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Design, synthesis and preliminary biological evaluation of zatebradine analogues as potential blockers of the hyperpolarization-activated current

Maria Novella Romanelli,[a,*] Elisabetta Cerbai,[b] Silvia Dei,[a] Luca Guandalini,[a] Cecilia Martelli,[a] Elisabetta Martini,[a] Serena Scapecchi,[a] Elisabetta Teodori[a] and Alessandro Mugelli[b]

[a]*Dipartimento di Scienze Farmaceutiche, Università di Firenze, Via Ugo Schiff 6, 50019 Sesto Fiorentino (FI), Italy*
[b]*Dipartimento di Farmacologia Preclinica e Clinica, Università di Firenze, Viale Pieraccini 6, 50139 Firenze, Italy*

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Abstract—A series of zatebradine analogues, differing in the basic moiety and in the methylene spacer, have been synthesized; their negative chronotropic activity has been determined in guinea pig atria. The most active compounds have been studied for their blocking properties on the hyperpolarization-activated current I_f (which is one of the main currents underlying automatic activity in the sinus node) measured on ventricular myocytes of old spontaneously-hypertensive rats (SHR) by means of the patch-clamp technique. The majority of the substances were able to block I_f, with one of them (**15**) being slightly more potent than zatebradine. Surprisingly one analogue (**6**), while showing good negative chronotropic activity, was found to inhibit I_f only at high concentration and to markedly reduce outward currents, suggesting for this substance a different mechanism of action responsible for the negative chronotropic effect.

1. Introduction

Pacemaker activity of the sinoatrial node (SAN) in mammalian cells is regulated both by neurotransmitters, such as norepinephrine and acetylcholine, and by ion channels, among which the T- and L-type Ca^{2+} channels, K^+ channels and f-channels play a crucial role.[1] I_f (also termed I_h or I_q in various tissues) is a cation current which is carried via channels with mixed permeability for K^+ and Na^+, it is activated following membrane hyperpolarization, and it is modulated by cAMP through a direct action on the channel.[2,3] These channels belong to the HCN (hyperpolarization-activated cyclic-nucleotide gated) family; four different isoforms (HCN1–HCN4) have been cloned in mammalians, whose distribution varies, among species, according to several factors such as tissue, pathological conditions and age.[1,4–6]

I_f can be blocked by inorganic cations, such as Cs^+, and by organic substances, such as ZD7288[7,8] and zatebradine[9] (Chart 1), which belong to different chemical classes but interact with the same biological target. Zatebradine (UL-SF-49) is a specific bradycardic agent, which reduces heart rate without negative inotropic or hypotensive effects. The bradycardic effect of zatebradine has been attributed to a use-dependent inhibition of the hyperpolarization-activated current, I_f.[10–13] However, electrophysiological studies demonstrated that zatebradine prolongs action potential duration in spontaneously beating rabbit sinoatrial cells,[14] in guinea pig papillary muscle and in rabbit Purkinje fibres,[15,16] with a mechanism that likely involves K^+ channel blockade: as a matter of fact, it inhibits the hKv1.5 channel (K_D 1.8 μM), behaving as a class III antiarrhythmic drug.[17] Zatebradine has been withdrawn from clinical studies due to its adverse side effects on vision,[18] and recently it has been shown to exert inhibitory effects on retinal rod photoreceptors, a finding which has been attributed to the low selectivity of this substance.[19]

Ivabradine (S-16257, Chart 1)[20] is a zatebradine analogue which blocks the f-channel with less effect on

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* Corresponding author. Tel.: +39 055 4573691; fax: +39 055 4573671; e-mail: novella.romanelli@unifi.it

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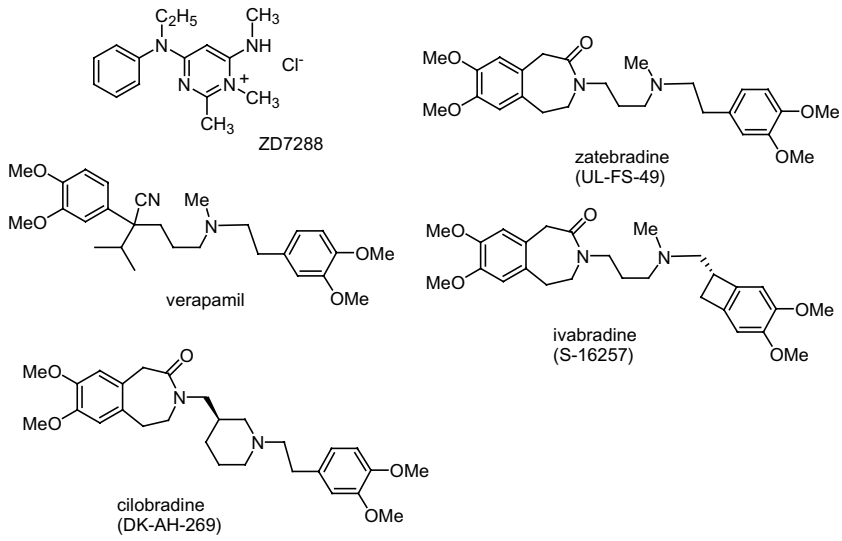


Chart 1.

action potential repolarization in isolated cardiac preparations.[15,21] Ivabradine has been reported to be less potent than zatebradine in blocking the hKv1.5 channel (K_D 29 μM)[22] and, therefore, seems safer than zatebradine. Ivabradine possesses a chiral centre in the *S* configuration; the stereoselectivity of its action has been characterized in vitro and in vivo,[23,24] as well as its interaction with f-channels in rabbit SAN.[25] Another chiral analogue, DK-AH-269 (cilobradine), the *R*(−) enantiomer of the racemate DK-AH-3, is reported to block I_f in heart and in cultured mouse dorsal root ganglion (DRG) neurons more potently than zatebradine,[26] and it has been recently patented for pain treatment and myocardial hypertrophy.[27,28]

The aim of this work was to find zatebradine analogues, able to reduce heart rate through a blockade of HCN channel, but with a safer pharmacological profile with respect to the lead. Only a few reports have appeared in the literature on zatebradine analogues:[9,29–31] the modifications made on the lead structure were mainly in the homoveratrylamino moiety, that is shortening or lengthening of the methylene chain with introduction of heteroatoms,[29,30] introduction of different substituents on the aromatic group[9] or replacement of this ring with different heterocycles,[29] modification of the *N*-methyl group.[9] Changes on the other side of the molecule led to the 7,8-dimethoxybenzazepinone ring as the optimal group, and only small modifications of the trimethylene chain have been reported.[9] Recently, the bradycardic activity of some analogues in which the benzazepinone ring has been replaced with a tetrahydroisoquinoline moiety has also been disclosed.[32] Despite the high number of synthesized analogues, for only a few of them the activity on HCN channel has been reported.

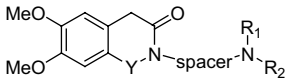
In a series of papers produced over the past decade, we have shown that modifications of the structure of the well-known calcium antagonist and multidrug resistance (MDR) modulator verapamil can change its cardiovascular and MDR-modulating pharmacological profile.[33–39] We modified the substituent on the nitrogen atom and the conformational freedom of the molecule, obtaining some dissociation between negative chronotropic and inotropic or vasorelaxant activity. Moreover, we were also able to obtain a clear-cut dissociation of calcium antagonism and MDR inhibitory activities. Taking advantage of the experience acquired in this research, we have decided to make similar molecular modulation on the molecule of zatebradine, which is structurally related to verapamil. As a consequence, we have designed several derivatives of zatebradine where the conformational freedom of the molecule has been reduced by introducing double and triple bonds, or where the homoveratryl moiety has been replaced by different groups, such as adamantyl, 9-fluorenyl, 5,6-dimethoxyindanyl or 6,7-dimethoxytetrahydroisoquinoline, which in the previous work on verapamil had led to compounds showing an increase in the negative chronotropic activity with respect to the negative inotropic and vasorelaxant effects. Moreover, since it is known that the dehydro- and the desmethyl analogues of zatebradine show potency similar to that of the parent compound,[9,30] we have applied some of our modifications also to these series (see structures in Table 1). This paper reports the synthesis and preliminary pharmacological evaluation of these derivatives.

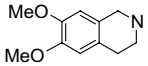
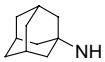
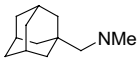
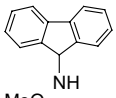
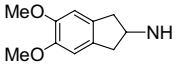
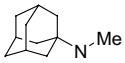
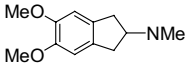
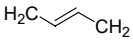
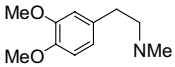
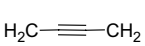
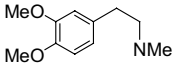
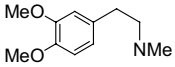
2. Chemistry

Compounds **2**–**10** were prepared according to Scheme 1. The halogenated derivatives **1a**,[9] **1b**[29] or **1c**[30] were reacted with the suitable amine to give compounds **2**, **3**, **6**–**8**. Compounds **4** and **5** were obtained through catalytic hydrogenation of the corresponding unsaturated amines **2** and **3**, respectively; methylation of compounds **5** and **8** with formaldehyde and formic acid gave compounds **9** and **10**, respectively.

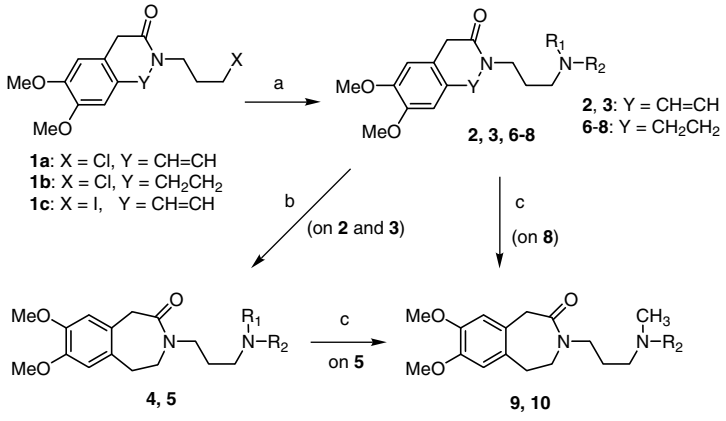
Compounds **15**–**17** were prepared according to Scheme 2: 6,7-dimethoxy-1,3-dihydro-2*H*-3-benzazepin-2-one

Table 1. Chemical structure and negative chronotropic activity of the synthesized compounds



N	Y	Spacer	NR_1R_2	EC_{50} (μM)	Heart rate (% over control at 10 μM)
2	CH=CH	$(CH_2)_3$		30.0 ± 3.0	65.5 ± 6.3
4	CH_2CH_2	$(CH_2)_3$		220 ± 90	78.8 ± 9.2
3	CH=CH	$(CH_2)_3$		45.0 ± 25.6	68.4 ± 6.6
5	CH_2CH_2	$(CH_2)_3$		130 ± 40	93.0 ± 7.1
6[a]	CH_2CH_2	$(CH_2)_3$		23.0 ± 17.9	54.7 ± 12.5
7	CH_2CH_2	$(CH_2)_3$		100 ± 30	75.3 ± 12.4
8	CH_2CH_2	$(CH_2)_3$		129 ± 79	92.0 ± 1.6
9	CH_2CH_2	$(CH_2)_3$		129 ± 31	91.7 ± 3.9
10	CH_2CH_2	$(CH_2)_3$		20.6 ± 18.7	59.7 ± 14.9
15	CH=CH			11.3 ± 3.3	49.6 ± 9.1
16	CH_2CH_2			149 ± 39	81.7 ± 11.9
17	CH_2CH_2			59.5 ± 20.7	87.4 ± 3.8
Zatebradine[a]	CH_2CH_2	$(CH_2)_3$		13.4 ± 8.7	44.6 ± 9.2

Numbers are mean ± SEM of four experiments.
[a] $p < 0.05$ (EC_{50} and % heart rate).



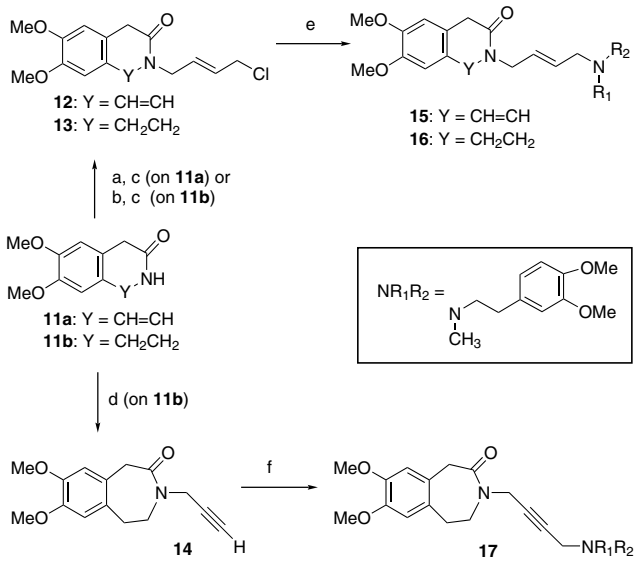
Scheme 1. Reagents and conditions: (a) HNR_1R_2; (b) H_2/Pd/C; (c) CH_2O, HCOOH. For the meaning of R_1 and R_2 see Table 1.

11a[9] and its tetrahydroderivative **11b**[29] were reacted with *trans* 1,4-dichlorobutene, obtaining compounds **12** and **13**, respectively, while reaction of **11b** with propargyl bromide gave compound **14**. Treatment of **12** and **13** with *N*-methylhomoveratrylamine gave the final compounds **15** and **16**. Compound **17** was obtained through Mannich reaction of the propyne derivative **14**.

3. Pharmacological studies

3.1. Bradycardic effect

The synthesized compounds were tested on guinea pig spontaneously beating atria to evaluate their negative chronotropic activity. Compounds were tested at



Scheme 2. (a) *t*-BuOK; (b) NaH; (c) 1,4-dichlorobutene; (d) NaH, propargyl bromide; (e) *N*-methylhomoveratrylamine; (f) HCHO, *N*-methylhomoveratrylamine.

increasing doses (10^{-9}–10^{-4} M) to measure the decrease in atrial rate (see Section 5.2.2. for details). The potency of the drug is defined as EC_{50} and it was evaluated by fitting of the concentration–effect curve with the Hill equation.

Table 1 summarizes data on the negative chronotropic potency of all derivatives. All the synthesized compounds, when used at 10^{-5} M, were able to reduce the heart rate in guinea pig spontaneously-beating isolated atria, although the extent of heart rate reduction was different. A direct comparison of three derivatives, among those with the lowest EC_{50}, is plotted in Figure 1. Values have been normalized with respect to controls: the concentration–response curves for **2**, **6** and **15** lie

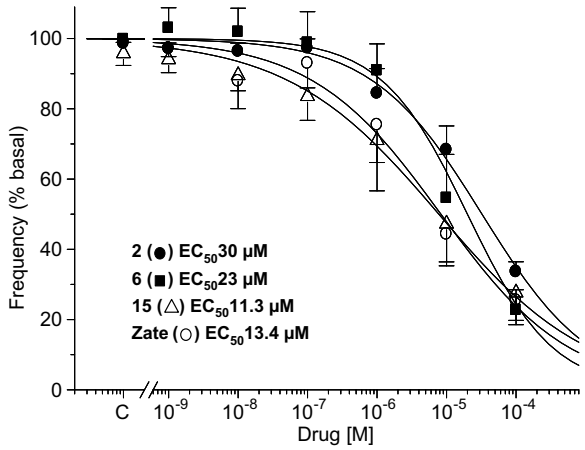


Figure 1. Concentration–response curves for the negative chronotropic activity of compounds **2**, **6**, **15** compared with zatebradine. Points represent mean ± SEM of four experiments.

near that obtained with the reference compound zatebradine, **15** being the most active one.

3.2. Effect on I_f

To verify that the bradycardic effect of derivatives was due to inhibition of the hyperpolarization-activated current I_f, we performed patch-clamp experiments on isolated ventricular cardiomyocytes of old spontaneously-hypertensive rats. Previous data from some of us demonstrated that these myocytes re-express I_f, whose biophysical and pharmacological properties closely resemble those reported for the sinoatrial node f-current.[5,6] Selected compounds (zatebradine, **2**, **4**–**7** and **15)** were tested at a 10^{-5} M concentration to evaluate the reduction of the current amplitude.

As shown in Figure 2A, superfusing with 10^{-6} M zatebradine progressively reduced the amplitude of the f-current

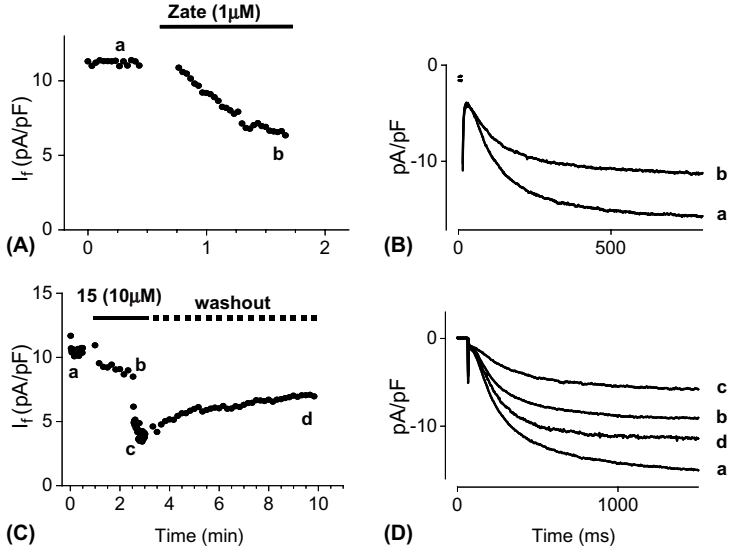


Figure 2. Effect of zatebradine (panels A, B) and compound **15** (panels C, D) on I_f. Panels B and D show the current recordings corresponding to different moments (a–d) during the experiment.

elicited by steps to −140 mV (see traces in Fig. 2B). At the concentrations of 10^{-6} M and 10^{-5} M, zatebradine reduced I_f to 72.7 ± 6.6% ($n = 6$) and 41.7 ± 11.7% ($n = 4$), respectively.

Compounds **2**, **4**, **5**, **7** and **15** show similar features to zatebradine. At 10^{-5} M, a concentration close to the EC_{50} measured for the bradycardic effect of these compounds, analogues **2**, **4**, **5**, **7** and **15** reduced I_f amplitude within 1 min of stimulation at 1 Hz, an effect which was partially reversed upon washout. Figure 2C shows the reduction of f-current by **15** (10^{-6} M). From this figure it is possible to see that, as expected,[10–12] this effect was markedly use-dependent: in fact, the f-current was minimally affected when pulses were applied at low frequency (0.1 Hz, trace b, Fig. 2D), but the blocking effect developed rapidly when the frequency was increased to 1 Hz (trace c, Fig. 2D). Again, washing out the compound partially reversed the effect, thus suggesting that the reduction of I_f was specifically due to the presence of the drug and not to current run-down.

Conversely, **6** at the same concentration did not affect I_f at either low or high stimulation frequency (Fig. 3A): in fact, an effect was observed only at 10-times higher concentration (data not shown). Panel B shows that, in the same cell, **6** caused a clear-cut reduction of outward currents, thus suggesting that mechanisms other than I_f reduction can account for the bradycardic effect of this substance. The percentage reduction of the f-current by the derivatives, tested with patch-clamp protocols in single SHR myocytes, is summarized in Figure 4: all

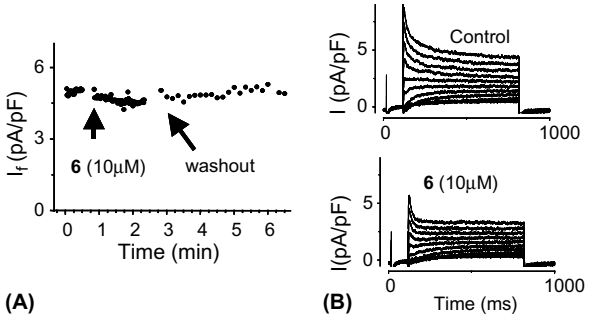


Figure 3. Effect of compound **6** at 10^{-5} M on I_f (A) and on outward currents (B).

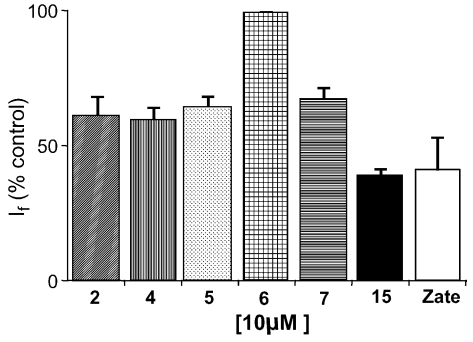


Figure 4. Effect of the tested compounds on I_f. Each column represents the mean ± SEM amplitude of I_f in the presence of the tested drug, normalized with respect to the pre-drug amplitude.

but one compound (**6**) reduced f-current, thus suggesting that the bradycardic effect observed in beating atria was due, at least in part, to the blockade of the hyperpolarization-activated current.

4. Discussion

All the compounds synthesized for this study were tested first for their negative chronotropic activity on guinea pig spontaneously-beating isolated atria; the absence of bradycardic activity can reasonably rule out a possible interaction with HCN channels. We reasoned that, before testing the compounds on I_f, a screening test was necessary in order to avoid expensive and time-consuming electrophysiological experiments on inactive substances. It was found that all compounds could lower heart rate, although with different potency, as shown by their EC_{50} values. The reduction of the conformational flexibility of the phenylethyl moiety into an indane ring (compound **10**) maintains a negative chronotropic activity similar to zatebradine, while the incorporation of the basic part into a tetrahydroisoquinoline ring (compound **4**) reduces the activity by one order of magnitude. The introduction of bulky lipophilic groups such as the fluorene or the adamantane rings gives substances whose potency is 7-fold (compound **7**) and 10-fold (compounds **5** and **9**) lower than the lead. Insertion of a methylene unit between the basic nitrogen and the adamantane ring (compound **6**) restores the negative chronotropic activity, as **6** is only twice less potent than zatebradine. Methylation of secondary amine does not have an appreciable effect for the adamantane derivatives (compounds **5** and **9**), while it increases the activity of the indane derivatives (compound **8** compared to **10**).

Introduction of a double bond into the benzazepinone ring brings an increase in potency; in fact, compounds **2**, **3** and **15** are 7-, 3- and 13-times more potent than their hydrogenated analogues (compounds **4**, **5** and **16**, respectively). With respect to zatebradine, compounds **2** and **3** are only 2-to 3-times less potent, while compound **15** is slightly more potent than the lead.

A few synthesized substances were then tested on ventricular cardiomyocytes of old spontaneously-hypertensive rats (SHR) to see if they could interact with the HCN channel. In fact, negative chronotropic activity may be due to several different mechanisms, including calcium antagonism and K^+-channel blockade, in addition to I_f blockade. Compounds **2**, **4**, **5**, **7** and **15** were able to block the ventricular f-current at a concentration of 10^{-5} M, a concentration close to the EC_{50} values; compound **15**, which is more potent than zatebradine as bradycardic agent, was the most active among the tested substances also in this test, being able to reduce I_f to about 40% of the control value.

With the patch-clamp approach, the effect of structural changes seems different with respect to negative chronotropic activity: in fact, compounds **2**, **4**, **5** and **7** are almost equiactive on I_f, while they show a different rank order of potency on isolated atria. This may suggest that the

negative chronotropic activity of these substances is due to a combination of different mechanisms, among which the HCN channel blockade plays an important role: indeed, also the bradycardic activity of zatebradine results from different mechanisms of action. Another possible explanation may be that compounds interact with different selectivity with the HCN isoforms expressed in the SAN tissue, which contains both HCN1 and HCN4, and those found in ventricular hypertrophied myocytes of old SHR, where the predominant isoform is HCN2.[40] More work is obviously necessary to clarify this point.

Surprisingly, compound **6**, while being one of the most potent substances as far as the negative chronotropic activity is concerned, was able to block the f-current only at a concentration 10-times higher than the other compounds tested. This means that small structural changes in the molecule can target the interaction towards different channels. Compounds **5** and **6** are close structural analogues, with **6** carrying a slightly longer substituent on the nitrogen. Their different behaviour seems to suggest that the volume of the substituent on the nitrogen greatly affects the interaction with the ventricular HCN channel, implying that on this protein the binding site has a limited space for accommodating bulky ligands. In fact, it was possible to show that the negative chronotropic activity of **6** was independent from I_f blockade, and possibly due to the marked reduction of outward K^+ current (Fig. 3B), suggesting an interaction with a voltage-dependent K^+ channel. These findings indicate that the interaction with the two channels seems to have different structural requirements, and therefore, it should be possible to achieve a better selectivity of action for zatebradine by suitably modifying the basic part of the molecule, as is suggested also by the pharmacological profile of ivabradine, discussed in the introduction.

Work is underway to clarify the structural requirements for interaction with these molecular targets, and to better characterize the pharmacological profile of these substances.

5. Experimental

5.1. Chemistry

5.1.1. General considerations. All melting points were taken on a Büchi apparatus and are uncorrected. Infrared spectra were recorded with a Perkin–Elmer 681 spectrophotometer in a Nujol mull for solids and neat for liquids. Unless otherwise stated, NMR spectra were recorded on a Gemini 200 spectrometer, at 200 MHz for ^{1}H and 50 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 otherwise stated. Where analyses are indicated by symbols, the analytical results are within ±0.4% of the theoretical values.

Amines were either commercially available (1-aminoadamantane, 9-aminofluorene, 6,7-dimethoxytetrahydroisoquinoline, *N*-methylhomoveratrylamine) or prepared through literature procedures (5,6-dimethoxy-2-aminoindane[41] and *N*-methylaminomethyladamantane[42]).

5.1.2. General procedure for the synthesis of amines 2, 3, 6–8, 15, 16. A mixture of the alkyl halide (1 equiv) and the amine (2 equiv) in the suitable solvent was heated for 3–48 h; after cooling at room temperature, it was diluted with $CHCl_3$ and treated with water. The organic phase was collected, anhydrified and the solvent was removed under vacuum, leaving a residue which was purified by column chromatography. The synthetic details are reported in Table 2, while the ^{1}H NMR spectra are shown in Table 3. Compounds were transformed into the oxalate salt using 1 equiv of oxalic acid in ethyl acetate.

5.1.3. Catalytic hydrogenation. The amine (0.2–0.4 g) was dissolved in acetic acid (20 mL), Pd/C (0.05–0.1 g) was added and the mixture was hydrogenated in a Parr apparatus for 24 h at 75 psi. After filtration and removal of solvent, the residue was dissolved in a saturated solution of $NaHCO_3$ and extracted with $CHCl_3$. After anhydrification, the solvent was removed under vacuum leaving a residue which was transformed into the oxalate salt using 1 equiv of oxalic acid in ethyl acetate (see Table 3 for the ^{1}H NMR spectra of the free bases).

Compound **4**: 67% yield. The oxalate melted at 111–114 °C. Anal. ($C_{28}H_{36}N_2O_9$) C, H, N.

Compound **5**: 51% yield. The oxalate melted at 178–181 °C. Anal. ($C_{27}H_{38}N_2O_7$) C, H, N.

Table 2. Reaction details for the synthesis of compounds **2**, **3**, **6**–**8**, **15**, **16**

N	Alkyl halide	Solvent	Time (h)	T (°C)	Eluent[a]	Yields (%)	Mp[b](°C)	Anal.[b]
2	**1a**	Et_3N	3	80	A	80	140–141	$C_{28}H_{34}N_2O_9$
3	**1c**	CH_3CN	4	25	B	33	214–216	$C_{27}H_{36}N_2O_7$
6	**1b**	Et_3N	48	80	C	50	155–157	$C_{29}H_{42}N_2O_7$
7	**1b**	None	6	100	D	25	175–180	$C_{30}H_{32}N_2O_7$
8	**1b**	Et_3N	6	80	E	28	203–205	$C_{28}H_{36}N_2O_9$
15	**12a**	Et_3N	5	60	E	53	139–141	$C_{29}H_{36}N_2O_9$
16	**12b**	Et_3N	7	60	E	40	115–118	$C_{29}H_{38}N_2O_9$

[a] A, $CHCl_3$/MeOH 95:5; B, CH_2Cl_2/petroleum ether/absolute ethanol/ammonium hydroxide 150:50:25:4; C, $CHCl_3$/petroleum ether/absolute ethanol/ammonium hydroxide 340:60:65:8; D, $CHCl_3$/MeOH 97:3; E, $CHCl_3$/MeOH 90:10.

[b] Melting points and analyses are referred to the oxalate salts.

Table 3. ^{1}H NMR data for the final compounds prepared through general procedures

N	^{1}H NMR ($CDCl_3$) ppm
2	1.70–1.90 (m, 2H, C–CH_2–C); 2.40 (t, 2H, CH_2); 2.60 (t, 2H, CH_2); 2.75 (t, 2H, CH_2); 3.40 (s, 2H, CH_2–CO); 3.45 (s, 2H, Ar–CH_2–N); 3.62 (t, 2H, CH_2–N–CO); 3.82 (s, 3H), 3.83 (s, 3H), 3.86 (s, 3H) and 3.90 (s, 3H) (4 × OCH_3); 6.25 (dd, 2H, CH=CH); 6.48 (s, 1H) and 6.56 (s, 1H) (tetrahydroisoquinoline aromatic protons); 6.71 (s, 1H) and 6.78 (s, 1H) (benzazepinone aromatic protons)
3	1.35–1.74 (m, 14H, CH_2 adam. + C–CH_2–C); 1.85–2.00 (br s, 4H, NH + CH adam.); 2.38 (t, 2H, CH_2); 3.44 (s, 2H, CH_2–CO); 3.72 (t, 2H, CH_2–N–CO); 3.90 (s, 6H, 2 × OCH_3); 6.13 (d, 1H) and 6.32 (d, 1H) (CH=CH); 6.68 (s, 1H) and 6.76 (s, 1H) (aromatic protons)
4	1.75–1.95 (m, 2H, C–CH_2–C); 2.41–2.54 (m, 2H, CH_2); 2.59–2.72 (m, 2H, CH_2); 2.72–2.83 (m, 2H, CH_2); 3.05 (t, 2H, CH_2–N–CO); 3.40–3.52 (m, 4H, N–CH_2–Ar + CH_2–CO); 3.65–3.75 (t, 4H, Ar–CH_2–CH_2–N); 3.80 (s, 12H, 4 × OCH_3); 6.40–6.60 (m, 4H, aromatic protons)
5	1.45–1.75 (m, 14H, adam. CH_2 + C–CH_2–C); 1.90–2.10 (br s, 4H, NH + adam. CH); 2.50 (t, 2H, CH_2); 3.00 (t, 2H, CH_2–N–CO); 3.45 (t, 2H, CH_2); 3.68 (t, 2H, CH_2); 3.75 (s, 2H, CH_2–CO); 3.80 (s, 6H, OCH_3); 6.51 (s. 1H) and 6.58 (s, 1H) (aromatic protons)
6	1.40–1.78 (m, 14H, adam. CH_2 + C–CH_2–C); 1.85–2.00 (m, 5H, adam. N–CH_2 + adam. CH); 2.20 (s, 3H, NCH_3); 2.30–2.40 (m, 2H, CH_2); 2.98–3.10 (t, 2H, CH_2–NCO); 3.39–3.49 (m, 2H, CH_2); 3.66–3.76 (m, 2H, CH_2); 3.80 (s, 2H, CH_2–CO); 3.82 (s, 6H, 2OCH_3); 6.52 (s, 1H) and 6.61 (s, 1H) (aromatic protons)
7	1.55–1.70 (m, 2H, C–CH_2–C); 2.25 (t, 2H, CH_2); 2.51–2.70 (br s, 1H, NH); 2.95 (t, 2H, CH_2); 3.41–3.65 (m, 4H, 2 CH_2); 3.80 (s, 6H, 2 × OCH_3); 3.86 (s, 2H, CH_2–CO); 4.95 (s, 1H, NCH); 6.49 (s, 1H) and 6.61 (s, 1H) (benzazepinone aromatic protons); 7.20–7.40 (m, 4H) and 7.50–7.70 (m, 4H) (fluorene aromatic protons)
8	1.98–2.12 (m, 3H, C–CH_2–C + NH); 2.65–2.95 (m, 4H, 2 × CH_2); 3.02–3.20 (m, 4H, 2 × CH_2); 3.50–3.60 (t, 2H, CH_2); 3.65–3.85 (m, 3H, CH_2 + CH–N); 3.82 (s, 2H, CH_2CO); 3.85 (s, 12H, 4 × OCH_3); 6.58 (s, 2H) and 6.68 (s, 2H) (aromatic protons)
9	1.40–1.80 (m, 14H, adam. CH_2 + C–CH_2–C); 1.92–2.10 (m, 3H, adam. CH); 2.19 (s, 3H, NCH_3); 2.30–2.45 (m, 2H, CH_2); 3.01–3.10 (m, 2H, CH_2–N–CO); 3.39–3.51 (m, 2H, CH_2); 3.68–3.78 (m, 2H, CH_2); 3.79 (s, 2H, CH_2–CO); 3.82 (s, 6H, 2 × OCH_3); 6.52 (s, 1H) and 6.61 (s, 1H) (aromatic protons)
10	1.75–1.90 (m, 2H, C–CH_2–C); 2.25 (s, 3H, NCH_3); 2.38–2.52 (m, 2H, CH_2); 2.67–2.96 (m, 4H, 2 × CH_2); 3.00–3.15 (m, 2H, CH_2–N–CO); 3.20–3.35 (m, 1H, N–CH); 3.41–3.52 (m, 2H, CH_2); 3.70–3.85 (m, 4H, CH_2–CO + CH_2); 3.85 (s, 12H, 4 × OCH_3); 6.58 (s, 1H), 6.62 (s, 1H) and 6.68 (s, 2H) (aromatic protons)
15	2.20 (s, 3H, NCH_3); 2.46–2.75 (m, 4H, CH_2CH_2); 2.92–3.03 (m, 2H, =CCH_2N); 3.39 (s, 2H, CH_2CO); 3.81 (s, 6H) and 3.86 (s, 6H) (4OCH_3); 4.06–4.20 (m, 2H, $CONCH_2C$=); 5.23–5.66 (m, 2H, butene CH=CH); 6.14 (d, 1H) and 6.27 (d, 1H) (Ar–CH=CH–N); 6.63–6.80 (m, 5H, aromatic protons)
16	2.30 (s, 3H, NCH_3); 2.59–2.75 (m, 4H, CH_2CH_2); 2.96–3.04 (m, 2H, $ArCH_2$–CH_2N); 3.07 (d, 2H, =CCH_2N); 3.60–3.69 (m, 2H, $ArCH_2$–CH_2N); 3.83 (s), 3.84 (s), 3.85 (s) and 3.88 (s) (14H, CH_2CO + 4 × OCH_3); 4.05 (d, 2H, $CONCH_2C$=); 5.53–5.68 (m, 2H, butene CH=CH); 6.53 (s, 1H) and 6.61 (s, 1H) (benzazepinone aromatic protons); 6.69–6.81 (m, 3H, aromatic protons)

5.1.4. Methylation of secondary amines. A mixture of the secondary amine (0.10–0.15 g) in ethanol (2–4 mL), formic acid (1.5–2.0 mL) and formaldehyde (40% water solution, 0.8–1.0 mL) was kept under reflux for 4–8 h; it was then concentrated under vacuum, treated with a saturated solution of $NaHCO_3$ and extracted with chloroform. After anhydrification and removal of solvent, the residue was purified (when necessary) by flash chromatography and transformed into the oxalate salt by treatment with 1 equiv of oxalic acid in ethyl acetate (see Table 3 for the ^{1}H NMR spectra of the free bases).

Compound **9**: 64% yield. The oxalate melted at 167–170 °C. Anal. ($C_{28}H_{40}N_2O_7$) C, H, N.

Compound **10**: 78% yield. The oxalate melted at 189–192 °C. Anal. ($C_{29}H_{38}N_2O_9$) C, H, N.

5.1.5. *trans* 3-(4-Chloro-2-butenyl)-7,8-dimethoxy-1,3-dihydro-2*H*-benzo[*d*]azepin-2-one (12). To a suspension of **11a**[9] (0.5 g, 2.3 mmol) in anhydrous DMSO (10 mL), *t*-BuOK (2.3 mmol) and 0.25 mL (1 equiv) of *trans*-1,4-dichloro-2-butene were added, and the resulting brown solution was left stirring at room temperature for 1 h. The mixture was poured into ice and extracted with CH_2Cl_2. After anhydrification and removal of the solvent, the residue was purified by column chromatography (ethyl acetate/cyclohexane 50:50) obtaining the title compound in 48% yield. ^{1}H NMR ($CDCl_3$) δ 3.48 (s, 2H, CH_2CO); 3.89 (s, 3H, OCH_3); 3.91 (s, 3H, OCH_3); 4.03 (d, 2H, *J* 6.4 Hz, CH_2Cl); 4.15–4.23 (m, 2H, CH_2N); 5.32–5.80 (m, 2H, butene CH=CH); 6.20 (d, 1H, *J* 9.7 Hz) and 6.40 (d, 1H, *J* 9.7 Hz) (benzazepinone CH=CH); 6.75 (s, 1H) and 6.81 (s, 1H) (aromatic protons) ppm. ^{13}C NMR 43.50 (t), 44.55 (t), 48.78 (t), 56.39 (q), 109.87 (d), 111.56 (d), 117.79 (d), 124.95 (s), 126.66 (s), 127.97 (d), 128.92 (d), 129.68 (d), 148.38 (s), 150.24 (s), 167.92 (s) ppm. Anal. ($C_{16}H_{18}ClNO_3$) C, H, N.

5.1.6. *trans* 3-(4-Chloro-2-butenyl)-7,8-dimethoxy-1,3,4,5-tetrahydro-2*H*-benzo[*d*]azepin-2-one (13). A mixture of **11b**[29] (0.43 g, 1.9 mmol), *trans*-1,4-dichloro-2-butene (0.41 mL, 2 equiv) and 0.08 g of NaH (60% dispersion in mineral oil) in anhydrous toluene (10 mL) was heated at 110 °C for 7 h. After cooling, ice was added and the organic phase was separated and collected. After anhydrification, the solvent was removed under vacuum and the residue was purified by column chromatography. The title compound was obtained in 10% yield. ^{1}H NMR ($CDCl_3$) δ 2.95–3.05 (m, 2H, CH_2–CH_2–N); 3.64–3.70 (m, 2H, CH_2–CH_2–N); 3.81 (s, 8H, CH_2CO + 2OCH_3); 4.01–4.08 (m, 4H, NCH_2–C=C–CH_2Cl); 5.52–5.82 (m, 2H, CH=CH); 6.55 (s, 1H) and 6.59 (s, 1H) (aromatic protons) ppm. ^{13}C NMR δ 32.57 (t), 42.50 (t), 44.63 (t), 46.37 (t), 47.99 (t), 56.33 (q), 113.51 (d), 114.33 (d), 123.71 (s), 127.86 (s), 129.15 (d), 130.48 (d), 147.58 (s), 148.27 (s), 172.20 (s) ppm. Anal. ($C_{16}H_{20}ClNO_3$) C, H, N.

5.1.7. 7,8-Dimethoxy-3-(3-propinyl)-1,3,4,5-tetrahydro-2*H*-benzo[*d*]azepin-2-one (14). Following the same procedure as for **13**, starting from 0.15 g (0.68 mmol) of **11b**, 0.10 g (0.84 mmol) of propargyl bromide and 0.03 g (1.1 equiv) of NaH (60% dispersion in mineral oil), compound **14** was obtained after purification by column chromatography (cyclohexane/ethyl acetate 3:7) in 17% yield. ^{1}H NMR ($CDCl_3$) δ 2.23 (t, 1H, J 2.2 Hz, ≡CH); 3.09–3.15 (m, 2H, CH_2–CH_2–N); 3.83 (s, 8H, $CH_2CO + 2 \times OCH_3$); 3.78–391 (m, 2H, CH_2–CH_2–N); 4.28 (d, 2H, J 2.2 Hz, NCH_2C≡); 6.58 (s, 1H) and 6.60 (s, 1H) (aromatic protons) ppm. ^{13}C NMR δ 32.28 (t), 35.48 (t), 42.60 (t), 46.01 (t), 56.26 (q), 72.43 (d), 79.40 (s), 113.42 (d), 114.18 (d), 123.29 (s), 128.10 (s), 147.40 (s), 148.16 (s), 171.84 (s) ppm. Anal. ($C_{15}H_{17}NO_3$) C, H, N.

5.1.8. 3-[[4-[2-(3,4-Dimethoxyphenyl)ethyl]methylamino]-2-butinyl]-7,8-dimethoxy-1,3,4,5-tetrahydro-2*H*-benzo[*d*]-azepin-2-one (17). To a solution of **14** (0.1 g, 0.39 mmol) in 96% ethanol (4 mL), formaldehyde (0.04 mL), water (4 mL), *N*-methylhomoveratrylamine (0.10 g, 0.5 mmol) and $CuSO_4$ (0.01 g) were added and the pH was adjusted to 8 with 3 M H_2SO_4. The mixture was heated at 80 °C for 26 h, then it was made alkaline with NH_4OH and extracted with ether. After anhydrification and removal of the solvent, the residue was purified by column chromatography (abs ethanol/CH_2Cl_2/pet. ether/NH_4OH 25:150:50:4). IR (neat): 2260 cm^{-1} (C≡C). ^{1}H NMR ($CDCl_3$) δ 2.35 (s, 3H, NCH_3); 2.59–2.81 (m, 4H, CH_2CH_2); 3.06–3.14 (m, 2H, $ArCH_2$–CH_2N); 3.39 (s, 2H, ≡CCH_2N); 3.73–3.96 (m, 16H, ArCH_2–CH_2N + $CH_2CO + 4 \times OCH_3$); 4.32 (s, 2H, CON-CH_2C≡); 6.55 (s, 1H) and 6.60 (s, 1H) (benzazepinone aromatic protons); 6.68–6.82 (m, 3H, aromatic protons). ^{13}C NMR δ 32.41 (t), 34.19 (t), 35.79 (t), 42.31 (q), 42.80 (t), 45.90 (t), 46.30 (t), 56.30 (q), 58.13 (t) 78.79 (s), 80.61 (s), 111.60 (d), 112.33 (d), 113.42 (d), 114.24 (d), 120.79 (d), 123.47 (s), 127.99 (s), 132.93 (s), 147.51 (s), 147.69 (s), 148.22 (s), 149.13 (s), 171.75 (s) ppm. The title compound was obtained in 57% yield and transformed into the oxalate salt, which melted at 100–102 °C (dec). Anal. ($C_{29}H_{36}N_2O_9$) C, H, N.

5.2. Pharmacology

5.2.1. Animals. This investigation conforms to the Guide for the Care and Use of Laboratory Animals published by the US National Institutes of Health (NIH Publication No. 85–23, revised 1996). The chronotropic and electrophysiological effects were studied, respectively, in spontaneously-beating atria of male guinea pig hearts (Pampaloni, Italy) and in single ventricular myocytes isolated from the heart of 18-month-old male spontaneously-hypertensive rats (SHR; Charles River, Italy).

5.2.2. Negative chronotropic activity. Guinea pigs were anesthetized with ether and killed. The heart was rapidly removed, the atria separated from ventricles and vertically mounted in a 50 mL chamber containing oxygenated (95% O_2, 5% CO_2) Tyrode's solution at 30 °C (see Solutions).

The atrial contraction was measured by an isometric transducer (mod. 83/F Narishige, Japan), amplified (Battaglia Rangoni, Italy), visualized on an oscilloscope (Tektronix TDS 210) digitized by a DAC/ADC converter (Digidata 1200B, Axon Inst., USA) and stored on a PC using a dedicated software (pClamp vers-6.0, Axon Inst., USA) for off-line analysis.

After 1 h for stabilization of atrial rate and twitch, a cumulative dose–response curve was obtained from each preparation by stepwise increasing the concentration of the tested drug (from 10^{-9} to 10^{-4} M) in 20-min intervals.

5.2.3. Patch-clamp recordings. Single left ventricular myocytes were isolated from SHR using a protocol based on previously described procedures.[5] After anesthetizing the rat with ether and killing it, the heart was rapidly excised, mounted in a Langendorff apparatus and perfused for 20 min with a low-calcium solution (LCS) prewarmed to 37 °C and equilibrated with 100% O_2. The solution was then quickly changed to LCS plus 1 mg/mL collagenase (Type I, Worthington, USA), 0.03 mg/mL dispase (Boehringer, Italy), 1 mg/mL albumin (Fatty Acid Free Fraction V, Sigma, Italy) for 20 min. The left ventricle and the septum were removed with fine scissors, chunked and the pieces were stirred in the LCS. Cardiomyocytes that appeared in the supernatant were purified by gravity sedimentation, collected and stored in Tyrode's solution, supplemented with 0.5 mM $CaCl_2$ and 4% penicillin/streptomycin (Gibco BRL, Italy), at room temperature and used within the day.

The experimental set-up for patch-clamp (whole-cell) recording and data acquisition was similar to that described previously.[5,6] The patch-clamped cell was superfused by means of a temperature-controlled microsuperfusor, which allowed rapid changes of the solution bathing the cell, with normal or modified Tyrode's solutions (see Solutions). Temperature was maintained in the range of 36 ± 0.5 °C. Patch-clamp pipettes had a resistance of 1.5–2.5 MOhm when filled with the internal solution (see Solutions).

The presence of I_f (i.e., of a hyperpolarization-activated, time-dependent inward current blocked by addition of 4 mM CsCl) was assessed by application of a hyperpolarizing step to −120 from a holding potential of −40 mV. Current–voltage relations were generated by use of clamping from a holding potential of −40 to more negative voltages (−60 to −140 mV) in 10 mV increments. To investigate whether I_f was blocked in a use-dependent manner by the drug, current was evoked by a series of 30 consecutive hyperpolarizing steps, applied at a frequency of 1 Hz. I_f amplitude was measured as the difference between the steady-state current and the peak current, measured at the end and the beginning of the hyperpolarizing step, respectively, as previously described in detail.[5]

Outward potassium currents were measured in normal Tyrode's solution, containing 0.5 mM $CdCl_2$ to block L-type calcium current, by applying depolarizing steps to −40/+70 mV from a holding potential of −70 mV.

5.2.4. Solutions. The composition of solutions used was the following (in mmol): Tyrode's solution for spontaneously-beating atria: NaCl 137, KCl 2.7, $CaCl_2$ 1.8, $MgCl_2$ 1.05, NaH_2PO_4 0.42, $NaHCO_3$ 25, D(+)-glucose 5.6; LCS for myocyte isolation: NaCl 120, KCl 10, KH_2PO_4 1.2, $MgCl_2$ 1.2, D(+)-glucose 10, taurine 20, HEPES–NaOH, 10 (pH 7.0). Tyrode's solution for patch-clamp recordings: NaCl 140, KCl 5.4, $CaCl_2$ 1.5, $MgCl_2$ 1.2, glucose 10, HEPES–NaOH 5 (pH 7.35). To measure the hyperpolarization-activated current, Tyrode's solution was modified by adding $BaCl_2$ (5), $MnCl_2$ (2), 4-aminopyridine (0.5), and increasing KCl to 25 mmol; this solution allowed for the reduction of interference from other currents, that is, L-type calcium current, inward rectifier-like current and transient outward potassium current. Pipette solution: K-aspartate 130; Na_2-ATP 5, $MgCl_2$ 2, $CaCl_2$ 5, EGTA 11, HEPES–KOH 10 (pH 7.2; pCa 7.0).

From stock solutions (10^{-2} M) in water, drugs were diluted in Tyrode's solution to reach the final concentration.

5.2.5. Analysis and Statistics. Data were analyzed using pClamp software and Origin software (vers. 4.1, MicroCal Software Inc.). The rate of spontaneous beating was expressed in beats/min and plotted as a function of the tested drug concentration to obtain dose-effect curves, which were fitted using the Hill equation:

$$y = E_{\max} \cdot \frac{x}{k + x}$$

where $E_{\max}$ is the maximum effect, k corresponds to the concentration at which 50% $E_{\max}$ is obtained (EC_{50}) and x is the concentration of drug.

All data are expressed as mean ± SEM. Statistical analysis was performed by means of the Graph-Pad Instat program (version. 3.05, GraphPad Software), using one-way analysis of variance followed by the Student–Newman–Keuls test. A probability value of less than 0.05 was considered significant.

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Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.bmc.2004.11.017.

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