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Brief Article

Novel, Potent, and Selective Quinoxaline-Based 5-HT$_3$ Receptor Ligands. 1. Further Structure−Activity Relationships and Pharmacological Characterization[¥]

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We investigated the pharmacological profile of a novel series of quinoxaline-based 5-HT$_3$ receptor ligands bearing an extra basic moiety on the piperazine N-4. High affinity and selectivity were dependent on the electronic properties of the substituents, and at cardiac level **3a** and **3c** modulated chronotropy but not inotropy. In von Bezold−Jarisch reflex test **3a**−**c** were partial agonists while **3i** was a full agonist. Preliminary pharmacokinetic studies indicated that **3a** is a brain penetrating agent.

Introduction

Serotonin (5-HT[a]) is a neurotransmitter involved in many physiological functions and pathological disorders. It has been clearly established that 5-HT plays a significant role in sensory reflexes and peristalsis in the gastrointestinal tract, in regulation of cardiovascular system (vasomotricity, positive chronotropic, and inotropic effects), and in platelet aggregation.[1,2] Evidence is emerging for a potential role of 5-HT in the pathophysiology of disorders such as the irritable bowel syndrome and cardiac arrhythmias.[1] Although the consequences of selective 5-HT$_3$ receptor (5-HT$_3$R) blockade are well-known, a more complete understanding of the effects of 5-HT$_3$R agonism is still needed. Indeed, 5-HT$_3$R functionality is modulated by different structural class of compounds. To date, five 5-HT$_3$R antagonists are available for clinical use primarily used for controlling chemotherapy- and radiotherapy-induced nausea and vomiting. Furthermore 5-HT$_3$Rs are also modulated by antidepressants and antipsychotics (non-competitive antagonists).[3] Most of 5-HT effects are mediated by specific receptors, and unlike all other known receptors for 5-HT, whose actions are mediated via G-proteins,[4] the 5-HT$_3$R is a pentameric ligand-gated cation channel that is found in the central and peripheral nervous systems and in extraneuronal locations such as lymphocytes, monocytes, and fetal tissues. Five monomer subtypes, the 5-HT3A−E subunits, have been identified that show differences in the amino terminal and the transmembrane regions.[5] 5-HT$_3$R modulates visceral afferent information and visceral reflexes, participates in nociception and cognition, and has been suggested to play a role in the biology of drugs of abuse.[6,7] Furthermore, antiinflammatory and immunomodulatory properties have been observed for 5-HT$_3$R antagonists which might explain key findings in systemic sclerosis and other immunological conditions.[5]

5-HT modulates chronotropy and inotropy at cardiac level by activating parasympathetic and sympathetic pathways. These effects are mediated via 5-HT$_1$R, 5-HT$_2$R, and 5-HT$_3$R.[8] 5-HT$_{1A}$R activation mediates central sympatho-inhibition and bradycardia, while 5-HT$_{2A}$R activation mediates sympatho-excitation leading to a rise in blood pressure and tachycardia.[8] On the other hand, 5-HT can produce a tachycardic effect mediated by activation of 5-HT$_3$R, with positive inotropic and chronotropic effects, mimicked by the agonists quipazine (**1**, Chart 1) and 2-Me-5-HT.[9] Furthermore 5-HT$_3$R mediates the von Bezold−Jarisch (von B-J) reflex in humans. When 5-HT$_3$R on vagal afferent nerve endings is stimulated, this reflex gives rise to an abrupt but transient bradycardia resulting in a hypotensive response, which is followed by reflex tachycardia and blood pressure elevation.[8] Substances experimentally used to elicit the von B-J reflex include veratridine, 5-HT, and 1-phenylbiguanide (PBG), a selective 5-HT$_3$R agonist.[8] 5-HT regulation of the cardiac function is also mediated by 5-HT$_3$R, and these receptors may play a role in transduction of an electrical stimulus into mechanical responses and in perception of cardiac pain during ischemia in humans.[8] 5-HT also mediates positive chronotropic and inotropic effects on the myocardium via the 5-HT$_4$ receptors which are present on cardiac myocytes.[8]

We recently described a series of pyrroloquinoxalines (PQXs) (exemplified by **2a**, Chart 1 and Table 1) as 5-HT$_3$R ligands[2] characterized by an acidic chain at the distal piperazine nitrogen to prevent crossing of the blood−brain barrier

[¥]This paper is dedicated to the memory of Professor Vito Nacci deceased on October 6, 2009.

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[a]Abbreviations: 5-HT, serotonin; 5-HT$_3$R, 5-HT$_3$ receptor; BBB, blood−brain barrier; von B-J, von Bezold−Jarisch; PQX, pyrroloquinoxaline; PBG, 1-phenylbiguanide; DCM, dichloromethane; THF, tetrahydrofuran.

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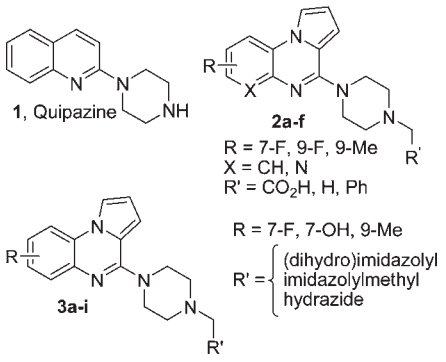
Chart 1. Reference and Title Compounds



(BBB). To further exploit the therapeutic potential of peripheral 5-HT_3R modulation, we further explored the effect on PQXs of extra protonatable functions with specific electronic properties to develop novel modulators of peripheral cardiac 5-HT_3R.

After exploitation of acidic functions[2] we improved the binding affinity to central 5-HT_3R by introducing a further basic chain at the piperazine N-4 (**3a**−**i**).[10,11] The novel 5-HT_3R ligands showed high affinity and selectivity when tested against a panel of receptors. We also explored the effect of the basic lateral molecular extension in in vitro functional studies and in the von B-J reflex test (in vivo). All tested compounds (**3a**−**c**,**i**) showed agonist properties in the von B-J reflex test, while **3a** and **3c** proved to be antagonists on guinea pig spontaneously beating right atrium (Table 2 and Figure 1). No inotropic effect was detected, indicating no activity at the left atrium 5-HTR. In addition, brain to plasma concentrations of **3a** were measured after systemic injection (Table 3).

Chemistry

The synthesis of **3a**−**i** is described in Schemes 1 and 2. Starting from our previously reported piperazinylpyrroloquinoxalines **4a**−**c**[11] (Scheme 1), compounds **3a**,**b**,**d** and **5** were obtained applying a standard reductive amination protocol by exposure to the appropriate aldehyde. Benzyl group removal, by catalytic hydrogenation of **5**, provided **3c**. Compound **3e** was obtained by the N-alkylation of **4a** carried out with 2-(chloromethyl)imidazoline. Compound **3f** (Scheme 2) was prepared by reduction of the carboxyl function of **6**. Amide **6** was synthesized by means of a classical coupling reaction between **4a** and (imidazol-4-yl)acetic acid. Intermediate **7b** was synthesized as previously described for **7a**,[11] and both were treated with methanesulfonyl chloride and imidazole sodium salt to provide **3g** and **3h**, respectively. Treatment of the ester **8**[2] with hydrazine provided the hydrazido derivative **3i**.

Biological Assays

5-HT_3R affinity was assessed, as previously described,[2] in binding studies, and corresponding data are given in Table 1. 5-HT_3R efficacy was measured in functional assays using guinea pig myocardium and aortic strips (Table 2). In vivo studies consisted of the von B-J reflex test for assessing the 5-HT_3R agonist/antagonist properties of a subset of selective ligands (Table 1). Some of the compounds were further examined for their selectivity toward serotonin (5-HT_{1A}R, 5-HT_{2A}R, and 5-HT_4R), adrenergic (α_1 and α_2), dopamine (DA_1 and DA_2), and muscarinic receptors (Table 1 SI of the Supporting Information)). At $\geq 10\,\mu$M, the tested compounds did not bind to any of the receptors listed in the panel, thus proving their high selectivity for 5-HT_3R. Additionally, brain distribution studies of **3a** were performed in rats after systemic injection (Table 3). In these studies **2a** and **3a** were extracted from plasma and brain homogenate and quantified by reversed-phase HPLC with UV detection as previously described.[2]

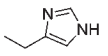
Structure−Activity Relationships (SAR)

As demonstrated for previous 5-HT_3R PQX ligands,[2] affinity is modulated by the nature of the substituent at the distal piperazine N-4. The original series[2] was characterized by an internal proton exchange equilibrium between the "acidic" lateral chain and the basic distal piperazine nitrogen. To exploit the therapeutic potential of modulating cardiac 5-HT_3R, according to the 5-HT_3R binding site features, we developed PQXs **3a**−**i** bearing extra protonatable functions to explore receptor affinity, selectivity, and biological properties with respect to the previously described "acidic" ligands.[2] The SARs were investigated with respect to (i) PQX system functionalization (7-F, 9-Me, 7-OH) and (ii) piperazine N-4 substitution with protonatable moieties (imidazol-5-ylmethyl (**3a**−**c**), imidazol-2-ylmethyl (**3d**), 4,5-dihydroimidazol-2-ylmethyl (**3e**), imidazol-5-ylethyl (**3f**), imidazol-1-ylethyl (**3g**,**h**), and acetylhydrazide (**3i**)). Binding studies indicated that the affinity of compounds bearing extra basic functions depends on the PQX nucleus functionalization, in line with the previously identified trend in the series of piperazine N-4 benzyl substituted PQXs (Chart 1 SI),[11] following the trend 7-F ≥ 9-Me > 7-OH (Table 1, **3a** vs **3b**, and **3g** vs **3h**). Data shown in Table 1 highlight that the basic piperazine N-4 functionalization (imidazole as a proton exchange system) is well tolerated at the binding site level. In fact, the introduction of the imidazole ring caused a 10-fold improvement in affinity with respect to phenyl[11] or other five-membered heterocyclic rings[2] previously reported by us. Imidazol-5-ylmethyl derivatives (**3a**−**c**) were the most potent compounds of the series. The shift of the imidazole junction from C5 to C2 lowered the activity of about 8-fold (**3a** vs **3d**). Partial saturation of the five-membered ring generated a further reduction of affinity (**3d** vs **3e**). Steric tolerance in proximity to the N-4 piperazine was explored by means of methylene homologation (imidazolylethyl vs imidazolylmethyl), and **3f** was 500 times less potent than the counterpart **3a**. The N-1 imidazole substituted derivatives **3g** and **3h**, characterized by an ethyl spacer, were less potent as well. The effects of different N-4/imidazole spacing tethers are probably due to the electronic effects on the whole heterocyclic system. The hydrazidoacetyl derivative bearing an amphiphilic moiety was the least potent compound of the series.

Functional in Vitro Assays. Assessment of Cardiovascular Activity on Isolated Atria

Cardiac properties of a set of newly developed analogues was determined in functional studies on isolated atrial preparation in the presence or absence of 5-HT. Their effect was compared to a series of PQXs substituted at N-4 by a methyl or a benzyl group (**2b**−**f**)[10,11] and characterized by different heteroaryl systems. The tests were performed as previously described.[2] Modulation of cardiovascular parameters by tested compounds and reference 5-HT_3R ligands is reported

Table 1. 5-HT_3R Binding Activity (K_i nM), in vivo Effects on the von B-J Reflex Test (ED_{50}, ID_{50}, μg/kg, iv) of a series of 5-HT_3R Ligands

cmpd	R	R'	X	K_i[a]	von B-J reflex test		
					ED_{50}[b]	ID_{50}[c]	Activity[d]
3a	7-F		-	0.68 (0.13)		10 (3.0)	pa
3b	9-Me		-	0.49 (0.07)	37 (6)	3.5 (1.0)	pa
3c	7-OH		-	2.25 (0.35)	28 (3)	6.5 (2.5)	pa
3d	7-F		-	5.60 (0.95)			
3e	7-F		-	30.0 (7.5)			
3f	7-F		-	330.2 (109)			
3g	7-F		-	22.21 (2.6)			
3h	9-Me		-	17.84 (1.78)			
3i	7-F		-	44.3 (7.1)	57 (6)		ag
2a	7-F	CO_2H	CH	11.7 (1.0)[e]	36 (4)[e]		ag[e]
2b	7-F	H	CH	0.81 (0.05)[f]		600 (75)[f]	ant[f]
2c	9-F	H	CH	0.44 (0.05)[f]		120 (51)[f]	pa[f]
2d	9-Me	H	CH	0.79 (0.1)[g]			
2e	H	H	N	2.4 (0.5)[g]			
2f	H	Ph	N	2.2 (0.5)[g]			
1	-	-	-	0.96 (0.11)			

[a] K_i nM: each value is the mean (± S.E.M.) of three determinations and represents the concentration giving half-maximal inhibition of [^{3}H]LY278584 binding to rat cortical homogenate; [b] ED_{50}: concentration that produces half of the maximal bradycardia evoked by the same compound; [c] ID_{50}: concentrations that produces 50% inhibition of the agonist maximal response (conf. lim. 95%); [d] pa = partial agonist; ag = agonist; ant = antagonist; [e] from ref 2; [f] from ref 10; [g] from ref 11.

in Figure 1 and Table 2. All tested compounds were devoid of positive inotropic intrinsic activity and did not induce vasoconstriction. Compounds **3a** and **3c**, as **2a**,[2] behaved as antagonists at the right atrium. They antagonized the positive chronotropic effect of 5-HT in a noncompetitive manner with IC_{50} of 0.27 (**3a**), 2.63 (**3c**), and 1.88 (**2a**) μM. Compounds **2b** and **2e** behaved as agonists at the right atrium with intrinsic activity, relative to 5-HT, of 0.6 and 0.8, respectively. The C9-substituted **2c** and **2d** were competitive antagonists, while the benzyl derivative **2f** was found inactive on atrial preparation. These data suggest that in the series of the N-4-methyl substituted compounds, substitution at the C7 or C9 position of the tricyclic system may be responsible for a shifting from agonist to competitive antagonist properties, respectively. The presence of a conjugated "charged" system at the distal piperazine N-4 (**2a**, **3a**, and **3c**) determined a noncompetitive antagonist behavior, suggesting that a charged lateral chain at the distal piperazine N-4 may be of key importance for activity and potency in right atrial preparations (**2a**, **3a**, **3c** vs **2f**).

Functional in Vivo Assays. von Bezold−Jarisch Reflex Test

In vivo, the new compounds **3a**−**c** and **3i** were tested on the 5-HT_3R-dependent von B-J reflex in urethane-anesthetized rats (Table 1).[12] The von B-J reflex is evoked from cardiopulmonary chemoreceptive afferents and causes hypopnea, bradycardia, and vasodilation.[13] The von B-J reflex can be activated by a variety of chemical stimuli delivered by the circulation to the heart, including veratrum alkaloids, nicotine, and 5-HT_3R agonists such as 5-HT and PBG.

PBG (agonist) produced a rapid fall of heart rate, and this effect could be completely prevented by zacopride (antagonist) injected 5 min before. Through this test, we have been able to establish the agonist and/or the antagonist effect of the new compounds as follows: (i) agonist effect was defined as the intrinsic capability of the tested compounds to induce the von B-J reflex; (ii) the potential antagonist effect was assessed iv 5, 15, 30 min prior to PBG, and their ability to reduce the effect of PBG was evaluated.[2] When **3a** was injected at 1 μg/kg iv, neither agonist nor antagonist effects were observed. Some discrete bradycardic effect was obtained when **3a** was administered at a dose of 10 μg/kg, but at this dose, the antagonist effect prevailed. Antagonist effect was more pronounced at 50 μg/kg and a clear-cut transient bradycardia was observed. Finally, at 100 μg/kg, both a marked reduction in heart rate (−40%) and a prevention of PBG-evoked bradycardia were observed. Clearly, the reduction of heart rate was mediated by 5-HT_3R, since it was completely prevented by pretreatment with zacopride (1 mg/kg, before **3a**). These data indicate that **3a** behaves as partial 5-HT_3R agonist with an ID_{50} of 10 μg/kg iv when tested against PBG as an agonist. Already at 1 μg/kg, **3b** produced a clear-cut attenuation of the bradycardic effect of PBG, but on its own, **3b** did not significantly alter the heart rate in urethane-anesthetized rats. At 10 μg/kg, **3b** alone still did not show any agonist effect but reduced by 60% the PBG-evoked bradycardic effect. However, at 50 and at 100 μg/kg, **3b** produced a significant decrease in heart rate (50% of that produced by PBG) and markedly reduced PBG-evoked bradycardia. These data indicate that **3b** behaves as a high affinity partial agonist at the 5-HT_3R mediating the von B-J reflex. At 10 μg/kg, **3c** also produced a significant decrease in heart rate (45% of that evoked by PBG) and a partial blockade of PBG-evoked bradycardia (62% reduction of PBG effect). **3c** at 50 μg/kg exerted a clear bradycardic effect (50% of that evoked by PBG) and almost completely (−90%) prevented PBG-evoked bradycardia. At 100 μg/kg, the agonist effect of **3c** was only slightly increased and its

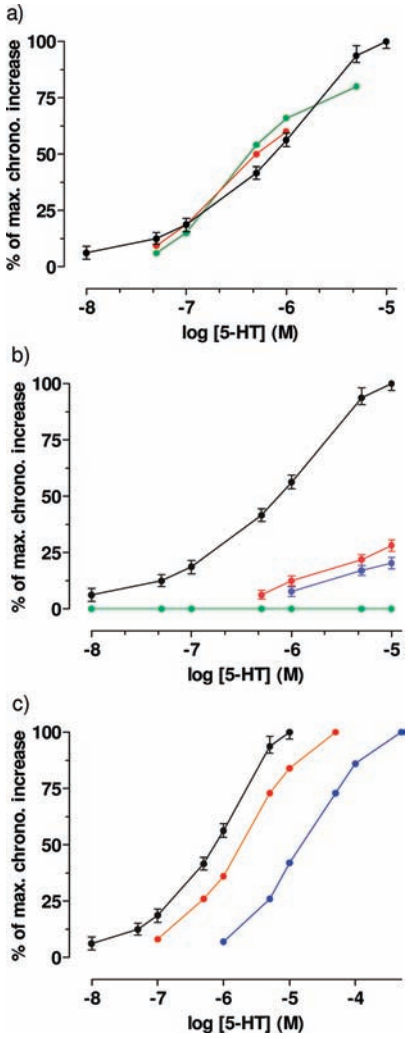


Figure 1. Guinea pig spontaneously beating right atria: (a) agonist activity indicated by cumulative log concentration−response curves to 5-HT (black line), **2b** (red line), and **2e** (green line); (b) non-competitive antagonistic activity indicated by cumulative log concentration−response curves to 5-HT of control (black line) and after exposure to **3a** (green line), **3c** (red line), and **2a** (blue line) at 10 μM for 30 min; (c) competitive antagonist activity indicated by cumulative log concentration−response curves to 5-HT of control (black line) and after exposure to **2d** (red line) or **2c** (blue line) at 1 μM for 30 min. In all cases the results are expressed as the mean ± SEM of five to seven different observations. Where error bars are not shown these are covered by the point itself.

antagonist effect was not larger but lasted longer. These data indicate that **3c** also acts as a partial agonist in the von B-J reflex test. On the other hand, **3i** did not show any agonist or antagonist effect (vs PBG) when administered iv at a dose of 10 μg/kg, but at 50 and 100 μg/kg this compound exerted a clear agonist effect reaching, at 100 μg/kg, 80% of the effect produced by PBG. These data indicate that **3i** acts as a pure agonist in the von B-J reflex test.

Brain-to-Plasma Distribution Studies

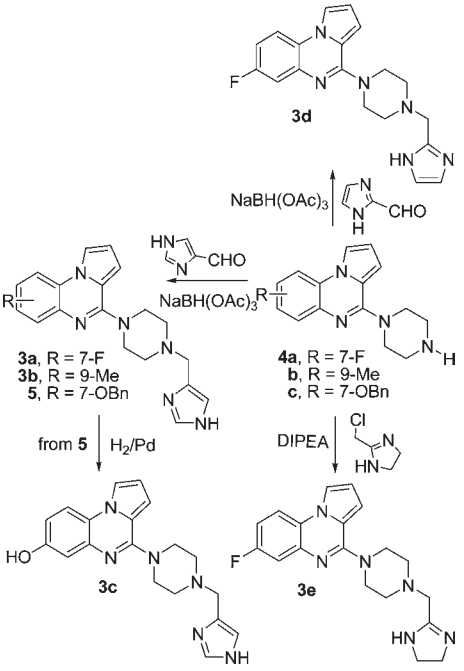
Compound **3a** and the reference **2a** were subjected to brain-to-plasma partition studies. While **2a** was characterized by poor BBB permeability, for **3a** the mean brain concentrations paralleled those in plasma, indicating that it rapidly and freely distributes across the BBB.

Table 2. In vitro Functional Behavior on Guinea-pig Spontaneously Beating Right Atrium (EC_{50}, IC_{50} μM, and pA_2), of a Series of 5-HT_3R Ligands

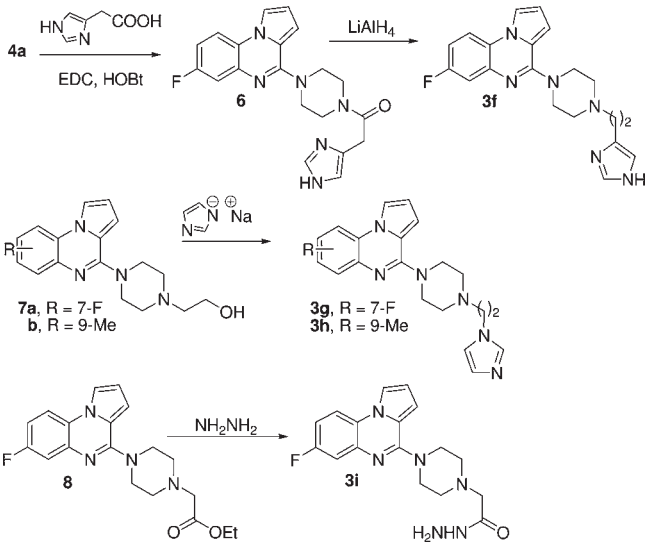
compd	Agonism EC_{50}[a]	Non Comp. Antag. IC_{50}[a]	Comp. Antag. pA_2[a]
3a		0.27 (0.21–0.35)	
3c		2.63 (1.75–3.85)	
2a		1.88 (1.37–2.57)[b]	
2b	0.16 (0.11–0.20) $\alpha = 0.6$		
2c			7.19 ± 0.03
2d			6.02 ± 0.02
2e	0.34 (0.27–0.43) $\alpha = 0.8$		
2f	inactive	inactive	inactive

[a] See Table 3 footnote in ref 2; [b] from ref 2.

Scheme 1. Synthesis of Compounds **3a−e**



Scheme 2. Synthesis of Compounds **3f−i**



In other studies rats were given different iv doses of **3a** and **2a** to obtain information on plasma concentrations, in relation

Table 3. Plasma and Brain Concentrations of Compounds **2a** and **3a** after Intravenous Injection in Rats

dose	concn (±SEM),[a] μM plasma	brain	brain/plasma[b]
	Compound **2a**		
0.25	0.54(0.12)	*c*	ND[d]
0.5	1.16(0.19)	*c*	ND[d]
1	2.46(0.43)	0.08(0.01)	0.03
	Compound **3a**		
0.25	*c*	*c*	ND[d]
0.5	0.07(0.01)	0.09(0.03)	1.3
1	0.21(0.04)	0.21(0.01)	1.0

[a] Each value is the mean ± SEM of three animals (sacrificed 30 min after the injection). [b] Ratio between brain and plasma (assuming 1 g of tissue equivalent to 1 mL of water). [c] Below the level of quantification. [d] ND = not determinable.

with those in the brain. Table 3 shows the mean plasma and brain concentrations of the compounds after 0.25−1 mg/kg administration. The mean plasma concentrations rose with the dose, although at 0.25 mg/kg of **3a** the concentrations were below the limits of quantification by our analytical procedure. Even at high doses, **3a** plasma concentrations were markedly lower than those of **2a** at 30 min. This may be the result of differences in clearance and/or in distribution, which could not be established in these preliminary distribution studies. We previously evaluated the ability of PQX 5-HT_3R agonists to cross the BBB in vivo in rodents.[11] Most of them rapidly entered the brain, achieving whole-brain concentrations higher than in plasma, although with differences in brain-to-plasma partition.[10,11] This is because of their lipophilicity which allows free, rapid diffusion across the BBB. In contrast, for the 7-F analogue **2a**, a derivative with a hydrophilic carboxylic group on the piperazine N-4 (log $P = 1.4$, ACD/Labs, version 12.0, Toronto Canada), we found a brain-to-plasma distribution ratio of about 0.03 after 1 mg/kg. By contrast, for the more lipophilic derivative **3a** (calculated log $P = 2.1$) brain concentrations were comparable to those in plasma 30 min after 0.5 and 1 mg/kg in rats. Like in plasma, these brain concentrations appeared to dose-dependently increase within these dose ranges (Table 3).

Conclusions

We developed new, potent, and selective 5-HT_3R ligands, and their cardiovascular activity was assessed in vitro and in vivo. According to functional assays, **3a** and **3c** showed a clear-cut selectivity for right atrium 5-HT_3R, specifically modulating chronotropy and not inotropy. In vivo **3a** is able to elicit the von B-J reflex. Preliminary brain-to-plasma distribution studies indicated that **3a**, prototype of the imidazole containing PQX 5-HT_3R ligands, rapidly crosses the BBB, yielding mean brain-to-plasma concentration ratios of about 1 after iv doses in rats. **3a** represents a new and useful tool to further investigate the role of 5-HT_3R at the cardiac level.

Acknowledgment. The authors thank MIUR (Roma) for financial support (PRIN).

Supporting Information Available: Chart 1 SI and Tables 1 SI and 2 SI, experimental procedures, and elemental analysis results for compounds **3a**−**i**. This material is available free of charge via the Internet at http://pubs.acs.org.

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