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Brief Article

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Design, Synthesis, and Preliminary Biological Evaluation of New Isoform-Selective f-Current Blockers

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New I_f blockers have been designed and tested on HEK293 cells stably expressing the HCN1, HCN2, and HCN4 channels to find compounds able to discriminate among the channel isoforms. Among the synthesized compounds, the *cis*-butene derivative (*R*)-**5** shows some preference for HCN2 while the pseudodimeric product (*R*)-**6** shows selectivity for HCN1. These compounds can be important pharmacological tools to study the channels in native tissues and may be useful to design safe drugs.

Introduction

The "funny current" (I_f) is a mixed Na^+/K^+ inward current, activated upon hyperpolarization, directly modulated by cAMP and regulated by neurotransmitter receptors coupled to cyclic nucleotide second messengers. (I_f)[1] is carried by the HCN[a] (hyperpolarization-activated cyclic nucleotide gated) channels; they belong to the superfamily of cyclic nucleotide-gated (CNG) channels and consist of four subunits, each showing six transmembrane domains (S1−6), a voltage sensor (S4), and a cyclic nucleotide-binding site.[2] In mammals, the HCN channel family is formed by four members (HCN1−4) that are differently distributed within tissues such as central and peripheral nervous system and in the heart.[3] When expressed in heterologous cells, the subunits form homomeric channels displaying the main biophysical properties of native I_f but differing from each other mainly with regard to their speed of activation and the extent by which they are modulated by cAMP.[4] There is evidence that in vivo the four HCN subunits can combine into heterotetrameric channels whose stoichiometry is not known (see ref 4 and references therein).

I_f plays a major role in the generation of pacemaker activity and in the regulation of heart rate, and it can be blocked by specific bradycardic agents, a group of structurally diverse drugs among which are **1** (ZD7288)[5] and the phenylalkylamines reported in Chart 1: zatebradine, cilobradine, and ivabradine. While cilobradine recently entered clinical trials,[6] the development of zatebradine stopped because of adverse side effects on vision,[7] probably caused by inhibition of HCN channels in retinal rod.[8] Ivabradine, whose pharmacological profile is safer than that of zatebradine,[9,10] has been approved in 2005 by EMEA for the treatment of stable angina pectoris and is the only specific bradycardic agent that is currently available for clinical use.

The interest in HCN channels modulators has recently grown because of the emerging evidence that these channels are involved in the function of sensory neurons and in pain perception,[11] making them attractive targets for the discovery of drugs not only for heart diseases[12] but also for the management of neuropathic pain.[13,14] In addition, the analysis of transgenic HCN knockout mice has shed more light into the physiological role of HCN channels.[3] Besides heart, HCN channels regulate rhythmic activity in other excitable cells such as neurons and photoreceptors. They have a role in dendritic integration, long-term potentiation, synaptic transmission, and the control of working memory, and they have been found in pancreatic β-cells, although their role is still unknown.[4,15] The different isoforms vary in their distribution within tissues; therefore, isoform-selective substances could be important pharmacological tools to study the channels in native tissues and may be useful to design safe drugs.

The design of selective HCN blockers is hampered by the fact that most of the phenylalkylamines synthesized so far have been tested in vivo or in vitro mainly for their ability to reduce heart rate, and only a few compounds have been tested on different HCN isoforms. Since the negative chronotropic activity may be due to several mechanisms,[16] it is obviously quite difficult to derive structure−activity relationships for the interaction with the molecular target of interest (i.e., HCN) from the data available in the literature. Zatebradine, cilobradine, ivabradine, and **1**, the most studied compounds, have a different ability to block I_f, but they are not able to discriminate among HCN channel isoforms.[17,18] Regarding the site of interaction of these substances on the channel, there is evidence that it is located into the channel pore and that the compounds must cross the cell membrane to interact with it;[1,19,20] therefore, an important property of HCN blockers is lipophilicity. In addition, at the site of interaction some differences exist within the four isoforms that can be exploited to design selective ligands.[19−21]

To search for subtype-selective I_f blockers, we decided to design new phenylalkylamines by applying classical approaches

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[a] Abbreviations: HCN, hyperpolarization-activated cyclic nucleotide gated; CNG, cyclic nucleotide-gated; HEK, human embryonic kidney.

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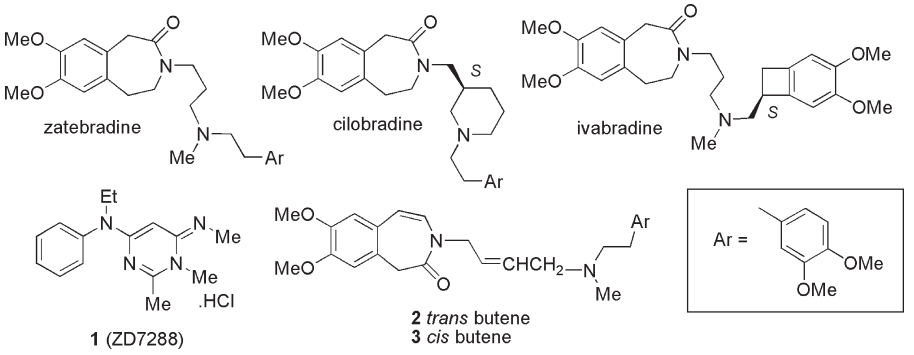
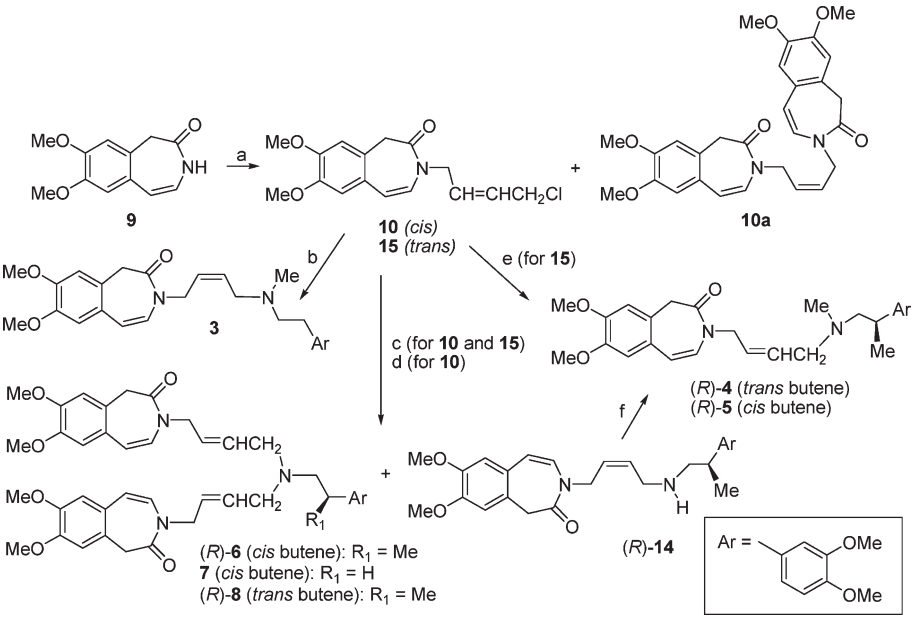
Chart 1



Scheme 1[a]



[a] Reagents and conditions: (a) *t*-BuOK, *cis*- or *trans*-1,4-dichlorobutene; (b) *N*-methylhomoveratrylamine; (c) (*R*)-**11**; (d) homoveratrylamine; (e) (*R*)-**13**; (f) CH_2O, HCOOH. The *S* enantiomers were prepared in the same way, using (*S*)-**11** (step c) and (*S*)-**13** (step e).

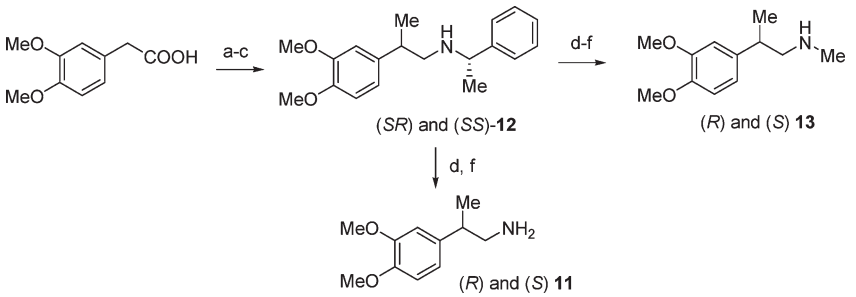
of medicinal chemistry. In a previous paper we disclosed a structural analogue of zatebradine (**2**, Chart 1) that was able to reduce heart rate in guinea pig spontaneously beating right atria and to block I_f in ventricular cardiomyocyte of old spontaneously hypertensive rats, with a potency comparable to that of the lead.[22] When this compound and its *cis* isomer **3** were tested on HCN isoforms expressed in HEK293 cells, it was found that, contrary to what happens for ivabradine, they show some preference in the blockade of channel isoforms (see Results and Discussion). These findings prompted us to make some additional structural manipulations to see if selectivity could be achieved. Therefore, a chiral center was placed on the phenethyl moiety of **2** and **3**, giving the enantiomers of **4** and **5**. The activity of the serendipitously discovered **6** prompted us to synthesize also the nonchiral analog **7** and the *trans*-derivative **8**. The structures of the new compounds are shown in Scheme 1.

Chemistry

The compounds were prepared starting from **9**[23] according to the synthetic pathway reported in Scheme 1. N-Alkylation of **9** with commercially available *cis*-1,4-dichlorobutene gave alkene **10**; the low yield of the reaction can be accounted for the competition with the double addition of **9** to the *cis*-dichlorobutene, yielding **10a**. Changes in the ratio between reactants or reverse addition of the anion to the halogen derivative did not stop the formation of the byproduct. The formation of the dimeric product was not observed when the reaction was carried out on *trans*-1,4-dichlorobutene.[22] Reaction of **10** with commercially available *N*-methylhomoveratrylamine gave **3** in good yields.

(*R*)- and (*S*)-2-(3,4-Dimethoxyphenyl)propan-1-amine (**11**, Scheme 2) were prepared following the procedure reported by Riggs,[24] with a slight modification. The mixture of *N*-((*S*)-1-phenylethyl)-2-(*RS*)-(3,4-dimethoxyphenyl)propan-1-amine was separated by column chromatography, yielding the two diastereoisomers (*SR*)-**12** and (*SS*)-**12** which were catalytically hydrogenated to yield the (*R*)- and (*S*)-**11**. The enantiomeric excess was the same as for the commercially available (*S*)-1-phenylethylamine (98%). In fact, after chromatographic separation, in the ^{1}H NMR spectrum of (*SR*)-**12** or (*SS*)-**12** no trace of the other diastereoisomer was detectable. Treatments of compounds (*SR*)-**12** and (*SS*)-**12** with formaldehyde and formic acid followed by catalytic hydrogenation afforded (*R*)- and (*S*)-**13**.[25,26]

Scheme 2[a]



[a] Reagents and conditions: (a) LDA, THF, MeI; (b) $SOCl_2$, $CHCl_3$, (*S*)-1-phenylethylamine; (c) $BH_3 \cdot THF$; (d) chromatographic separation; (e) HCHO, HCOOH; (f) $H_2/Pd(OH)_2/C$.

Table 1. Effect of Compounds **1**−**7** and Ivabradine on mHCN1 and hHCN4[a]

compd (5 μM)	mHCN1[b]	hHCN4[c]
2	35 ± 17	53 ± 5
3	53 ± 1	59 ± 0.5
(*R*)-**4**	85 ± 7	72 ± 4
(*S*)-**4**	85 ± 0.3	71 ± 9
(*R*)-**5**	63 ± 1	83 ± 0.1
(*S*)-**5**	76 ± 3	91 ± 2
(*R*)--**6**	27 ± 3	93 ± 1
(*S*)-**6**	76 ± 0.2	92 ± 1
7	82 ± 3	75 ± 1
(*R*)-**8**	60 ± 6	73 ± 18
(*S*)-**8**	93 ± 13	83 ± 13
ivabradine	47 ± 3	49 ± 2

[a] Results are expressed as percentage of f-current amplitude at −120 mV after application of a 5 μM drugs. Values are normalized to control and represent the mean ± SEM of three to five experiments. [b] Mouse HCN channel expressed in HEK 293 cells. [c] Human HCN channel expressed in HEK 293 cells.

Reaction of **10** with (*R*)-**11** in 1:1 ratio (Scheme 1) gave a mixture of compounds (*R*)-**6** and (*R*)-**14**, which were separated by column chromatography. (*R*)-**14** was then methylated using formaldehyde and formic acid, obtaining (*R*)-**5**. With the same procedure, starting from **10** and (*S*)-**11**, (*S*)-**6**, (*S*)-**14**, and after methylation, (*S*)-**5** were obtained. Compounds (*R*)- and (*S*)-**4** were prepared by reaction of **15**[22] with (*R*)- and (*S*)-**13**. Reaction of **10** with commercially available homoveratrylamine in 2:1 ratio gave **7** in good yield. (*R*)-**8** and (*S*)-**8** were obtained in the same way starting from **15** and (*R*)- or (*S*)-**11**.

Pharmacology

To measure the inhibition of the hyperpolarization-activated current, patch-clamp experiments were performed on mouse HCN1 and human HCN4 isoforms heterologously expressed in HEK293 cells. As a preliminary screening, the compounds were tested at 5 μM to check their ability to block f-current (Table 1). For the most interesting compounds, the EC_{50} has been determined on mHCN1 and hHCN4 and also on mHCN2 heterologously expressed in HEK293 cells (Table 2). Unfortunately HCN3 was not available. The selectivity ratios have been calculated as the ratio between the EC_{50} values and are reported in Figure 1. Ivabradine was taken as control. This compound has been previously tested on this cell line, transfected with hHCN1−4 cDNA[18] or with mHCN1 and hHCN4 cDNA.[27] It was found to block I_f with EC_{50} values in the micromolar range, although it was not able to discriminate among the isoforms.

Selected compounds were tested on guinea pig spontaneously beating atria to evaluate their negative chronotropic activity on a tissue expressing native HCN channels. Compounds were tested at increasing doses (10^{-9}−10^{-4} M) to measure the decrease in atrial rate. The potency of the drug is defined as EC_{50} (Table 2), and it was evaluated by fitting of the concentration−effect curve with the Hill equation.

Results and Discussion

The I_f blocking ability of the compounds at 5 μM is reported in Table 1. The synthesized compounds were able to block I_f at 5 μM, with the exception of (*S*)-**5**, (*R*)-**6**, and (*S*)-**6** on HCN4 and (*S*)-**8** on HCN1. Some compounds were more active on HCN1 than on HCN4 (**2**, (*R*)-**5**, (*S*)-**5**, (*R*)-**6**, (*S*)-**6**, and (*R*)-**8**), while (*R*)-**4**, (*S*)-**4**, **7**, and (*S*)-**8** were more active on HCN4. On HCN1, **2** and (*R*)-**6** were able to inhibit f-current to a larger extent with respect to ivabradine, which, as expected, did not show selectivity, the extent of the inhibition being similar on both HCN isoforms. The stereogenic center is important for the interaction with the channel, since for **5**, **6**, and **8** the *R* forms were more active than their *S* enantiomers. Also, the configuration of the double bond affected the biological activity, since the *cis*-butene derivatives were more active than their *trans* isomers (compare **5** and **6** with **4** and **8**, respectively). In this respect, **2** and **3** were an exception. In fact, the *trans*-derivative **2** was more active than its *cis* isomer **3** on HCN1, while the two compounds showed the same activity on HCN4.

In order to better evaluate the potency and the selectivity of our compounds, for some of them the EC_{50} was determined on the three available isoforms (Table 2) and used to calculate the selectivity ratio (Figure 1). Compounds **2**, **3**, and (*R*)-**5** were able to block I_f, with potency similar to ivabradine at least on one isoform. Compound **2** preferentially blocked the HCN1 channel, its EC_{50} values being, on this subtype, 3 and 7 times lower than on HCN4 and HCN2, respectively. The shift of the configuration of the double bond from *trans* (**2**) to *cis* (**3**) changed the preference for HCN isoforms, since **3** was equally active on HCN1 and HCN4 and 4 times less active on HCN2. The introduction of a methyl group on the phenylethyl moiety changed the selectivity profile of **3**: (*R*)-**5** was 4 and 10 times more potent on HCN2 than on HCN1 and HCN4, respectively. The duplication of the benzazepinylbutenyl group gave interesting results. While **7** showed low potency on the three isoforms, (*R*)-**6** was 7 times more potent than ivabradine on HCN1. In addition, its potency on HCN1 was 170 times higher than on HCN4 and 30 times higher than on HCN2, therefore displaying high selectivity for the HCN1

Table 2. I_f Blocking Potency and Negative Chronotropic Activity of Selected Compounds in Comparison with Ivabradine and Zatebradine

compd	EC_{50} (μM) mHCN1[a]	ER[b]	EC_{50} (μM) mHCN2[a]	ER[b]	EC_{50} (μM) hHCN4[c]	ER[b]	EC_{50} (μM) guinea pig atria[d]
2	2.31 ± 0.37		17.22 ± 1.74		7.23 ± 2.60		11.3 ± 3.3[e]
3	5.60 ± 0.26		24.58 ± 4.89		7.14 ± 0.11		15.8 ± 1.7
(*R*)-**5**	9.41 ± 0.25	3	2.3 ± 0.60	11	24.94 ± 0.10	1	7.1 ± 1.8
(*S*)-**5**	31.27 ± 6.69		25.47 ± 3.7		31.59 ± 3.49		nd
(*R*)-**6**	0.60 ± 0.07	30	18.3 ± 0.14	3	103.78 ± 29.80	1	113.2 ± 36.1
(*S*)-**6**	17.94 ± 0.48		50.11 ± 11.9		135.73 ± 74.70		nd
7	106.9 ± 49.9		147.4 ± 0.62		70.67 ± 23.80		nd
ivabradine	4.50 ± 0.44		4.52 ± 2.82		4.28 ± 0.40		nd
zatebradine	16.78 ± 0.9		5.45 ± 1.04		8.57 ± 1.85		13.4 ± 8.7[e]

[a] Mouse HCN channel expressed in HEK 293 cells. [b] Eudismic ratio (ER): ratio between the activity of the two enantiomers. [c] Human HCN channel expressed in HEK 293 cells. [d] Decrease of heart rate in spontaneously beating isolated guinea pig atria. Values represent the mean ± SEM of three to four experiments. nd: not determined. [e] From ref 22.

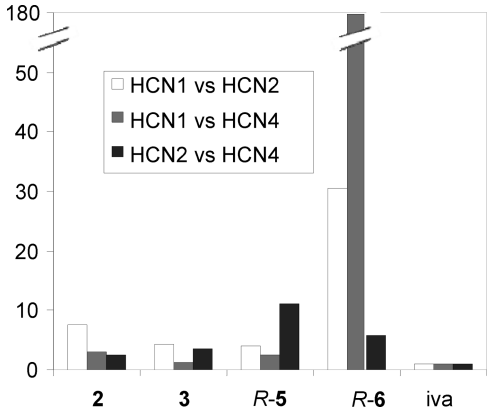


Figure 1. Ratio between the EC_{50} values on the three channel isoforms of selected compounds.

isoform (Figure 1). The study of the enantiomers of **5** and **6** shows that the interaction was enantioselective on those isoforms where the compounds showed higher potency, such as HCN2 for **5** (ER = 11) and HCN1 for **6** (ER = 30). In both cases, the *R* isomer is the eutomer. This is interesting, since the stereogenic center is located at the same position as in ivabradine, which has the *S* configuration. This enantiomer has been developed as drug because of its higher specificity for HCN channels with respect to other channels, since its *R*-isomer (R-60) also interacts with K^+ channels.[9,28,29]

Compounds **3**, (*R*)-**5**, and (*R*)-**6** were further evaluated on spontaneously beating guinea pig right atria to test their activity on a native tissue; the results are reported in Table 2. Zatebradine and compound **2** were used as a reference in this test. Although the distribution of HCN channel isoforms in guinea pig heart is not known, in the sinoatrial node of all the species so far examined the most abundant isoform is HCN4,[30] and the bradycardic effect of the compounds likely relies on their activity on this subtype. Indeed, **2** and **3** are almost equipotent with zatebradine in both their negative chronotropic activity and HCN4 blocking potency, while the poor activity of (*R*)-**6** on heart reflects its low potency on the HCN4 isoform, suggesting that the selectivity found in cellular test is maintained also in native tissues. The negative chronotropic potency of (*R*)-**5** is unexpectedly high if compared to its HCN4 blocking ability. In this regard, however, it must be mentioned that a recent report showed evidence that in mouse heart HCN2 protein coassembles with HCN4 to form functional heteromeric HCN channels.[31]

Selective compounds may be important tools to study the physiology and the stoichiometry of the channels in native tissues and to study the channel under pathological conditions. Moreover, they may be useful as drugs, providing they have high specificity for HCN with respect to other ion channels. For instance, a HCN1 selective blocker may be useful for the treatment of neuropathic pain,[14] while a HCN4 selective inhibitor can be useful to control sinus node rhythm and ventricular arrhythmia.[32] Our data show that it is possible to achieve selectivity, at least on the homomeric channels, by manipulating the chemical structure of phenylalkylamines related to zatebradine. Compounds **2**−**8** represents analogues with different steric and conformational characteristics, and some of them (**2**, (*R*)-**5**, and (*R*)-**6**) show a different preference for the homomeric HCN channel isoforms. Selectivity may be related to the ability of these compounds to adopt different conformations, to the presence of specific interactions due to the introduction of additional moieties, or to the different shape or volume of the molecules. Work is underway to derive sound structure−activity relationships in this class of compounds in order to optimize their potency and selectivity and to test them for their activity toward other ion currents.

Experimental Section

Chemistry: General Information. All melting points were taken on a Büchi apparatus and are uncorrected. NMR spectra were recorded on a Gemini 200 spectrometer (200 MHz for ^{1}H NMR, 50 MHz for ^{13}C) or on a Bruker Avance 400 spectrometer (400 MHz for ^{1}H NMR, 100 MHz for ^{13}C). Chromatographic separations were performed on a silica gel column by gravity chromatography (Kieselgel 40, 0.063−0.200 mm; Merck) or flash chromatography (Kieselgel 40, 0.040−0.063 mm; Merck). Yields are given after purification, unless differently stated. Where analyses are indicated by symbols, the analytical results are within 0.4% of the theoretical values. The synthesis of (*R*)-**6** is described below as representative. The syntheses of all other compounds and the NMR spectra of (*R*)-**6** are reported in the Supporting Information.

(*R*)-*N*,*N*-Bis[(*Z*)-4-(7,8-dimethoxy-2-oxo-1,3-dihydrobenzo-[*d*]azepin-3-yl)but-2-enyl]-2-(3,4-dimethoxyphenyl)propanamine [(*R*)-6]. To a solution of **10** (0.25 g, 0.82 mmol) in anhydrous CH_3CN (10 mL) were added anhydrous triethylamine (0.083 g, 0.82 mmol) and (*R*)-**11** (0.82 mmol, 0.16 g). The mixture was left stirring under nitrogen at room temperature overnight. Then the solvent was removed by rotary evaporation, and the residue was dissolved in dichloromethane and washed with 2 M NaOH (3 × 15 mL). The organic layers were collected, dried on Na_2SO_4, and the solvent was evaporated under vacuum. The residue was then purified by flash chromatography (dichloromethane/methanol/ammonia, 95/5/0.5). The first eluting compound is (*R*)-**6** (R_f = 0.71, 12% yield, mp 97−98 °C) The second eluting compound is (*R*)-**14** (R_f = 0.42, yellow oil, 8% yield). (*R*)-**6** (hydrochloride): $[\alpha]^{20}_D$ −22.25° (*c* 0.5, CH_3OH). Anal. ($C_{43}H_{52}ClN_3O_8$) C, H, N.

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Supporting Information Available: Synthetic procedures and biological methods. This material is available free of charge via the Internet at http://pubs.acs.org.

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