 3440, \text{m} \text{C=O} = 1721, \text{m} \text{C} = \text{C} = 1618, \text{m} \text{C} - \text{O} = 1266, \text{m} \text{C} - \text{H} = 750, \text{m} \text{C} - \text{H} = 695 \text{~cm}^{-1}.\)
183–184 °C (abs EtOH/ EtO); 92% ee ([3 H NMR); [α]D20 = +73 (c = 2, CHCl₃); [3 H NMR (300 MHz, D₂]ujOSO)] θ = 0.13 (d, J = 6.0 Hz, 3H, CH₃). 2.48 (br s, 1H, NH); 2.62 (s, 3H, CH₃N); 3.25–3.45 (m, 2H, CH₂), 4.95–5.15 (m, 1H, CH₂), 7.09 (d, J = 7.7 Hz, 1H, Ar), 7.38–7.58 (m, 4H, Ar), 7.82–7.90 (m, 1H, Ar), 8.25–8.35 (m, 1H, Ar), 9.12 ppm (br s, 1H, NH); [3 C NMR (75 MHz, CDCl₃); δ = 15.8 (13C), 33.0 (13C), 53.6 (13C), 70.3 (13C), 107.6 (13C), 121.5 (13C), 125.3 (13C), 125.7 (13C), 126.4 (13C), 127.4 (13C), 135.1 (13C), 152.2 ppm (13C); Anal. calcd for C₃H₁₁NO·HCl: C 66.7, H 7.21, N 5.56, found: C 66.6, H 7.37, N 5.58.

(−)(−)-N-Methyl-2-(1-naphthyl)propan-1-amine Hydrochloride [(+)−(−)-22 b·HCl]: The title compound was prepared from (+)−R)-19 b using the procedure described in the preparation of (+)−20 a·HCl (See Supporting Information). Compound (−)−(−)-22 b (93%): [α]D20 = −62 (c = 2.6, CHCl₃); [3 H NMR (300 MHz); δ = 1.40 (d, J = 6.0 Hz, 3H, CH₃), 2.19 (s, 3H, CH₃), 2.86 (dd, J = 12.4, 3.5 Hz, 1H, CH₂), 3.05 (dd, J = 12.4, 7.8 Hz, 1H, CH₂), 4.72–4.88 (m, 1H, CH₂), 6.90 (d, J = 7.4 Hz, 1H, Ar), 7.35–7.55 (m, 4H, Ar), 7.75–7.85 (m, 1H, Ar), 8.20–8.30 ppm (m, 1H, Ar); MS (70 ev): m/z (%) 215 [M]+ = 72 (100). Salt (−)−(−)-22 b·HCl (52%): mp: 184–185 °C (abs EtOH/ EtO); 97% ee ([3 H NMR); [α]D20 = −70 (c = 2, CHCl₃); [3 H NMR (300 MHz, CDCl₃); δ = 1.39 (d, J = 6.0 Hz, 3H, CH₃), 2.82 (s, 3H, CH₃N), 3.38–3.55 (m, 2H, CH₂), 4.98–5.12 (m, 1H, CH₂), 7.08 (d, J = 7.4 Hz, 1H, Ar), 7.42 (t, 1H, Ar), 7.45–7.55 (m, 3H, Ar), 7.78–7.88 (m, 1H, Ar), 8.28–8.36 ppm (m, 1H, Ar); 13 C NMR (75 MHz, CDCl₃); δ = 15.8 (13C), 33.1 (13C), 53.6 (13C), 70.3 (13C), 107.6 (13C), 121.4 (13C), 122.0 (13C), 125.3 (13C), 126.4 (13C), 126.7 (13C), 127.4 (13C), 135.1 (13C), 152.2 ppm (13C); Anal. calcd for C₃H₁₁NO·HCl: C 66.0, H 7.25, N 5.50, found: C 66.14, H 6.92, N 5.61.

(−)(+)-5-(2,4-Dichloro-phenyl)tetrahydropyridine Hydrochloride [(−)(+)-20 a·HCl]: A solution of (S)−20 a (1.10 g, 3.18 mmol) in 95% formic acid (6 ml) was stirred at 0 °C for 3 h and then at RT for 2.5 h. The solvent was removed in vacuo and the residue neutralized by addition of saturated aq K₂CO₃ and extracted with EtOAc (3 × 30 ml). The combined extracts were dried (Na₂SO₄), filtered, and concentrated in vacuo to give (−)(+)-20 a as a yellow oil (0.56 g, 72%): [α]D20 = +6.4 (c = 2.6, CHCl₃); IR (neat): ν = 3346 cm⁻¹ (NH); [3 H NMR (300 MHz); δ = 1.55–2.05 (m, 4H, NCH₂CH₂N); 2.11 (s, 3H, exd O, NH), 2.90–3.16 (m, 2H, NCH₂), 3.50–3.68 (m, 1H, CH₂), 3.88–4.08 (m, 2H, CH₂O), 6.78–6.92 (m, 1H, Ar), 7.02–7.20 ppm (m, 2H, Ar); MS (70 ev): m/z (%): 244 [M]+ (%) <1%; 70 (100). The salt [(−)(+)-5-(3,5)-a-acetyliminophenyl]-pyrrolidine Hydrochloride [(−)(+)-31 a·HCl]: Compound (−)(+)-31 a (92%): [α]D20 = −90 (c = 2.2, CHCl₃); [3 H NMR (300 MHz, CDCl₃); δ = 1.64–2.12 (m, 4H, NCH₂CH₂CH₂N, 1H, N), 2.95–3.17 (m, 2H, CH₂), 3.65 (quintet, 1H, CH₂O), 4.03–4.16 (m, 2H, CH₂O), 6.82 (d, J = 7.4 Hz, 1H, Ar), 7.36 (t, 1H, Ar), 7.38–7.54 (m, 3H, Ar), 7.76–7.84 (m, 1H, Ar), 8.22–8.32 ppm (m, 1H, Ar); MS (70 ev): m/z (%): 227 [M]+ (%) 4%; 227 (70). Salt [(−)(+)-31 b·HCl (80%): mp: 226–227 °C (abs EtOH/ EtO); >98% ee ([3 H NMR); [α]D20 = +52 (c = 2.2, CHCl₃); [3 H NMR (300 MHz, CDCl₃); δ = 1.92–2.42 (m, 4H, NCH₂CH₂CH₂N, 1H, N), 2.72, 2.78 (m, 2H, CH₂O), 4.14–4.25 (m, 1H, CH₂O), 4.30 (dd, J = 10.5, 8.5 Hz, 1H, CH₂O), 4.51 (dd, J = 10.5, 3.3 Hz, 1H, CH₂O), 6.97 (d, J = 7.5 Hz, 1H, Ar), 7.40 (t, 1H, Ar), 7.45–7.56 (m, 3H, Ar), 7.78–7.87 (m, 1H, Ar), 8.28–8.36 ppm (m, 1H, Ar); 13 C NMR (75 MHz, CDCl₃); δ = 23.9 (1C), 26.5 (1C), 46.0 (1C), 59.4 (1C), 67.1 (1C), 105.0 (1C), 121.2 (1C), 121.6 (1C), 125.3 (1C), 125.7 (1C), 126.4 (1C), 127.4 (1C), 134.9 (1C), 153.7 ppm (1C); Anal. calcd for C₁₅H₁₇NO·HCl: C 68.30, H 6.88, N 5.31, found: C 67.98, H 6.63, N 5.24.

Physicochemical data

Physicochemical data of compounds shown in Table 1 were obtained by a pH-meter technique using a Glpkp apparatus (Sirius Analytical Instruments Ltd., Forrest Row, East Sussex, UK).41–46 Because of the low solubility of the investigated compounds in aqueous medium, methanol was used as a cosolvent for pKₐ measurements. Three separate solutions of test compound in CH₃OH/H₂O (10–30% w/w) were prepared (concentration – 10⁻⁶ M) and subse-

quently acidified to pH 4 using aq HCl (0.5 M). The solutions were then titrated with aq KOH (0.5 M) to pH 12. Initial pKₐ values, which are the apparent ionization constants relative to the mixture of the solvents, were obtained by Bjerrum Plot, that is, the curve obtained by the difference between the curve of titration of the ionizable substance and that of the blank solution. These values were then optimized by a weighted nonlinear least-squares procedure (Refinemint Pro 1.0 software) to obtain pKₐ values in the absence of cosolvent, by extrapolation using the Yasuda–Shedlovsky equation.⁴⁴

To obtain LogP data, at least three separate titrations were performed on each compound. The concentration of the analyte was approximately 10⁻⁷ M, in mixtures of HO₃ (7.5 mL) and n-octanol, (0.1–10 mL). The biphasic solutions were acidified to pH 4 with aq HCl (0.5 M) and then titrated with aq KOH (0.5 M) to pH 12. The results were optimized as described above, and the average of these data gave the LogP value for each compound.⁴⁵ All titrations were carried out at 25 ± 0.1 °C under N₂ atmosphere to exclude CO₂.

Pharmacology

Human cloned 5-HT₁₆ receptor proteins and [³H]-5-CT were purchased from PerkinElmer Life Science (Monza, Italy). 5-HT₁₆ was obtained from Tocris (Bristol, UK). [³H]Sumatriptan was purchased from Amersham (Piscataway, USA), sumatriptan was purchased from Kemprotec Ltd (Middlesbrough, UK).

Guinea pigs used in the isolated guinea pig ileum assay were obtained from Harlan (San Pietro al Natisone, Italy). Each animal weighed 250–300 g. Male Swiss albino mice (24–26 g) from Morini (San Polo d’Enza, Italy) were used in the hot-plate assay. All experiments were carried out in accordance with the NIH Guide for the Care and Use of Laboratory animals. All efforts were made to minimize animal suffering, and to reduce the number of animals used.

Serotonergic 5-HT₅ receptor binding assay

This experiment was carried out as described by Sternfeld et al. with minor modifications.⁴⁶ The test compound at several concentrations (from 10⁻¹⁰ M to 10⁻⁶ M), [³H]-5-CT (0.56 nM), and human cloned receptor proteins were placed in incubation buffer (50 mM Tris-HCl, EDTA 1 mM, 12.5 mM MgCl₂, 0.1% ascorbic acid, pH 7.7) to give a final volume of 500 µL. The samples were incubated for 60 min at 25 °C. The suspension was filtered on GF/B filters (pre-soaked in PEI for 30 min) washing twice with the same incubation buffer. The nonspecific binding was determined in the presence of 10 µM 5-HT.

Serotonergic 5-HT₁₀ receptor binding assay

Guinea-pig striatum membranes and experimental conditions were as described by Audinot et al. with minor modifications.⁴⁸ The test compound at several concentrations (from 10⁻¹⁰ M to 10⁻⁶ M), [³H]sumatriptan (1.80 nM), and striatum membranes were placed in incubation buffer (50 mM Tris-HCl, 10 mM Pargylin, 4 mM CaCl₂, 0.1% ascorbic acid, pH 7.7) to give a final volume of 500 µL. The samples were incubated for 1 h at 25 °C in the presence of 8-OH-DPAT (1 µM) and mesulergine (10 µM) to mask 5-HT₁₆ D₂ and 5-HT₂ receptors, respectively. The suspension was filtered through glass fiber filter plates (GF/B), presoaked in PEI for 30 min, washing twice with the same incubation buffer. The nonspecific binding was determined in the presence of 10 µM sumatriptan.

[³H]Cytisine binding

[³H]Cytisine binding assays were performed as described by Ji et al.⁴⁹ with few adjustments. Samples containing 100–200 µg of protein from frozen mouse brains, 0.75 mM [³H]cytisine and the test compounds were incubated in 50 mM Tris-HCl buffer (containing 120 mM NaCl, 5 mM KCl, 2 mM CaCl₂, 2 mM MgCl₂, pH 7.4) in a final volume of 500 µL for 75 min at 4 °C. For equilibrium competition binding assays, the concentration of unlabeled compounds varied from 10⁻¹⁰ M to 10⁻⁶ M. Bound radioactivity was isolated by vacuum filtration onto presoaked GF/B using a 96-well filtration apparatus, and were then rapidly rinsed with 2 mL of the same ice-cold buffer. MicroScint-20 scintillation cocktail was added to each well and radioactivity determined using a Perkin–Elmer TopCount instrument. The nonspecific binding was measured in the presence of 10 µM nicotinic bitartrate.

Isolated guinea pig ileum assay

Guinea pigs were anesthetized, decapitated and the proximal ileum removed. The intestine was carefully flushed several times with warm Krebs–Henseleit solution (118 mM NaCl, 25 mM NaHCO₃, 4.7 mM KCl, 0.6 mM MgSO₄, 1.2 mM KH₂PO₄, 1.2 mM CaCl₂, 11.2 mM glucose, pH 7.4). Whole ileal segments (~3 cm in length) were suspended under 1.0 g tension in Krebs solution gassed with 95% O₂ and 5% CO₂ and maintained at 37 °C. Following a literature procedure with minor modification, the bath organ medium contained 1 µM atropine to antagonize cholinergically-mediated contractions due to activation of 5-HT₁ and 5-HT₁₃ receptors, 1 µM ketanserin to block 5-HT₂A receptors, 1 µM prazosin to block α₁ adrenoceptors and 1 µM indomethacin to preserve the organ integrity. Changes in tension of the tissue were recorded by Fort 10 Original WPI isometric transducer 2B(biological Instruments; Besozzo, Italy) connected to a PowerLab/400 workstation. Tissue responses were recorded as gram changes in isometric tension and expressed as percentage of reduction in the height of the contraction. Tissue was contracted by substance P (200 nM). This value was preliminary determined by concentration–response curves (1–200 nM). 200 nM substance P elicited 80% of maximum contraction. The reference compound, sumatriptan, was added 5 min before substance P addition and noncumulative concentration–response curves were constructed (0.5–5 µM). Sumatriptan induced relaxation with maximal response (52%) at 3 µM. Analogously, each test compound (0.5–3 µM) was added 5 min before substance P addition and a noncumulative concentration–response curve was constructed. The study of agonists was consequently repeated after an equilibration time (75 min) in the presence of 3 µM sumatriptan in order to obtain the desensitization of 5-HT₁₃ receptors and to establish the contribution of other receptors.

Hot-plate test

Fifteen male Swiss albino mice (24–26 g) were housed per cage. The cages were placed in the experimental room 24 h before the test for acclimatization. The animals were fed a standard laboratory diet and tap water ad libitum and kept at 23 ± 1 °C with a 12 h light/dark cycle (lights on at 07:00). Mice were placed inside a stainless steel container, which was set thermostatically at 52.5 ± 0.1 °C in a precision water bath (KW Mechanical Workshop, Siena, Italy). Reaction times (s) were measured with a stopwatch before and 15, 30, 45, and 60 min after administration of the analgesic drug. The endpoint used was the licking of thefore or hind paws.
Those mice scoring less than 12 and more than 18 s in the pretest were rejected (30%). An arbitrary cut-off time of 45 s was adopted.

**Statistical analysis**

All experimental results are given as the mean ± SEM (standard error of the mean). Analysis of variance ANOVA, followed by Fisher’s protected least significant difference procedure for post-hoc comparison, were used to verify significance between two means. Data were analyzed with the StatView software for Macintosh. P values of less than 0.05 were considered significant.

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