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New Arylpiperazine Derivatives as Antagonists of the Human Cloned 5-HT$_4$ Receptor Isoforms

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New derivatives of arylpiperazine **9** were designed from ML 10302, a potent 5-HT$_4$ receptor agonist in the gastrointestinal system. Compounds were synthesized by condensation of a number of available arylpiperazines or heteroarylpiperazines with 2-bromoethyl 4-amino-5-chloro-2-methoxybenzoate. They were evaluated in binding assays on the recently cloned human 5-HT$_{4(e)}$ isoform stably expressed in C6 glial cells with [^{3}H]GR 113808 as the radioligand. The affinity values (K_i) depended upon the substituent on the aromatic ring. A chlorine atom produced a marked drop in activity (K_i > 100 nM), while a *m*-methoxy group gave a compound with nanomolar affinity (K_i = 3 nM). The most potent compounds were the heterocyclic derivatives with pyrimidine, pyrazine, pyridazine, or pyridine moieties (compounds **9r**, **9t**, **9u**, **9x**, respectively). K_i values for **9a** and **9r** were determined for the 5-HT$_{4(a)}$, 5-HT$_{4(b)}$, 5-HT$_{4(c)}$, and 5-HT$_{4(d)}$ receptor isoforms transiently expressed in COS cells. The results indicated that the compounds were not selective. They produced an inhibition of the 5-HT-stimulated cyclic AMP synthesis in the C6 glial cells stably expressing the 5-HT$_{4(e)}$ receptor and shifted the 5-HT concentration−effect curve on adenylyl cyclase activity with pK_D values of 7.44 and 8.47, respectively. In isolated human atrial myocytes, **9r** antagonized the stimulatory effect of 5-HT on the L-type calcium current (I_{Ca}) with a K_D value of 0.7 nM.

Introduction

The 5-HT$_4$ receptor is a member of the seven transmembrane-spanning G protein-coupled family of receptors.[1−3] Initially, it was characterized by Dumuis[4] in neuronal cells and shown to be linked positively to adenylyl cyclase. This neuronal receptor was similar to that discovered by Clarke[5] in the gastrointestinal (GI) system where it was shown to be responsible for stimulating motility. Considerable interest has developed in this receptor because it provided a mechanism of action for the gastric prokinetic drugs, a number of which were found to be 5-HT$_4$ receptor agonists.[6] Many of these compounds are members of the generic benzamide family, derived from metoclopramide.[7] They are amides or esters of 4-amino-5-chloro-2-methoxybenzoic acid derivatives such as renzapride (**2**),[8a] SC-53116 (**3**),[8b] ML 10302[8c] (**1**), cisapride (**4**),[8c] prucalopride (**5**),[8d] and mosapride (**6**) (Chart 1).[8e] The pharmacological characterization of this receptor was facilitated by the synthesis of potent and selective antagonists such as GR 113808 (**7**)[9a] and SB 207710 (**8**).[9b]

5-HT$_4$ receptors are expressed in a wide variety of tissues: brain, heart, bladder, intestine, and kidney[10,11] and have been implicated in a number of pathological disorders: memory deficits,[12a] irritable bowel syndrome, gastroparesis, urinary incontinence,[11] and cardiac atrial arrhythmias.[12b] Recently, several groups reported the cloning of the receptors from different species.[1−3] They are characterized by several splice variants which differ in the length and sequence of their C termini. We described[2] the cloning and pharmacological characterization of four splice variants of the human 5-HT$_4$ receptor: 5-HT$_{4(a)}$, 5-HT$_{4(b)}$, 5-HT$_{4(c)}$, and 5-HT$_{4(d)}$. More recently another isoform (5-HT$_{4(e)}$) has been characterized.[13] Other 5-HT receptor subtypes also have splice variants, in particular the 5-HT$_7$ receptor which has four isoforms[14] produced by alternative splicing in the carboxyl terminus. The expression of 5-HT$_4$ receptor isoforms depends on the tissue: three or four variants were expressed in heart atrium, brain, and intestine, while only one was found in kidney and bladder.[2] On the other hand, the 5-HT$_{4(d)}$ variant was only present in the intestine.[2]

The availability of the cloned 5-HT$_4$ receptors stimulated more interest in the search for new ligands. In particular the potential use of 5-HT$_4$ receptor antagonists in the treatment of the human atrial arrhythmias has become an emerging concept[12b,15,16] since the 5-HT$_4$ receptor stimulates two potentially arrhythmogenic ion channel currents in human atrial tissue: the L-type calcium current (I_{Ca})[17] and the pacemaker *f* channel current (I_f).[15] A very recent study has demonstrated the potential therapeutic value of a selective 5-HT$_4$ receptor antagonist (RS-100302) as an antiarrhythmic drug.[16b] Therefore, there is much interest in the clinical evalu-

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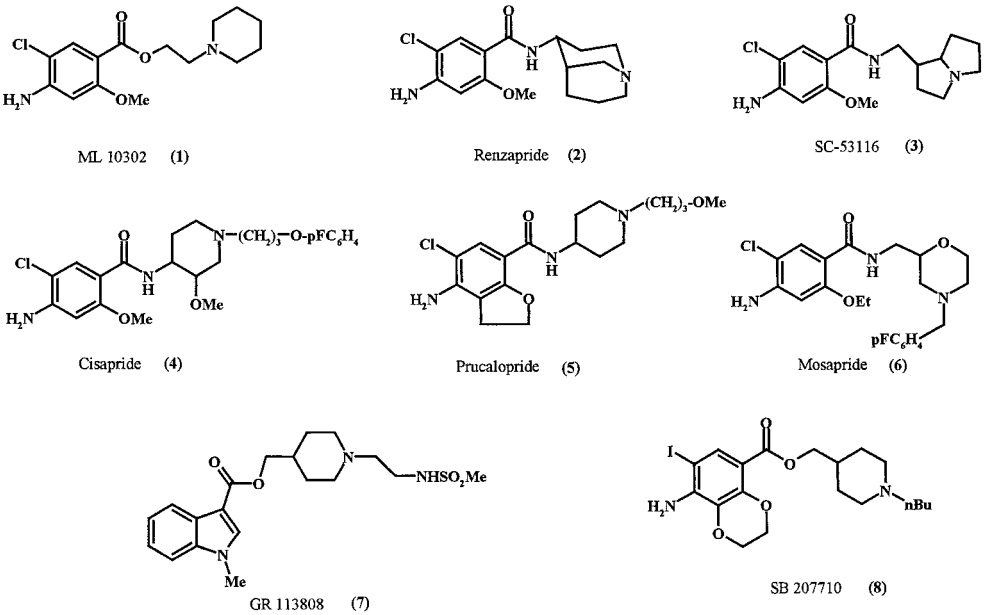
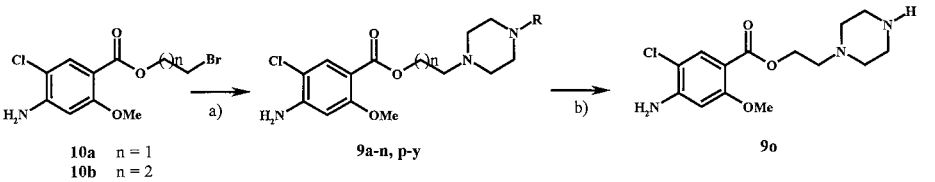
Chart 1



Scheme 1[a]



[a] Reagents and conditions: (a) piperazine derivative, K_2CO_3, MeCN or DMF, 18−24 h, rt (method A); DIPEA, MeCN, reflux, 24 h (method B); (b) R = *t*-BOC, anisole, TFA, CH_2Cl_2, rt.

ation of compounds with cardiac 5-HT_4 receptor antagonist properties.[18] The design of molecules to be evaluated on the available human cloned 5-HT_4 receptors as well as on native 5-HT_4 receptors expressed in human atrial myocytes constituted an original approach to identify new leads capable of treating atrial arrhythmias.

We report herein the preliminary results of this approach where the piperazine derivatives **9** (Scheme 1) were synthesized and evaluated on the cloned and native human 5-HT_4 receptors.

Chemistry

ML 10302 (**1**) has been characterized as a potent 5-HT_4 receptor agonist in the GI system[8c] where it has been shown to be more potent than cisapride while its efficacy as an agonist on I_{Ca} in isolated human atrial myocites was very weak.[19] Moreover it antagonized the stimulatory effect of 100 nM 5-HT on I_{Ca} with an IC_{50} value of 17.1 nM.[19] Therefore, **1** seemed to be an excellent lead for the search for new molecules with the ability to block cardiac 5-HT_4 receptors. As SARs (structure−activity relationships) reported by us[20] have shown that various substituents could be introduced on the 4 position of the piperidine ring of **1**, the synthesis of piperazine derivatives **9** in particular could constitute a promising method for the design of new molecules. They were prepared according to the synthetic pathway reported in Scheme 1.

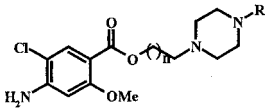
2-Bromoethyl and 3-bromopropyl 4-amino-5-chloro-2-methoxybenzoate (**10a** and **10b**, respectively) were synthesized by the process already reported[20] and were then condensed with various arylpiperazines which were commercially available or were synthesized by methods already described.[21a−c] Initially, the reaction was carried out in DMF or acetonitrile in the presence of K_2CO_3 at room temperature, and the compounds were isolated after column chromatography as hydrochloride salts (method A). Generally the yield of the pure compound was low. A clear improvement of the yield of the reaction was obtained with acetonitrile as solvent at refluxing in the presence of diisopropylethylamine (method B).

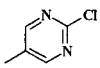
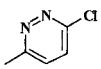
The biological activity of compound **9a** (Table 1) was compared to that of the corresponding amide 2-[*N*-(4-phenyl)piperazino]ethyl 4-amino-5-chloro-2-methoxy benzamide (**11**), which was synthesized by the condensation of 2-amino-5-chloro-2-methoxybenzoic acid and *N*-phenylpiperazineethylamine (see Experimental Section).

Biological Results and Discussion

The compounds were evaluated routinely by binding assays on the newly described human 5-$HT_{4(e)}$ isoform stably expressed in C6 glial cells, using [^{3}H]GR 113808 as the radioligand.[2] These receptors have been characterized in the human atrium,[13] and consequently they were relevant to select active compounds with therapeutic potential for the treatment of human atrial arrhythmias. As shown in Table 1, substitution of the piperidine ring of ML 10302 by an arylpiperazine moiety gave compounds with good affinities for these receptors. However, the potency was largely influenced by the

Table 1. Binding Data for Compounds **9a**−**y** on the Cloned Human 5-$HT_{4(e)}$ Receptor Isoform



Compound	n	R	[a]h5-$HT_{4(e)}$ K_i (nM)
9a	1	C_6H_5	14.3 [9.7-21.2]
9b	1	4-ClC_6H_4	715[374-1365]
9c	1	3-ClC_6H_4	162[119-221]
9d	1	2-ClC_6H_4	296[191-457]
9e	1	4-$MeOC_6H_4$	17.7 [11-28]
9f	1	3-$MeOC_6H_4$	3 [2.1-4.3]
9g	1	2-$MeOC_6H_4$	64 [42-97]
9h	1	3-$CF_3C_6H_4$	199.2[146.6-270.5]
9i	1	4-FC_6H_4	23.1 [16.3-32.9]
9j	1	2-FC_6H_4	16.8 [11.8-24.1]
9k	1	1-naphthyl	>1000
9l	1	COOEt	23 [16-32]
9m	1	*t*-BOC	>1000
9n	1	Me	>1000
9o	1	H	>1000
9p	2	C_6H_5	11.5[7.8-17]
9q	1	C_6H_5-CH_2	240 [153-375]
9r	1		1[0.47-2]
9s	1		9.2 [7.3-11.6]
9t	1		1.4 [1-1.9]
9u	1		1.4 [1-1.9]
9v	1		9.8 [5.7-6.7]
9w	1		2 [1.5-2.7]
9x	1		1.5 [1.1-2]
9y	1		3.5 [2.5-4.9]
ML 10302			5.6 [3.4-7.9]
GR 113808			0.1 [7.8-17]

[a] Membranes from stably transfected C6 glial cells were incubated with 0.2 nM [^{3}H]GR 113808. Nonspecific binding was defined with 10 μM ML 10375 (see Experimental Section). The data are the result of two or three experiments. K_i values are expressed in nM and were calculated using the Cheng−Prussof equation from the IC_{50} values with the PRISM program; 95% confidence limits are in brackets.

substituent on the aromatic ring and the position of the substitution. A *p*-methoxy group and *o*- and *p*-fluorine atom gave compounds **9e**, **9i**, and **9j** equipotent to the unsubstituted compound **9a** (K_i = 14.3 nM). A chlorine atom on different positions (**9b**, **9c**, **9d**), a *m*-trifluoromethyl substituent (**9h**), and an *o*-methoxy group (**9g**) brought about a drop in activity which was particularly marked with the chlorine atom while the *m*-methoxy group provided a compound with nanomolar affinity (**9f**, K_i = 3 nM, respectively). The naphthalene derivative **9k** was inactive, indicating the unfavorable influence of steric hindrance in this part of the receptor site. The affinity was not influenced by the lengthening of the ethyl chain (**9p**, K_i = 11.5 nM) which was equipotent to that for **9a**. As seen earlier with the esters derived from piperidine,[20,22] the presence of the ester function was essential for recognition by the binding site of 5-HT_4 receptors. Indeed, the corresponding amide **11** was inactive (K_i > 1000 nM). On the other hand, substitution of the piperazine by heteroaryl rings provided very potent compounds; thus pyrimidine, pyrazine, pyridazine, and pyridine derivatives **9r**, **9t**, **9u**, **9x**, and **9y** were compounds with nanomolar affinity.

Table 2. Binding Data for Compounds **9a** and **9r** on the Cloned Human 5-$HT_{4(a)}$, 5-$HT_{4(b)}$, 5-$HT_{4(c)}$, and 5-$HT_{4(d)}$ Receptors Expressed in COS-7 Cells

	K_i (nM)[a]			
compound	h5-$HT_{4(a)}$	h5-$HT_{4(b)}$	h5-$HT_{4(c)}$	h5-$HT_{4(d)}$
9a	23 [17−31]	55 [44−68]	12.5 [6.7−21]	32 [17−57]
9r	3.5 [2.8−4.5]	8.1 [6.55−9.9]	2.47 [1.8−3.3]	7.03 [3.2−15.4]
ML 10302	8.4 [6.9−9.9]	10.7 [7.8−13.6]	7.98 [5.2−10.7]	3.69 [2.44−4.94]
GR 113808	0.33 [0.29−0.36]	0.53 [0.43−0.63]	0.41 [28−0.54]	0.078 [0.054−0.1]

[a] Membranes from transiently transfected COS-7 cells were incubated with 50% of the K_D value of [^{3}H]GR 113808 for each h5-HT_4 receptor isoform (see ref 2). Nonspecific binding was defined with 10 μM ML 10375 (see Experimental Section). Data are the result of two or three experiments. K_i values are expressed in nM and were calculated using the Cheng−Prussof equation from the IC_{50} values with the PRISM program; 95% confidence limits are in brackets.

An increase in the basicity of the second nitrogen atom was not a favorable parameter as the N-Me and NH derivatives **9n** and **9o** were inactive, while the corresponding ethyl carbamate **9l** had high affinity (K_i = 23 nM). This point was confirmed with the *N*-benzyl derivative **9q** which was 1 order of magnitude less potent than **9a**.

K_i values for compounds **9a** and **9r** for the 5-$HT_{4(a)}$, 5-$HT_{4(b)}$, 5-$HT_{4(c)}$, and 5-$HT_{4(d)}$ receptor isoforms were evaluated in binding assays on the different splice variants of the human receptors transiently expressed in COS-7 cells.[2] The results are reported in Table 2. The affinity values for the receptors were in the same range as those obtained with the stable line where the 5-$HT_{4(e)}$ isoform was expressed. Similar to the reference compounds, **9a** and **9r** did not show any selectivity for the different isoforms.[2,13]

Previous results[20] have shown that the pharmacological profile of the molecule derived from ML 10302 depended upon the structure of the basic moiety; therefore the pharmacological activities of compounds **9a** and **9r** were determined on the activity of adenylyl cyclase, the effector of the 5-HT_4 receptors. The production of cAMP was measured in C6 glial cells stably expressing the human 5-$HT_{4(e)}$ receptor, using a radioimmunoassay technique as previously reported.[2,19] In contrast to 5-HT (10^{-6} M) which stimulated the synthesis of cAMP, **9a** and **9r** did not possess intrinsic activity at these receptors. On the contrary, both compounds produced an antagonist effect on the 5-HT-stimulated cAMP synthesis (Figure 1). **9r** (1 μM) clearly inhibited the effect of 5-HT (1 and 0.1 μM), while the effect of **9a** was less marked and only observed with 0.1 μM 5-HT. The competitive antagonist properties of both compounds were demonstrated on the 5-HT concentration−effect curve which was shifted to the right by the addition of **9a** and **9r** (70 and 5 nM, respectively). pK_D values of 7.54 and 8.47 were found respectively by calculation of the Schild plots (Figure 2).

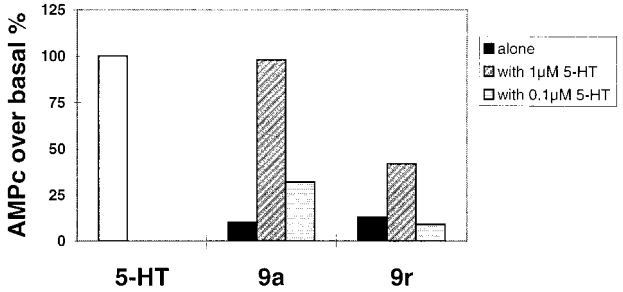


Figure 1. Evaluation of the antagonist effects of compounds **9a** and **9r** on cAMP synthesis in C6 glial cells stably transfected with the 5-$HT_{4(e)}$ receptor isoform. The data are represented as % over basal activity. 5-HT was used alone at 1 or 0.1 μM (maximum effect at both doses). **9a** and **9r** were used alone at 1 μM. Antagonist effects of **9a** (1 μM) and **9r** (1 μM) were evaluated in the presence of 1 and 0.1 μM 5-HT.

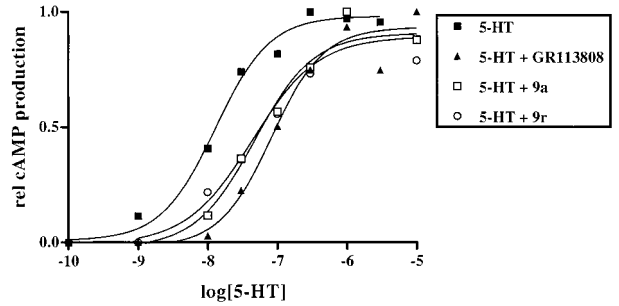


Figure 2. Concentration−effect curves for 5-HT on adenylyl cyclase activity in C6 glial cells stably transfected with the 5-$HT_{4(e)}$ receptor isoform: ■, 5-HT alone; ▲, 5-HT + GR 113808 (2 nM); □, 5-HT + **9a** (70 nM); ○, 5-HT + **9r** (5 nM).

The effect of **9r** (ML 10821) on the L-type calcium current (I_{Ca}) in isolated human atrial myocytes was examined using the whole-cell patch clamp technique.[19] As previously reported,[17,19] application of 5-HT to the human atrial myocytes resulted in a large stimulation of I_{Ca}. In the typical experiment of Figure 3, 10 nM 5-HT increased basal I_{Ca} almost 4-fold. In contrast, **9r** produced no effect on basal I_{Ca}. However, when it was tested in the presence of 5-HT, a potent antagonist effect was observed. The experiment of Figure 3 shows that as low as 1 nM **9r** produced an attenuation of the stimulatory effect of 10 nM 5-HT. Increasing the concentration of **9r** further reduced I_{Ca} until the effect of 5-HT was totally blunted at 100 nM. This effect was reversible, since the amplitude of I_{Ca} increased again upon washout of the drug (5-HT still present, Figure 3). Figure 4 summarizes the results of a number of experiments in which the effects of increasing concentrations of 5-HT on I_{Ca} were successively examined on the same cells in the absence (circles) or presence (squares) of 10 nM **9r**. The presence of **9r** induced a clear shift to the right of the concentration−effect curve for the response of I_{Ca} to 5-HT. Maximal response of I_{Ca} to 5-HT (E_{max}) and concentration of 5-HT necessary to produce half-maximal response (EC_{50}) were derived by fitting the experimental points to the Hill equation. Hill coefficients were close to unity in both conditions (0.71 ± 0.15 and 0.89 ± 0.14, respectively). The presence of **9r** increased the EC_{50} 15-fold, from 4.5 to 66 nM. Calculation of the Schild plot led to a K_D value of 7×10^{-10} for compound **9r** in human atrial cells. This value

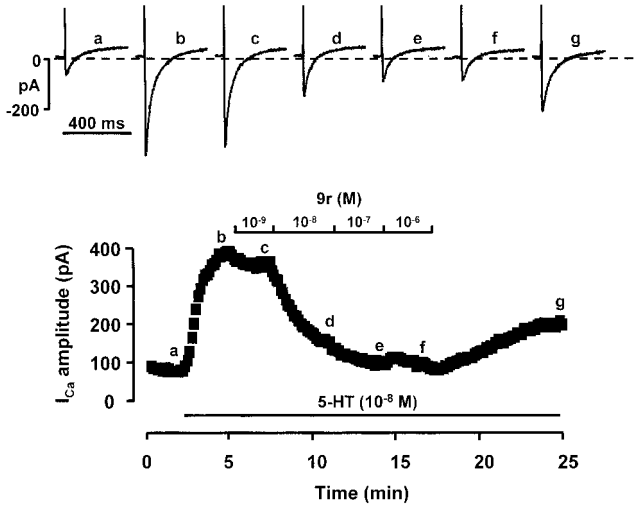


Figure 3. Representative experiment on the antagonism between 5-HT and **9r** (ML 10821) on the L-type Ca current (I_{Ca}) in a human atrial myocyte. Each symbol corresponds to a measure of I_{Ca} at 0 mV obtained every 8 s. The cell was first superfused with control solution and then exposed to 10 nM 5-HT alone or 5-HT in the presence of increasing concentrations of **9r** (1 nM to 1 μM) during the periods indicated by the solid lines. The individual current traces shown on the upper part were obtained at the times indicated by the corresponding letters in the bottom graph. The dotted line indicates the zero current level.

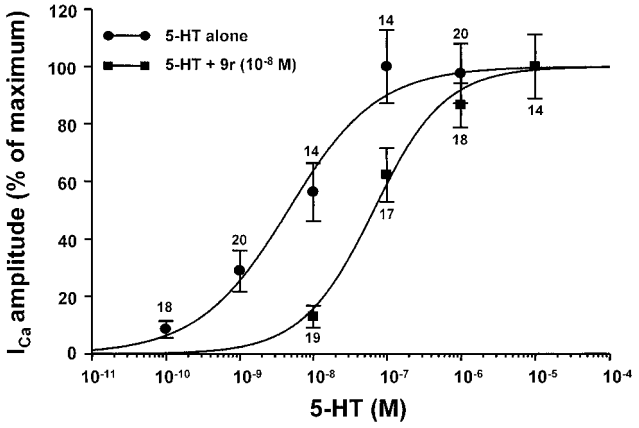


Figure 4. Concentration−effect curves for 5-HT on cumulative I_{Ca} alone (●) or in the presence of 10 nM **9r** (■) in human atrial myocytes. The symbols indicate the mean values in percentage stimulation of basal I_{Ca} (normalized to maximal response), and the vertical line indicates the SEM of the number of experiments indicated near the symbols. Data were fitted with the Hill equation program Origin V.5. The concentration−effect response of each cell was fitted with the Hill equation and used to calculate the mean values and standard errors. Maximal response of I_{Ca} to 5-HT (E_{max}) and concentration of 5-HT necessary to produce half-maximal response (EC_{50}) were derived from this analysis. EC_{50} values were 4.5 ± 1.5 nM for 5-HT alone and 66 ± 13 nM for 5-HT in the presence of 10 nM **9r**.

is in the range of those found previously by Kaumann[12b] for the antagonist reference compounds, and it confirmed the favorable role of the arylpiperazine and particularly that of the heteroaryl substitution in the design of new human 5-HT_4 receptor antagonists. These properties were in contrast to those observed with ML 10302 which was characterized as a potent agonist in the GI system[20] and a partial agonist in human atrium[19] and the recombinant receptors.[2]

Conclusions

We have reported here a new class of antagonists of the human 5-HT_4 receptor isoforms, and on the basis of the structural framework of ML 10302, we have shown that the introduction of the heteroarylpiperazine moiety gave compounds with high affinity for human 5-HT_4 receptor isoforms. The antagonist properties of **9a** and **9r** were evaluated with an adenylyl cyclase assay in C6 glial cells stably expressing the newly cloned human 5-$HT_{4(e)}$ receptor. The antagonist profile of **9r** was confirmed electrophysiologically in human atrial myocytes where it blocked the 5-HT-induced rise in I_{Ca} with high affinity. Important SAR studies are in course to evaluate the interest of these new compounds and their therapeutic potential in preventing atrial arrhythmia.

Experimental Section

Chemistry. Melting points were determined on a Mettler FP 61 melting point apparatus and are uncorrected. ^{1}H and ^{19}F NMR spectra were recorded on a Bruker AC 200 spectrometer at 200 MHz. Chemical shifts are reported in parts per million (δ) relative to tetramethylsilane as the internal standard, and signals are quoted as s (singlet), d (doublet), br s (broad singlet), or m (multiplet). Elemental analyses were performed at the CNRS Analysis Services in Châtenay-Malabry (France) and were within 0.4% of the theoretical values unless otherwise noted.

Materials. Acetonitrile, DMF, and the usual solvents were purchased from SDS (Paris, France). Column chromatography was performed on Merck silica gel 60 (70/230 mesh) and on Aldrich neutral aluminum oxide (150 mesh).

2-Bromoethyl 4-amino-5-chloro-2-methoxybenzoate **(10a)** was synthesized from 4-amino-5-chloro-2-methoxybenzoic acid by condensation with 1,2-dibromoethane and 1,8-diazobicyclo-[5.4.0]undec-7-ene (DBU) in dry THF.[20] 1-(1-Naphthyl)piperazine hydrochloride was prepared according to a previously reported procedure.[23] *N*-Benzylpiperazine and *N*-(2-pyrimidyl)-piperazine were commercially available. *N*-(2-Pyridyl)piperazine, *N*-(4-pyridyl)piperazine, *N*-(4-pyrimidyl)piperazine, *N*-(2-pyrazinyl)piperazine, *N*-(2-(6-chloro)pyridazinyl)piperazine, *N*-(3-pyridazinyl)piperazine and *N*-(4-(2-chloro)pyrimidyl)piperazine were prepared according to the methods already reported. [23a−c]

4-Phenyl-1-[2-(2-phthalimido)ethyl]piperazine. *N*-(2-Bromoethyl)phthalimide (5.6 g, 22 mmol), potassium carbonate (0.8 g, 6 mmol) and a catalytic quantity of KI were added sequentially to a solution of 1-phenylpiperazine (3 mL, 20 mmol) in DMF (100 mL). The resulting mixture was maintained at 65 °C for 17 h. The reaction mixture was evaporated in vacuo and partitioned between EtOAc and H_2O. The aqueous layer was extracted with EtOAc. The combined organic layers were dried over Na_2SO_4, filtered, and evaporated to give a yellow solid. Recrystallization from absolute EtOH yielded 3.7 g (55%) of product as gold needles: mp 154 °C; ^{1}H NMR ($CDCl_3$) δ 7.86−7.68 (m, 4H), 7.27−7.19 (m, 2H), 6.91−6.78 (m, 3H), 3.86 (t, J = 6.5 Hz), 3.16−3.11 (m, 4H), 2.73−2.65 (m, 6H); ^{13}C NMR ($CDCl_3$) δ 168.3, 151.2, 133.8, 132.1, 128.9, 123.1, 119.5, 115.8, 55.6, 53, 49.1, 35.2.

1-(2-Aminoethyl)-4-phenylpiperazine. A solution of 85% hydrazine hydrate (0.13 mL, 2.8 mmol) was added dropwise to a stirred suspension of the previous compound (0.46 g, 1.4 mmol) in absolute EtOH (15 mL). After completing the addition, the reaction mixture was heated for 1 h. The solution was allowed to cool at room temperature and the solid material was collected by filtration. The filtrate was evaporated under reduced pressure to yield a yellow solid (0.3 g) used without further purification: mp 203 °C; ^{1}H NMR ($CDCl_3$) δ 7.29−7.21 (m, 2H), 6.94−6.8 (m, 3H), 3.22−3.17 (m, 4H), 2.82 (t, J = 6.1 Hz, 2H), 2.63−2.58 (m, 4H), 2.47 (t, J = 6.1 Hz, 2H), 1.45 (br s, 2H); ^{13}C NMR ($CDCl_3$) δ 151.4, 129.1, 119.6, 116, 61.2, 53.3, 49.2, 38.9.

3-Bromopropyl 4-Amino-5-chloro-2-methoxybenzoate (10b). A mixture of 4-amino-5-chloro-2-methoxybenzoic acid (2.02 g, 10 mmol) in 1,3-dibromopropane (10.2 mL) and dry THF (20 mL) was heated to reflux. Then 1.5 mL of 1,8-diazabicyclo[5.4.0]undec-7-ene (DBU) was added dropwise over 2 h, and the resulting mixture was maintained under reflux overnight. The cooled mixture was then filtered, and the filtrate was concentrated in vacuo. The residue was diluted with CH_2Cl_2, washed with water, dried over $MgSO_4$, and the solvent evaporated. Purification by chromatography on silica gel (CH_2Cl_2) and recrystallization from cyclohexane gave a yellow solid (1.26 g, 39%): mp 104 °C; R_f 0.15 (CH_2Cl_2); ^{1}H NMR ($CDCl_3$) δ 7.80 (s, 1H), 6.28 (s, 1H), 4.47 (br s, 2H), 4.36 (t, J = 6 Hz, 2H), 3.84 (s, 3H), 3.54 (t, J = 6 Hz, 2H), 2.27 (m, 2H); ^{13}C NMR ($CDCl_3$) δ 164.6, 160.3, 148.1, 133.3, 110.0, 109.4, 98.3, 62.1, 56.1, 31.9, 29.8. Anal. ($C_{11}H_{13}BrNO_3$).

General Method for the Preparation of Compounds 9a−n,p−y. Method A. 2-[*N*-(4-Chlorophenyl)piperazino]-ethyl 4-Amino-5-chloro-2-methoxybenzoate Dihydrochloride Salt (9b). A mixture of **10a** (1.23 g, 16 mmol), 4-chlorophenylpiperazine (1.29 g, 4.8 mmol) and K_2CO_3 (2.21 g, 16 mmol) in dry CH_3CN or DMF (30 mL) was stirred for 18 h at room temperature. After concentration in vacuo, the residue was dissolved in CH_2Cl_2 (100 mL), washed with water, and dried over $MgSO_4$. Chromatography on a column of silica gel with Et_2O/CH_2Cl_2/MeOH (40:50:10) afforded an oil which was purified on neutral alumina with Et_2O/CH_2Cl_2/MeOH (45:50:5) to yield a pure white solid (1.19 g). This compound was transformed into the hydrochloride salt with a 3 N HCl ethyl acetate solution and recrystallized from MeOH/AcOEt/cyclohexane to give 0.110 g of a white solid (5%): mp 202 °C; ^{1}H NMR (CD_3OD) (δ) 7.82 (s, 1H, ArH), 7.23 (d, 2H, J = 8.69 Hz, PhH), 7 (d, 2H, J = 8.69 Hz, PhH), 6.44 (s, 1H, ArH), 4.6 (m, 2H, OCH_2), 3.8 (s, 3H, OCH_3), 3.82−3 (m, 8H, piperH), 3.64 (m, 2H, CH_2N). Anal. ($C_{20}H_{23}N_3O_3Cl_2\cdot 2HCl\cdot 3.5H_2O$).

2-[*N*-(Phenyl)piperazino]ethyl 4-Amino-5-chloro-2-methoxybenzoate (9a). It was prepared according to the process described for **9b** and isolated as the hydrochloride salt (11%) by crystallization from a MeOH/*i*-Pr_2O mixture: mp 206 °C; ^{1}H NMR (CD_3OD) δ 7.83 (s, 1H), 7.27 (m, 2H), 7.02 (m, 1H), 6.89 (m, 2H), 6.48 (s, 1H), 4.62 (m, 2H), 3.82 (s, 3H), 3.8−3.32 (m, 8H), 3.61 (m, 2H). Anal. ($C_{20}H_{24}N_3O_3Cl\cdot HCl\cdot 0.25H_2O$).

2-[*N*-(3-Chlorophenyl)piperazino]ethyl 4-Amino-5-chloro-2-methoxybenzoate (9c). It was prepared according to the process described for **9b** and isolated as the hydrochloride salt (19%) by crystallization from a MeOH/AcOEt/cyclohexane mixture: mp 208 °C; ^{1}H NMR (CD_3OD) δ 7.82 (s, 1H), 7.23 (m, 1H), 7.03 (m, 1H), 6.9 (m, 2H), 6.46 (s, 1H), 4.6 (m, 2H), 3.80 (s, 3H), 3.8−3.13 (m, 10H). Anal. ($C_{20}H_{23}N_3O_3Cl_2\cdot HCl\cdot 0.25H_2O$).

2-[*N*-(2-Chlorophenyl)piperazino]ethyl 4-Amino-5-chloro-2-methoxybenzoate (9d). It was prepared according to the process described for **9b** and isolated as the hydrochloride salt (6%) by crystallization from a MeOH/AcOEt/cyclohexane mixture: mp 234 °C; ^{1}H NMR (CD_3OD) δ 7.84 (s, 1H), 7.42 (m, 1H), 7.32 (m, 1H), 7.20 (m, 1H), 7.10 (m, 1H), 6.48 (s, 1H), 4.63 (m, 2H), 3.82 (s, 3H), 3.8 (m, 2H), 3.67 (m, 2H), 3.56 (m, 2H), 3.46 (m, 2H), 3.17 (m, 2H). Anal. ($C_{20}H_{23}N_3O_3Cl_2\cdot HCl\cdot H_2O$).

2-[*N*-(4-Methoxyphenyl)piperazino]ethyl 4-Amino-5-chloro-2-methoxybenzoate (9e). It was prepared according to the process described for **9b** and isolated as the hydrochloride salt (25%) by crystallization from a MeOH/AcOEt/cyclohexane mixture: mp 198 °C; ^{1}H NMR (CD_3OD) δ 7.86 (s, 1H), 7.31 (d, 2H, J = 7.18 Hz), 6.97 (d, 2H, J = 7.18 Hz), 6.53 (s, 1H), 4.66 (m, 2H), 3.9−3.69 (m, 10H), 3.8 (s, 3H), 3.78 (s, 3H). Anal. ($C_{21}H_{26}N_3O_4Cl\cdot 2HCl\cdot 2H_2O$).

2-[*N*-(3-Methoxyphenyl)piperazino]ethyl 4-Amino-5-chloro-2-methoxybenzoate (9f). It was prepared according to the process described for **9b** and isolated as the hydrochloride salt (13%) by crystallization from a MeOH/AcOEt/cyclohexane mixture: mp 180 °C; ^{1}H NMR (CD_3OD) δ 7.84 (s, 1H, ArH), 7.25−7.11 (m, 1H, PhH), 6.67−6.48 (m, 3H, PhH), 6.5 (s, 1H, ArH), 4.61 (m, 2H, OCH_2), 3.82 (s, 3H, OCH_3), 3.76 (s,

3H, OCH_3), 3.9−3.6 (m, 8H, piperH), 3.65 (m, 2H, CH_2N). Anal. ($C_{21}H_{26}N_3O_4Cl\cdot 2HCl\cdot H_2O$).

2-[*N*-(2-Methoxyphenyl)piperazino]ethyl 4-Amino-5-chloro-2-methoxybenzoate (9g). It was prepared according to the process described for **9b** and isolated as the hydrochloride salt (19%) by crystallization from a MeOH/AcOEt/cyclohexane mixture: mp 196 °C; ^{1}H NMR (CD_3OD) (δ) 7.81 (s, 1H), 7.75−6.82 (m,4H), 6.45 (s, 1H), 4.61 (m, 2H), 3.84 (s, 3H), 3.82 (s, 3H), 3.9−3 (m, 8H), 3.65 (m, 2H). Anal. ($C_{21}H_{26}N_3O_4Cl\cdot 2HCl\cdot 2H_2O$).

2-[*N*-(3-Trifluorophenyl)piperazino]ethyl 4-Amino-5-chloro-2-methoxybenzoate (9h). It was prepared according to the process described for **9b** and isolated as the hydrochloride salt (10%) by crystallization from a MeOH/AcOEt/cyclohexane mixture: mp 232−234 °C; ^{1}H NMR (CD_3OD) (δ) 7.84 (s, 1H), 7.46 (m, 1H), 7.28 (m, 2H), 7.19 (m, 1H), 6.48 (s, 1H), 4.62 (m, 2H), 3.82 (s, 3H), 3.8−3.4 (m, 10H); ^{19}F NMR ($CDCl_3$) δ 62.937 (s, 3F). Anal. ($C_{21}H_{23}N_3O_3F_3\cdot HCl\cdot 0.15H_2O$).

2-[*N*-(4-Fluorophenyl)piperazino]ethyl 4-Amino-5-chloro-2-methoxybenzoate (9i). It was prepared according to the process described for **9b** and isolated as hydrochloride salt (17%) by crystallization from a MeOH/AcOEt/cyclohexane mixture: mp 130 °C dec; ^{1}H NMR (CD_3OD) δ 7.86 (s, 1H), 7.16−6.94 (m, 4H), 6.54 (s, 1H), 4.64 (m, 2H), 3.83 (s, 3H), 3.92−3.5 (m, 8H), 3.65 (m, 2H); ^{19}F NMR ($CDCl_3$) δ 124.87 (s, 1F). Anal. ($C_{20}H_{23}N_3O_3F\cdot 2HCl\cdot 0.75H_2O$).

2-[*N*-(2-Fluorophenyl)piperazino]ethyl 4-Amino-5-chloro-2-methoxybenzoate (9j). It was prepared according to the process described for **9b** and isolated as the hydrochloride salt (16%) by crystallization from a MeOH/AcOEt/cyclohexane mixture: mp 235 °C; ^{1}H NMR (CD_3OD) δ 7.84 (s, 1H), 7.16−7 (m, 4H), 6.48 (s, 1H), 4.63 (m, 2H), 3.82 (s, 3H), 3.77 (m, 2H), 3.65 (m, 2H), 3.61(m, 2H), 3.46 (m, 2H), 3.16 (m, 2H); ^{19}F NMR ($CDCl_3$) δ 123.03 (s, 1F). Anal. ($C_{20}H_{23}N_3O_3F\cdot HCl\cdot 0.25H_2O$).

2-[*N*-(Naphthyl)piperazino]ethyl 4-Amino-5-chloro-2-methoxybenzoate (9k). It was prepared according to the process described for **9b** and isolated as the hydrochloride salt (21%) by crystallization from MeOH/AcOEt/Et_2O: mp 174 °C dec; ^{1}H NMR (CD_3OD) δ 8.22 (m, 1H), 7.84 (m, 2H), 7.63 (m, 1H), 7.49 (m, 2H), 7.41 (m, 1H), 7.21 (m, 1H), 6.75 (s, 1H), 4.65 (m, 2H), 3.82 (s, 3H), 3.94−3.27 (m, 10H). Anal. ($C_{24}H_{26}N_3O_3Cl\cdot HCl\cdot 0.5H_2O$).

2-[*N*-(Ethoxycarbonyl)piperazino]ethyl 4-Amino-5-chloro-2-methoxybenzoate (9l). The compound was synthesized according to the process used for **9b**. It was purified by chromatography (CH_2Cl_2/Et_2O 1:1) to give a colorless oil which was recrystallized in an acetone/pentane mixture to give a white solid (yield 28%): mp 92 °C; R_f 0.2 (CH_2Cl_2/*i*-PrOH 95:5); ^{1}H NMR ($CDCl_3$) δ 7.78 (s, 1H), 6.27 (s, 1H), 4.52 (s, 2H), 4.34 (t, J = 6 Hz, 2H), 4.11 (q, J = 7 Hz, 2H); 3.80 (s, 3H), 3.46 (t, J = 5 Hz, 4H), 2.73 (t, J = 6 Hz, 2H), 2.50 (t, J = 5 Hz, 4H), 1.24 (t, J = 7 Hz, 3H); ^{13}C NMR ($CDCl_3$) δ 164.5, 160.3, 155.5, 148, 133.3, 109.9, 109.5, 98.2, 62, 61.3, 56.8, 56, 53.1, 43.7,14.7. Anal. ($C_{17}H_{24}ClN_3O_5$).

2-[*N*-(*tert*-Butyloxycarbonyl)piperazino]ethyl 4-Amino-5-chloro-2-methoxybenzoate (9m). It was synthesized according to the previous process (yield 58%): mp 102 °C; ^{1}H NMR ($CDCl_3$) δ 7.80 (s, 1H), 6.28 (s, 1H), 4.48 (br.s, 2H), 4.36 (m, 2H), 3.83 (s, 3H), 3.43 (m, 4H), 2.74 (m, 2H), 2.47 (m, 4H), 1.45 (s, 9H). Anal. ($C_{19}H_{28}N_3O_5Cl\cdot 0.25H_2O$).

2-[*N*-(4-Methyl)piperazino]ethyl 4-Amino-5-chloro-2-methoxybenzoate (9n). A mixture of **10a** (1.2 g, 3.9 mmol), *N*-methylpiperazine (0.5 mL, 4.3 mmol) and diisopropylethylamine (1.4 mL, 7.8 mmol) in dry DMF (10 mL) was stirred for 24h at room temperature. The resulting mixture was concentrated in vacuo. The residue was dissolved in methylene chloride, washed successively with water and brine, and dried over $MgSO_4$. After evaporation of the solvent, the residue was purified by chromatography (eluant CH_2Cl_2/MeOH 9:1) to give 0.6 g (46%) of a white powder: mp 118 °C; R_f 0.10 (CH_2Cl_2/MeOH 9:1); ^{1}H NMR ($CDCl_3$) δ 7.78 (s, 1H), 6.25 (s, 1H), 4.50 (s, 2H), 4.34 (t, J = 6 Hz, 2H), 3.79 (s, 3H), 2.72 (t, J = 6 Hz, 2H), 2.70−2.40 (m, 8H), 2.27 (s, 3H); ^{13}C NMR ($CDCl_3$) δ 164.5, 160.2, 148.1, 133.3, 109.9, 109.4, 98.2, 62, 56.6, 55, 53.2, 45.9. Anal. ($C_{15}H_{22}ClN_3O_3\cdot 0.3H_2O$).

2-[*N*-Piperazino]ethyl 4-Amino-5-chloro-2-methoxybenzoate (9o). A solution of **9m** (1.3 g, 3.1 mmol) in CH_2Cl_2 (30 mL) was treated with anisole (0.1 mL) and trifluoroacetic acid (2.43 mL) for 6 h. The solvent was removed in vacuo, and the residue was taken up in CH_2Cl_2 and was made basic with a saturated aqueous $NaHCO_3$ solution and solid $NaHCO_3$. The organic phase was separated, washed with brine, dried with $MgSO_4$ and concentrated. Column chromatography on silica gel with MeOH/CH_2Cl_2 (10:90) gave 0.9 g of **9o** (96%) as a white foam solid. This compound was transformed into its hydrochloride salt with a 2 N HCl methanol solution and precipitated with Et_2O. Recrystallization from *i*-Pr_2O/MeOH afforded the dihydrochloride salt (14%): mp 244 °C; ^{1}H NMR (CD_3OD) δ 7.81 (s, 1H), 6.47 (s, 1H), 4.57 (m, 2H), 3.88 (s, 3H), 3.57 (br m, 10H). Anal. ($C_{14}H_{20}N_3O_3Cl\cdot 2HCl\cdot H_2O$).

2-[*N*-(4-Phenyl)piperazino]propyl 4-Amino-5-chloro-2-methoxybenzoate (9p). A mixture of **10b** (1.12 g, 3.4 mmol), *N*-phenylpiperazine (0.6 mL, 3.7 mmol) and diisopropylethylamine (1.2 mL, 6.8 mmol) in dry DMF (10 mL) was stirred for 24 h at room temperature. The resulting mixture was concentrated in vacuo. The residue was recristallized in AcOEt/cyclohexane to give 0.7 g (50%) of a pale pink solid: mp 116 °C; ^{1}H NMR ($CDCl_3$) δ 7.80 (s, 1H), 7.29−7.21 (m, 2H), 6.94−6.81 (m, 3H), 6.28 (s, 1H), 4.48 (s, 2H), 4.31 (t, J = 6.4 Hz, 2H), 3.83 (s, 3H), 3.25 (m, 4H), 2.71−2.57 (m, 6H), 2.00 (m, 2H); ^{13}C NMR ($CDCl_3$) δ 164.7, 160.3, 151.4, 147.8, 133.3, 129.1, 119.7, 116.1, 109.9, 98.3, 62.9, 56.1, 55.3, 53.3, 49.2, 26.3. Anal. ($C_{21}H_{26}ClN_3O_3$).

2-[*N*-(2-Pyrimidyl)piperazino]ethyl 4-Amino-5-chloro-2-methoxybenzoate (9r). It was prepared according to the process described for **9b** and isolated as the hydrochloride salt (17%) by crystallization from a MeOH/AcOEt/cyclohexane mixture: mp 244 °C; ^{1}H NMR (CD_3OD) δ 8.43 (m, 2H), 7.82 (s, 1H), 6.71 (m, 1H), 6.50 (s, 1H), 4.56 (m, 2H), 3.85 (s, 3H), 3.8−3.13 (m, 10H). Anal. ($C_{18}H_{22}N_5O_3Cl\cdot 4HCl\cdot 0.25H_2O$).

Method B. 2-[*N*-(4-Benzyl)piperazino]ethyl 4-Amino-5-chloro-2-methoxybenzoate (9q). A mixture of 2-bromoethyl 4-amino-5-chloro-2-methoxybenzoate **(10a)** (0.62 g, 3.9 mmol), *N*-benzylpiperazine (0.38 mL, 2.2 mmol), and diisopropylethylamine (0.7 mL, 4 mmol) in dry CH_3CN (10 mL) was refluxed for 24 h under argon. The resulting mixture was concentrated in vacuo. The residue was dissolved in methylene chloride, washed successively with water and brine, and dried over $MgSO_4$. After evaporation of the solvent, the residue was purified by chromatography (eluant CH_2Cl_2/*i*-PrOH 9:1) to give a solid which was recrystallized in an AcOEt/cyclohexane mixture to give 0.27 g (33%) of product as yellow needles: mp 117 °C; R_f 0.1 (CH_2Cl_2/*i*-PrOH 9:1); ^{1}H NMR ($CDCl_3$) δ 7.8 (s, 1H), 7.32−7.22 (m, 5H), 6.27 (s, 1H), 4.43 (s, 2H), 4.36 (t, J = 6 Hz, 2H), 3.82 (s, 3H), 3.51 (s, 2H), 2.75 (t, J = 6 Hz, 2H), 2.61−2.50 (m, 8H); ^{13}C NMR ($CDCl_3$) δ 164.4, 160.2, 147.8, 138.12, 133.3, 129.2, 128.2, 126.9, 110, 98.3, 63, 62, 56.7, 56.1, 53.4, 53. Anal. ($C_{21}H_{26}ClN_3O_3$).

2-[*N*-(4-Pyrimidyl)piperazino]ethyl 4-Amino-5-chloro-2-methoxybenzoate (9s). It was synthesized according to the previous process: the reaction between 2-bromoethyl 4-amino-5-chloro-2-methoxybenzoate **(10a)** (0.53 g, 1.71 mmol), *N*-(4-pyrimidyl)piperazine (306 mg, 1.9 mmol), and diisopropylethylamine (0.6 mL, 3.42 mmol) in dry CH_3CN (10 mL) gave, after purification by chromatography (eluant CH_2Cl_2/*i*-PrOH 9:1) 200 mg (33%) of product as a white solid: mp 218−220 °C; R_f 0.63 (eluant CH_2Cl_2/MeOH 9:1); ^{1}H NMR ($CDCl_3$) δ 8.58 (s, 1H), 8.18 (d, J = 5.3 Hz, 1H), 7.80 (s, 1H), 6.48 (d, J = 6 Hz, 1H), 6.28 (s, 1H), 4.48 (s, 2H), 4.39 (t, J = 6 Hz, 2H), 3.83 (s, 3H), 3.68−3.63 (m, 4H), 2.79 (t, J = 6 Hz, 2H), 2.66−2.61 (m, 4H); ^{13}C NMR ($CDCl_3$) δ 164.4, 161.6, 161.3, 160.3, 155.5, 147.8, 133.3, 110, 101.9, 98.3, 61.8, 56.7, 56.7, 52.9, 43.7. Anal. ($C_{19}H_{23}ClN_4O_3\cdot 2HCl\cdot 1H_2O$).

2-[*N*-(2-Pyrazinyl)piperazino]ethyl 4-Amino-5-chloro-2-methoxybenzoate (9t). It was synthesized according to the previous process: the reaction between 2-bromoethyl 4-amino-5-chloro-2-methoxybenzoate **(10a)** (0.63 g, 2 mmol), *N*-(2-

pyrazinyl)piperazine (373 mg, 2.3 mmol), and diisopropylethylamine (0.71 mL, 4.08 mmol) in dry CH_3CN (10 mL) gave, after purification by chromatography (eluant CH_2Cl_2/*i*-PrOH 9:1) 318 mg (40%) of product as a pale yellow solid: mp 163 °C; R_f 0.15 (CH_2Cl_2/*i*-PrOH 9:1); ^{1}H NMR ($CDCl_3$) δ 8.09 (s, 1H), 8.02−8.01 (m, 1H), 7.70 (d, J = 2.5 Hz, 1H), 7.77 (s, 1H), 6.24 (s, 1H), 4.56 (s, 2H), 4.39 (t, J = 6 Hz, 2H), 3.77 (s, 3H), 3.60−3.54 (m, 4H), 2.76 (t, J = 6 Hz, 2H), 2.70−2.60 (m, 4H); ^{13}C NMR ($CDCl_3$) δ 164.4, 160.2, 154.9, 147.9, 141.6, 133.2, 132.8, 130.9, 109.9, 109.5, 98.2, 61.8, 56.7, 56, 52.9, 44.5. Anal. ($C_{18}H_{23}ClN_5O_3\cdot 0.2H_2O$).

2-[*N*-(3-Pyridazinyl)piperazino]ethyl 4-Amino-5-chloro-2-methoxybenzoate (9u). It was synthesized according to the previous process: the reaction between 2-bromoethyl 4-amino-5-chloro-2-methoxybenzoate **(10a)** (0.62 g, 2 mmol), *N*-(3-pyridazinyl)piperazine (0.36 g, 1.8 mmol), and diisopropylethylamine (0.7 mL, 4 mmol) in dry CH_3CN (10 mL) gave, after purification by chromatography (eluant CH_2Cl_2/*i*-PrOH 9:1 then CH_2Cl_2/MeOH 9:1) and formation of the chlorhydrate 176 mg (20%) of product as a yellow solid: mp 210 °C dec; R_f 0.22 (CH_2Cl_2/*i*-PrOH 9:1); ^{1}H NMR ($CDCl_3$) δ 8.49 (dd, J = 3.3 Hz, J = 1.2 Hz, 1H), 7.75 (s, 1H), 7.14 (dd, J = 9.3 Hz, J = 4.4 Hz, 1H), 6.84 (dd, J = 9.4 Hz, J = 1.2 Hz, 1H), 6.27 (s, 1H), 4.71 (s, 2H), 4.35 (t, J = 5.7 Hz, 2H), 3.74 (s, 3H), 3.65−3.55 (m, 4H), 2.74 (t, J = 5.7 Hz, 2H), 2.66−2.60 (m, 4H). Anal. ($C_{18}H_{22}ClN_5O_3\cdot 3HCl$).

2-[*N*-(4-(2-Chloro)pyrimidyl)piperazino]ethyl 4-Amino-5-chloro-2-methoxybenzoate (9v). It was synthesized according to the previous process: the reaction between 2-bromoethyl 4-amino-5-chloro-2-methoxybenzoate **(10a)** (0.62 g, 2 mmol), *N*-(4-(2-chloro)pyrimidyl)piperazine (0.36 g, 1.8 mmol), and diisopropylethylamine (0.7 mL, 4 mmol) in dry CH_3CN (13 mL) gave, after purification by chromatography (eluant CH_2Cl_2/*i*-PrOH 9:1), 155 mg (20%) of product as a white solid: mp 97 °C; R_f 0.59 (CH_2Cl_2/*i*-PrOH 9:1); ^{1}H NMR ($CDCl_3$) δ 8.02 (d, J = 6 Hz, 1H, $CH_{(6')}$), 7.80 (s, 1H, ArH), 6.37 (d, J = 6 Hz, 1H), 6.28 (s, 1H), 4.46 (s, 2H), 4.39 (t, J = 5.8 Hz, 2H), 3.84 (s, 3H), 3.68−3.64 (m, 4H), 2.79 (t, J = 6 Hz, 2H), 2.66−2.60 (m, 4H); ^{13}C NMR ($CDCl_3$) δ 164.4, 162.6, 160.8, 160.3, 157.2, 147.8, 133.3, 109.09, 109.6, 101.1, 98.3, 61.7, 56.6, 56.1, 52.7, 42.8. Anal. ($C_{18}H_{21}Cl_2N_5O_3\cdot 0.9H_2O$).

2-[*N*-(3-(6-Chloro)pyridazinyl)piperazino]ethyl 4-Amino-5-chloro-2-methoxybenzoate (9w). It was synthesized according to the previous process: the reaction between 2-bromoethyl 4-amino-5-chloro-2-methoxybenzoate **(10a)** (0.62 g, 2 mmol), *N*-(3-(6-chloro)pyridazinyl)piperazine (0.36 g, 1.8 mmol), and diisopropylethylamine (0.7 mL, 4 mmol) in dry CH_3CN (10 mL) gave, after purification by chromatography (eluant CH_2Cl_2/*i*-PrOH 95:5 then CH_2Cl_2/*i*-PrOH 9:1), 0.5 g (65%) of product as a pale yellow solid: mp 179 °C; R_f 0.54 (CH_2Cl_2/*i*-PrOH 9:1); ^{1}H NMR ($CDCl_3$) δ 7.81 (s, 1H), 7.19 (d, J = 9.5 Hz, 1H), 6.87 (d, J = 9.5 Hz, 1H), 6.28 (s, 1H), 4.46 (s, 2H), 4.41 (t, J = 6 Hz, 2H), 3.84 (s, 3H), 3.67−3.62 (m, 4H), 2.80 (t, J = 6 Hz, 2H), 2.71−2.67 (m, 4H); ^{13}C NMR ($CDCl_3$) δ 164.6, 160.4, 159.7, 148, 146.8, 133.3, 128.8, 115.3, 109.9, 109.3, 98.2, 61.8, 56.7, 56, 52.8, 45. Anal. ($C_{18}H_{21}Cl_2N_5O_3$).

2-[*N*-(2-Pyridyl)piperazino]ethyl 4-Amino-5-chloro-2-methoxybenzoate (9x). It was synthesized according to the previous process: the reaction between 2-bromoethyl 4-amino-5-chloro-2-methoxybenzoate **(10a)** (0.62 g, 2 mmol), *N*-(2-pyridyl)piperazine (0.33 mL, 2.2 mmol), and diisopropylethylamine (0.7 mL, 4 mmol) in dry CH_3CN (10 mL) gave, after purification by chromatography (eluant CH_2Cl_2/*i*-PrOH 95:5 then CH_2Cl_2/*i*-PrOH 9:1) and recrystallization in an AcOEt/cyclohexane mixture, 0.33 g (42%) of product as a yellow powder: mp 143 °C; R_f 0.3 (CH_2Cl_2/*i*-PrOH 9:1); ^{1}H NMR ($CDCl_3$) δ 8.18 (dd, J_o = 4.8 Hz, J_m = 1.6 Hz, 1H), 7.82 (s, 1H), 7.46 (m, 1H), 6.66−6.58 (m, 2H), 6.27 (s, 1H), 4.47 (s, 2H), 4.41 (t, J = 6 Hz, 2H), 3.83 (s, 3H), 3.58−3.53 (m, 4H), 2.79 (t, J = 6 Hz, 2H), 2.70−2.66 (m, 4H); ^{13}C NMR ($CDCl_3$) δ 165.1, 160.9, 160.1, 148.6, 148.4, 138, 133.9, 110.6, 110.4, 107.7, 98.9, 62.6, 57.4, 56.7, 53.8, 45.8. Anal. ($C_{19}H_{23}ClN_4O_3\cdot 0.2H_2O$).

2-[*N*-(4-Pyridyl)piperazino]ethyl 4-Amino-5-chloro-2-methoxybenzoate (9y). It was synthesized according to the previous process: the reaction between 2-bromoethyl 4-amino-5-chloro-2-methoxybenzoate **(10a)** (0.64 g, 2 mmol), *N*-(4-pyridyl)piperazine (375 mg, 2.3 mmol), and diisopropylethylamine (0.72 mL, 4.1 mmol) in dry CH_3CN (10 mL) gave, after purification by chromatography (eluant CH_2Cl_2/MeOH 9:1) 200 mg (24%) of product as a white solid: mp °C; R_f 0.07 (CH_2Cl_2/MeOH 9:1); ^{1}H NMR ($CDCl_3$) δ 8.24 (d, J = 4 Hz, 1H), 7.82 (s, 1H), 6.65 (d, J = 5.5 Hz, 1H), 6.29 (s, 1H), 4.54 (s, 2H), 4.39 (t, J = 6 Hz, 2H), 3.82 (s, 3H), 3.38−3.33 (m, 4H), 2.78 (t, J = 6 Hz, 2H), 2.70−2.65 (m, 4H); ^{13}C NMR ($CDCl_3$) δ 163.9, 160.3, 155.1, 149, 147.9, 133.2, 110.1, 108.3, 98.3, 61.7, 56.6, 56, 52.7, 46. Anal. ($C_{19}H_{23}ClN_4O_3\cdot 0.4H_2O$).

2-[*N*-(4-Phenyl)piperazino]propyl 4-Amino-5-chloro-2-methoxybenzamide (11). A mixture of 4-amino-5-chloro-2-methoxybenzoic acid (0.7 g, 3.5 mmol), triethylamine (0.54 mL, 3.9 mmol) and DMF (30 mL) was cooled to 4 °C. Ethyl chloroformate (0.33 mL, 3.5 mmol) was added and the mixture stirred for 30 min at 4 °C. 1-(2-Aminoethyl)-4-phenylpiperazine (0.8 g, 3.9 mmol) was then added at room temperature and the mixture stirred overnight. The reaction mixture was washed with a 10% Na_2CO_3 solution and the aqueous layer was extracted several times with CH_2Cl_2. The combined organic layers were dried over $MgSO_4$ and concentrated in vacuo. Recrystallization from absolute EtOH yielded 0.52 g (38%) of product as yellow needles: mp 185 °C; ^{1}H NMR ($CDCl_3$) δ 8.22 (br s, 1H), 8.12 (s, 1H), 7.31−7.23 (m, 2H), 6.96−6.86 (m, 3H), 6.27 (s, 1H), 4.35 (s, 2H), 3.87 (s, 3H), 3.58 (m, 2H); 3.40−3.20 (m, 4H), 2.70−2.60 (m, 6H); ^{13}C NMR ($CDCl_3$) δ 176.8, 164.3, 151.1, 146.5, 132.9, 129.07, 119.7, 115.9, 111.5, 110.7, 97.8, 56.5, 56.1, 52.8, 49.3, 36.4. Anal. ($C_{20}H_{25}ClN_4O_2$).

Membrane Preparation and Radioligand Binding Assays. Briefly, cells grown to confluence were washed twice with phosphate-buffered-saline (PBS) and centrifuged at 300*g* for 5 min. The pellet was used immediately or stored at −80 °C. The pellet was resuspended in 10 volumes of ice-cold HEPES buffer (50 mM, pH 7.4) and centrifuged at 40000*g* for 20 min at 4 °C. The resulting pellet was resuspended in 15 volumes of HEPES buffer (50 mM, pH 7.4). The protein concentration were determined by the method of Lowry[24] using bovine serum albumin as the standard.

Radioligand binding studies were performed in 500 μL of buffer (50 mM HEPES, pH 7.4), 20 μL of competing agent (7 concentrations), 20 μL of [^{3}H]GR 113808 at a concentration of 0.2 nM for the 5-$HT_{4(e)}$ receptor isoform or at a concentration of half the value of the K_D values for the other isoforms expressed in the COS cell lines and 50 μL of membrane preparation (100−200 μg of protein). Nonspecific binding was defined with 10 μM ML 10375 2-(*cis*-3,5-dimethylpiperidino)-ethyl 4-amino-5-chloro-2-methoxybenzoate.[19] Tubes were incubated at 25 °C for 30 min, and the reaction was terminated by filtration through Whatman GF/B filter paper using the Brandel 48R cell harvester. Filters were presoaked in a 0.1% solution of polyethylenimine. Filters were subsequently washed with ice-cold buffer (50 mM Tris-HCl, pH 7.4) and placed overnight in 4 mL of ready-safe scintillation cocktail. Radioactivity was measured using a Beckman model LS 6500C liquid scintillation counter. Binding data (K_i) were analyzed by computer-assisted nonlinear regression analysis (Prism, Graphpad Software, San Diego, CA). The data are the results of two or three determinations.

Measurement of cAMP. For the measurement of intracellular cAMP accumulation, C6 glial cells stably transfected with human 5-$HT_{4(e)}$ receptors were grown to confluency and incubated with serum-free medium for 4 h before the beginning of the assay. Then the cells were preincubated for 15 min with serum-free medium supplemented with 5 mM theophylline and 10 μM pargyline. 5-HT (1 μM) and/or compounds **9a** and **9r** were added and incubated for an additional 15 min at 37 °C in 5% CO_2. The reaction was stopped by aspiration of the medium and addition of 500 μL of ice-cold ethanol. After 1 h at room temperature, the ethanol fraction was collected and lyophilized. The pellet was reconstituted and cAMP was quantified using a radioimmunoassay. The 5-HT concentra-

tion−effect curve was calculated using seven concentrations (10^{-8}−10^{-5} M) alone or in the presence of **9a** (70 nM) or **9r** (5 nM).

Experiments on Human Atrial Myocytes. Surgery: All protocols for obtaining human cardiac tissue were approved by the ethics committee of our institution (GREBB, Hôpital de Bicêtre, Université de Paris-Sud). Specimens of right atrial appendages were obtained from patients undergoing heart surgery for congenital defects, coronary artery diseases or valve replacement. Most patients received a pharmacological pretreatment (Ca channel blockers, digitalis, β-adrenergic antagonists, diuretics, ACE inhibitors, NO donors, and/or antiarrhythmic drugs). In addition, all patients received sedatives, anasthesia, and antibiotics. Dissociation of the cells was realized immediately after surgery.

Human atrial cell dissociation: Myocytes were isolated as desribed previously[25] with some modifications. Briefly, quickly after excision, the tissue was cut up and washed in a calcium-free Tyrode solution supplemented with 30 mM 2,3-butanedionemonoxine which was carefully removed after cutting. Small (∼1 mm^3) pieces of atria were then incubated in the same Tyrode solution containing 40 IU/mL collegenase, 15 IU/mL protease and 5 mg/mL BSA. After 30 min, this solution was removed and replaced by fresh enzymatic solution containing only collagenase (200 UI/mL) for 10−20 min until a satisfactory cell yield was obtained. All steps were carried out at 37 °C, with continuous stirring at 200 rpm and gassing with 95% O_2 and 5% CO_2. The cell suspension was filtered and centrifuged (1 min at 600−700 rpm) and the pellet resuspended in DMEM supplemented with 10% fœtal calf serum, nonessential amino acids, 1 nM insulin and antibiotics (penicillin, 100 IU/mL, and streptomycin, 01 μg/mL). For patch-clamp experiments 20−100 mL of this cell suspension was put in a Petri dish containing control external solution.

Electrophysiological experiments: The whole-cell configuration of the patch-clamp technique[26] was used to record the high-threshold calcium current (I_{Ca}) on Ca^{2+}-tolerant human atrial myocytes. In the routine protocols the cells were depolarized every 8 s from a holding potential of −50 to 0 mV for 400 ms. This holding potential was chosen to completely inactivate the fast Na current. K currents were blocked by replacing all K^+ ions with intracellular Cs^+ and extracellular Cs^+. Voltage-clamp protocol was generated by a challenger/09-VM programmable function generator (Kinetic Software, Atlanta, GA). The cells were voltage-clamped using a patch-clamp amplifier (model RK-400, Biologic, Claix, France). Currents were sampled at a frequency of 10 kHz using a 12-bit analogue-to-digital converter (DT2827, Data Translation, Malboro, MA) connected to a PC compatible computer (3865/33 System-pro, Compaq, Houston, TX). All experiments were done at room temperature (19−25 °C).

Solutions: Control external solution contained (in mM): 107.1 NaCl, 10 Hepes, 40 CsCl, 4 $NaHCO_3$, 0.8 NaH_2PO_4, 1.8 $CaCl_2$, 1.8 $MgCl_2$, 5 D-glucose, 5 sodium pyruvate, pH 7.4 adjusted with NaOH. Patch electrodes (0.8−1.5 MΩ) were filled with control internal solution that contained (in mM): 119.8 CsCl, 5 EGTA (acid form), 4 $MgCl_2$, 5 creatine phosphate disodium salt, 3.1 Na_2-ATP, 10 Hepes, 62 μM $CaCl_2$ (pCa 8.5), pH 7.3 adjusted with CsOH. Control or drug-containing solutions were applied to the exterior of the cell by placing the cell at the opening of 250-μm inner diameter capillary tubing flowing at a rate of ∼10 mL/min.

Materials: Collagenase type V and protease type XXIV and serotonin (5-HT) were from Sigma Aldrich (L'Isle d'Abeau, Chesnes, France). 5-HT was dissolved in ionic aqueous solutions, made fresh daily, and kept at 4 °C until use.

Data analysis: The maximal amplitude of I_{Ca} was measured as the difference between the peak inward current and the leak current, which was the current amplitude at the end of the 400-ms duration pulse.[26] Currents were not compensated for capacitive and leak currents. The on-line analysis was made possible by programming a PC compatible computer in Pascal language to determine, for each depolarization, peak and steady-state current values. The results are expressed as mean ± SEM. In each experimental condition, the effects of 5-HT on I_{Ca} are expressed in percent (%) change with respect to the values of the current under basal conditions, i.e., in the absence of any drug.

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