Discovery of Novel and Cardioselective Diltiazem-like Calcium Channel Blockers via Virtual Screening

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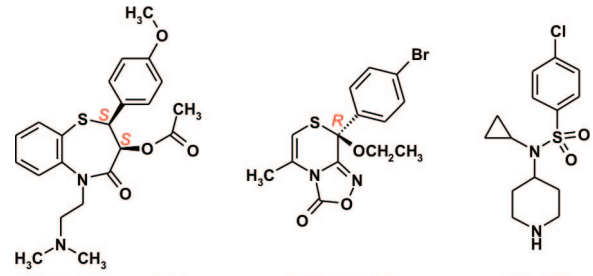
With the effort to discover new chemotypes blocking L-type calcium channels (LTCCs), ligand-based virtual screening was applied with a specific interest toward the diltiazem binding site. Roughly 50000 commercially available compounds served as a database for screening. The filtering through predicted pharmacokinetic properties and structural requirements reduced the initial database to a few compounds for which the similarity was calculated toward two template molecules, diltiazem and 4-chloro-*N*-cyclopropyl-*N*-(4-piperidinyl)benzene-sulfonamide, the most interesting hit of a previous screening experiment. For 18 compounds, inotropic and chronotropic activity as well as the vasorelaxant effect on guinea pig were studied "in vitro", and for the most promising, binding studies to the diltiazem site were carried out. The procedure yielded several hits, confirming in silico techniques to be useful for finding new chemotypes. In particular, *N*-[2-(dimethylamino)ethyl]-3-hydroxy-2-naphthamide, *N*,*N*-dimethyl-*N*′-(2-pyridin-3-ylquinolin-4-yl)ethane-1,2-diamine, 2-[(4-chlorophenyl)(pyridin-2-yl)methoxy]-*N*,*N*-dimethylethanamine (carbinoxamine), and 7-[2-(diethylamino)ethoxy]-2*H*-chromen-2-one revealed interesting activity and binding to the benzothiazepine site.

Introduction

Voltage-gated calcium channels constitute a large family of receptors, the best investigated class of which is represented by the L-type calcium channels (LTCCs)[a], formed by the Ca_v1 family of pore-forming α_1 subunit isoforms ($Ca_v1.1$, $Ca_v1.2$, $Ca_v1.3$, and $Ca_v1.4$).[1] LTCC blockers selectively inhibit the influx of extracellular calcium;[2] they are important drugs in use in the treatment of cardiovascular disorders such as hypertension, atrial arrhythmia, and angina pectoris.[3] LTCC are not only expressed in the cardiovascular system but also in a variety of electrically excitable cells such as smooth muscle cells, neurons, neuroendocrine cells, and even immune cells.[4] Therefore, isoform-selective LTCC modulators may extend the pharmacotherapeutic potential of these drugs beyond their established use in cardiovascular diseases.[5]

To date, among the three most important classes of LTCC blockers currently used in therapy, the class of benzothiazepines (BTZ) is least characterized and the availability of diltiazem(**1**)-like compounds is limited.[6–10] Classical structure−activity relationships (SAR), used in the past to find new structures and to define binding modes of new BTZ-like compounds,[11–13] are coupled, nowadays, to in silico techniques such as virtual screening, which could contribute to a significant extent. In fact, virtual screening procedures increasingly gain acceptance in the pharmaceutical research as an efficient strategy for analyzing very large chemical databases, screening thousands of compounds and searching for new chemotypes.[14]

Chart 1



Previous qualitative and quantitative structure−activity relationship studies[15,16] have shown that mapping the diltiazem binding site is a difficult task; some studies are in progress to better define the binding sites of some compounds recently synthesized by modifying 8-(4-bromophenyl)-8-ethoxy-8*H*-[1,4]thiazino[3,4-*c*][1,2,4]oxadiazol-3-one, coded as **5b** in ref 16 (**2**), or found by screening (Chart 1). In fact, we recently discovered by ligand-based virtual screening some novel LTCC blockers; one of the hits, 4-chloro-*N*-cyclopropyl-*N*-(4-piperidinyl)benzene-sulfonamide (**3**), was found to bind specifically to the diltiazem binding site and to exhibit significant negative inotropic activity. Among the former series of hits, **3** was the

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[a] Abbreviations: LTCCs, L-type calcium channels; DTZ, diltiazem; BTZ, benzothiazepines; SAR, structure−activity relationship; PK, pharmacokinetic; GPILSM, guinea pig ileum longitudinal smooth muscle; MIF, molecular interaction fields; PSS, physiological salt solution; LCS, low Ca^{2+} solution; HOCM, hypertrophic obstructive cardiomyopathy.

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Table 1. Details of the Screening Process

Sigma-Aldrich library from ZINC[a]	
starting compounds[b]	49006
pharmacokinetic filtering	1207
structural features	90
chemical stability and potential toxicity	36
purchased compounds	18
active hits[c]	**9**

[a] From ZINC database, downloaded on January 2005. [b] Although in ZINC some molecules have duplicates in all the statistics and numbers reported, each compound is considered only once. [c] Active hits are compounds with negative inotropic potency at least equivalent to diltiazem (EC_{50} = 0.79 μM).

only compound containing a basic nitrogen, which was hypothesized to be essential for its resemblance to diltiazem in terms of the LTCC interaction.[17]

Because of the continuing interest in the discovery of novel diltiazem-like compounds and with the final aim of finding new chemical candidates and to characterize their binding site, a fine-tuned search was carried out focused on diltiazem and the novel LTCC blocker **3**. To identify novel chemotypes and to provide hypotheses for their binding modes via virtual screening, the library of Sigma-Aldrich was selected out of the ZINC archive supplied via the WorldWide Web[18,19] and the program SHOP[20–22] was used for assessing the similarity between data set and reference compounds. The virtual screening was exclusively ligand-based because the existing homology models of calcium channel structures are mainly focused on the binding site of dihydropyridines such as nifedipine and nitrendipine.[23,24] Detailed knowledge or 3D-structures of complexes between diltiazem-like compounds and their binding sites are still unavailable, excluding to date the application of structure-based virtual screening strategies.

Results and Discussion

Virtual Screening. Overview. The Sigma-Aldrich library was selected among the collection assembled in the so-called ZINC database,[18,19] supplied via the WorldWide Web,[19] where tautomerism, chirality, and protonation/deprotonation states have already been treated.[18] A subset of roughly 50000 commercially available compounds,[25] covering a broad chemical landscape, was extracted from the ZINC database (release 5; January 2005).[18] Some compounds were provided as duplicates and could simultaneously approach the screening procedure with, for instance, diverse biologically relevant protonation states or different tautomers. Thereby, the 60862 molecules used for screening represented 49006 compounds commercially available from the Sigma-Aldrich vendor.[25]

The virtual screening procedure was based on several steps, some of which were highly selective. On the basis of pharmacokinetic profile and structural requirements (presence of a basic nitrogen), more than 99% of the compounds were filtered out. The screening procedure is summarized in Table 1 and Figure 1, whereas each single step is briefly presented below and detailed in the Experimental Section.

Pharmacokinetic Filtering. A good PK-profile of drug-likeness is the normal first filter for screening databases,[14,26,27] because selecting drug-like compounds enhances the possibility to achieve relevant hits. Therefore the database was restricted exclusively to compounds with drug-like properties. A set of physical and biological properties were predicted in silico for each database compound using the well-known VolSurf program[28] using the following automated procedure:

1. Derivation of VolSurf descriptors.

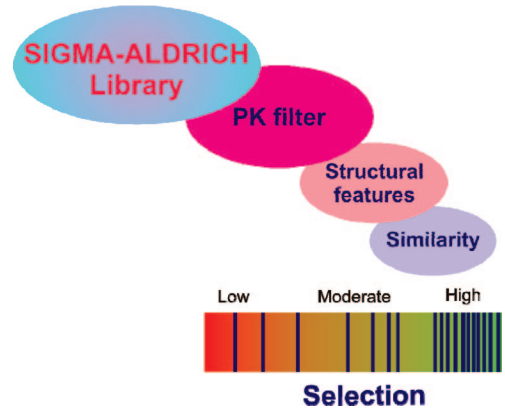


Figure 1. Pharmacokinetic and structural filters were applied to the Sigma-Aldrich library, followed by similarity calculation to rank the final set of compounds. A selection of some representative compounds with low, moderate, and especially high similarity toward the templates was carried out for biological tests.

2. Use of some common VolSurf descriptors, such as molecular weight and calculated logP, and prediction on precalculated VolSurf library models (blood−brain barrier, absorption, thermodynamic solubility, and metabolic stability)[14,29–31] to derive the PK-profile of the compound;

3. filtering according to the predefined PK-settings shown in Figure 2.

This PK profile guaranteed standard drug size properties and a broad lipophilicity range, sufficient solubility (at least 100 μM), and adequate absorption, whereas rapidly metabolized compounds and compounds crossing the blood−brain barrier were avoided. Via this filtering, the database size was significantly reduced from 49006 to 1207 structures, which equals an elimination of about 97.5% of compounds. Details are provided in Figure 2 and as Supporting Information.

Structural Features. SAR studies of diltiazem-like compounds[10] as well as our virtual screening experience[17] favor the hypothesis that compounds with a basic nitrogen are privileged for binding to the diltiazem site. Therefore, the presence of a protonated nitrogen was the basis for the next refinement: in silico prediction of pK_a was carried out for the data set compounds with the software Moka[32,33] and only 90 of the 1207 compounds, with a calculated pK_a of the nitrogen greater than 5, were accepted.

Chemical Stability and Potential Toxicity. Compounds that passed the PK filter and exhibited the desired structural features were finally checked for chemical stability and potential toxicity by visual inspection. Only 36 compounds remained after removing compounds with potential toxic and reactive functional groups, according to basic medicinal chemistry criteria as well as literature suggestions.[34,35]

SHOP Similarity. For 36 compounds that passed all filters, the similarity toward two reference compounds **1** and **3** was calculated. The similarity calculation was based on the recently presented procedure SHOP, which helps chemists in scaffold hopping, i.e., in finding new interesting structures by varying the central scaffold and maintaining those substituents considered relevant for the biological properties and activity.[20,21]

To date, the applicability of the program SHOP to ligand-based virtual screening has not been investigated. However, the presence of a basic nitrogen atom as a fixed structural requirement prompted us to try the method by defining the nitrogen as unique anchor point and the whole molecule as molecular scaffold. To deal with the molecular flexibility, a set of different

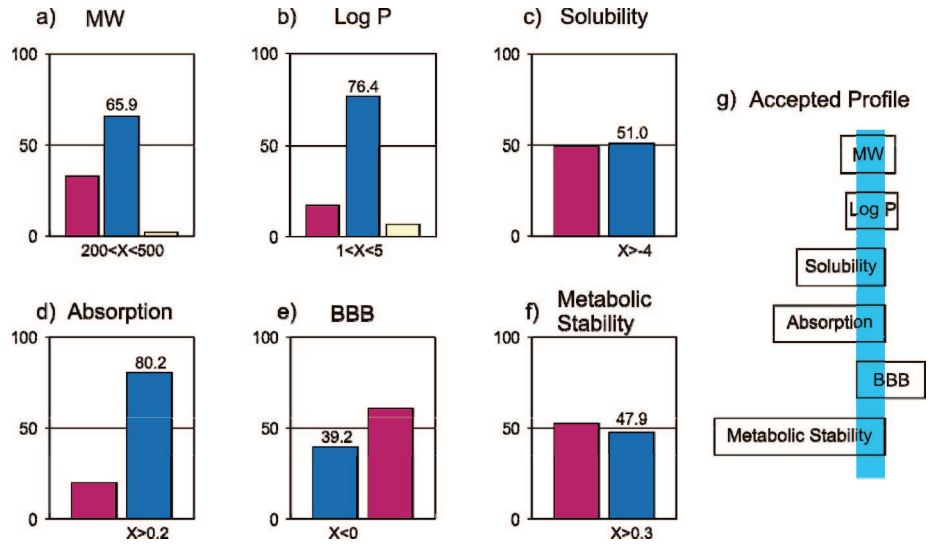


Figure 2. Results of PK-filtering are separately schematized for the six filters used (a−f), with the blue bars representing the amount of the selected compounds; (g) only a few compounds (cyan section) revealed a good in silico PK profile and passed all filters. (a) Standard drug size properties were selected via a molecular weight (MW) filter range between 200 and 500. (b) A broad lipophilicity profile was applied by accepting compounds with a calculated logP in the range of 1−5. (c) To select compounds with sufficient solubility (at least 100 μM), the Soly min (minimum acceptable solubility) value was set −4, where Soly is expressed as the log of the solubility, measured in mol/L. (d) The Caco2-min (minimum acceptable Caco2 passive permeability) value was set 0.2 to guarantee adequate absorption. (e) The BBB-max (maximum acceptable permeability across the blood−brain barrier) value was set zero in order to avoid data set compounds crossing the blood brain barrier. (f) The Metabolic Stability-min (metabolic stability due to the cytochrome P450 3A4) value was set to 0.3 to exclude rapidly metabolized compounds.

conformers is internally generated by SHOP for each reference and data set compound, and any molecular comparison is carried out over the entire set of conformations. For molecular description SHOP uses correlograms, which are based on the GRIND formalism,[36] that refer to distances between the anchor point and the GRID fields over the molecule. Comparing the correlograms of two molecules gives their similarity.

The preliminary results were intriguing because **3** was highly similar to diltiazem (**1**) by this measure (the corresponding similarity value was 0.834). The comparison of the molecular description for the molecules **1** and **3** is reported in Figure 3, together with the superposition of **3** over **1** proposed by SHOP on the basis of their molecular interaction fields.

Therefore, for each compound, similarity values were calculated toward **1** and **3**, and their average gave the final score: high scores corresponded to compounds with good similarity versus both templates, low scores to compounds with low similarity versus both templates, whereas moderate scores corresponded to all other cases. The 36 molecules were ranked accordingly and, finally, 18 compounds were selected for biological tests. Fifteen compounds were selected with moderate to high similarity toward the templates, whereas three others were selected among the compounds with low similarity. These latter three compounds were expected to have low if any biological activity and have been added to check the validity of the method. Similarity values and final scores for the selected candidates are reported in Table 2, where the chemical structures are also given: data set molecules **8** and **17** were tested as racemates, compound **20** as mixture of diastereoisomers, and **16** in its (*R*)-(+) form.

Literature Analysis of Selected Compounds. Within the series of top-ranked compounds, some well-known drugs and other already studied compounds were found. Therefore, it was investigated in the literature whether LTCC modulation was already known for the selected compounds.

Carbinoxamine (**8**) is a histamine-H_1 blocking agent clinically used in the treatment of both seasonal and perennial allergic rhinitis. The (−)-stereoisomer of carbinoxamine is the antihistaminic eutomer.[37] Compound **19** is another well-known H_1-antihistaminic drug, known with the common names mepyramine and pyrilamine. To our knowledge, no information is available to date concerning the LTCC blocking activity of **8** and **19**.

Compound **9**, also known as ML9, is able to inhibit agonist-induced Ca^{2+} entry in endothelial cells and catecholamine secretion in intact and permeabilized chromaffin cells.[38] ML9, known also as specific inhibitor for myosin light chain kinase, was recently tested for its anticancer activity by evaluating its ability to affect dimethylsphingosine-induced increase of pH in the U937 monocyte cell line.[39]

Propranolol (**16**) is a well-known β-blocker used for the treatment of systemic hypertension.[40] Evidence from the last 20 years suggests that propranolol: (i) produces relaxation of K^+-depolarized coronary artery,[41] (ii) exerts part of its therapeutic action by reducing Ca^{2+} influx into cells through a mechanism not related with β-adrenoreceptor blockade,[42] (iii) interacts with the dihydropyridine and benzothiazepine binding sites, inhibiting [^{3}H]diltiazem binding to rat cortical membranes,[43,44] (iv) is stereoselective because the (*R*)-(+) form is 60−100 times less active than the (*S*)-(−) form as β_1-blocker.[45]

Finally, **7** has been used for a study based on a probabilistic neural network multiple classifier system for predicting the genotoxicity of quinolone and quinoline derivatives,[46] whereas **13** was evaluated for its hypotensive activity.[47] No information is available to date about the activity of **7** and **13** as LTCC blockers.

In Vitro Screening of Virtual Hits. Functional Studies. The selected compounds were assayed on guinea pig left and right atria for negative inotropic and chronotropic action, while vasorelaxant activity was assessed on K^+-depolarized guinea pig aortic strips. In particular, compounds were checked at increasing doses to evaluate the percent decrease of developed tension on isolated left atrium driven at 1 Hz (negative inotropic activity), the percent decrease in atrial rate on spontaneously

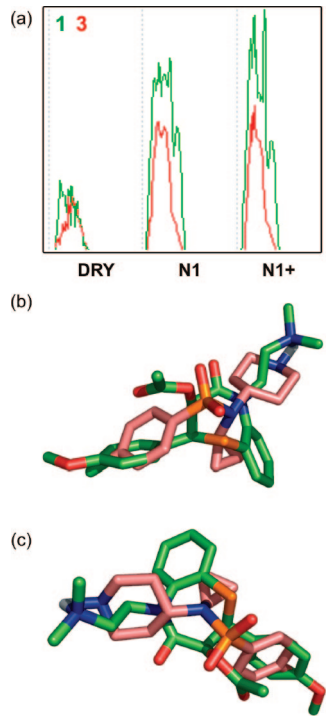


Figure 3. (a) Molecular description obtained for the two templates, **1** (green correlogram) and **3** (red correlogram). Each correlogram is composed of three blocks, obtained with the GRID hydrophobic probe (DRY) and with the nitrogen probes N1 and N1+, which reflect hydrogen bonding donor character (N1), even reinforced (N1+) by a charge contribution. The hydrophobic regions of the two molecules are comparable at higher distances to the nitrogen, whereas a little difference exists at lower distances to the nitrogen. The cyclopropyl ring of **3** in place of the phenyl ring of **1** produces a weaker interaction with the DRY probe and could be hypothesized to be mainly responsible for this difference. The profile with the probes N1 and N1+ is very similar for **3**, whereas some differences exist for **1**; both the probes N1 and N1+ interact with hydrogen bonding acceptor groups, but the additional electrostatic contribution for N1+ results in higher peaks in the correlogram obtained for **1**. (b−c) Two different views of the three-dimensional superposition proposed by SHOP for compounds **1** and **3**. Nitrogen atoms are represented in blue, oxygen atoms in red, sulfur atoms in orange; carbon atoms of **1** and **3** are differently colored (**1** = green, **3** = pink). Hydrogen atoms are not presented for clarity, whereas the white atom bonded to the nitrogen is the anchor used by SHOP.

beating right atrium (negative chronotropic activity), and the percent inhibition of calcium-induced contraction on K^+-depolarized (80 mM) aortic strips (vasorelaxant activity).[16] The functional activity determined was expressed as efficacy and potency.

Overview. Results are summarized in Table 3 together with those of reference compounds **1**, **2**, and **3**.[17] Overall, the compounds selected with low scores (**19**, **20**, and **21**) have very low negative inotropic potency, about 8, 2.7, and 1.4 fold with respect to **1** and 20, 7, and 3.6 with respect to **3**; compounds with medium scores have medium to high EC_{50} values, whereas all highly active compounds have high scores. This is evidenced in Figure 4, where the compounds are ranked according to their scores and the measured negative inotropic potency is reported on the *y* axis.

Eight of the compounds with high scores could be considered as hits, being at least as potent as **1**: **4**, **7**, **8**, **9**, **10**, **11**, **13**, and **14**. Four (**4**, **7**, **8**, and **13**) were very interesting, being equipotent to **3**.

In addition, all compounds exhibited cardiac selectivity higher than the references; they revealed intrinsic negative inotropic activity without chronotropic and vasorelaxant effects. In fact, references **1** and **3** have also chronotropic activity, not observed in this new series, whereas diltiazem (**1**) is also vasorelaxant, even if weaker than nifedipine.[49]

Structure−Activity Relationships of Novel L-type Calcium Channel Blockers. The diversity of the selected compounds excluded the derivation of classical structure−activity relationships; the profound differences existing between the virtual hit structures make it difficult to attribute distinct molecular features to corresponding differences in activity. Therefore, the series is described on the basis of the required structural feature, the basic nitrogen that varies along the series. Several features could influence the inotropic activity: the aminic nitrogen could be secondary or tertiary, part of a cycle, or along an aliphatic chain, and its position within the molecule could be lateral or central. This qualitative description is schematized in Figure 5.

Compounds with a secondary amine group, which are the reference **3** and compounds **9**, **11**, **16**, and **17**, lie on the left branch of the graph of Figure 5. All of them have lateral nitrogen atoms, whereas differences exist for the chain/cycle character. Compounds **16** and **17** have secondary nitrogen atoms part of an alkyl chain and potency in the 1−10 μM range (EC_{50} = 1.54 μM and EC_{50} = 3.59 μM, respectively), whereas the reference **3** as well as compounds **9** and **11**, with cyclic secondary nitrogen atoms, resulted at least 10-fold more potent (EC_{50} = 0.31 μM, EC_{50} = 0.43 μM, and EC_{50} = 0.52 μM, respectively). It is worth noting that not only the chain-type secondary nitrogen but also the presence of an aliphatic hydroxyl group could be hypothesized to be relevant for the lower activity of **16** and **17**, as discussed below.

Diltiazem and all of the virtual hits containing a tertiary nitrogen lie on the right branch of the graph (Figure 5); here, differences concern both the chain/cycle type and the lateral/central position of the nitrogen with respect to the molecule. In general, the low similarity to the templates observed for compounds with a central nitrogen (**20** and **21**) corresponded to potency greater than 1 μM (EC_{50} = 2.17 μM and EC_{50} = 1.11 μM, respectively).

All of the other compounds with the lateral nitrogen have high similarity and, very often, significant inotropic potency. The exception is compound **19**, which, despite its lateral nitrogen, has low similarity and is the weakest negative inotropic agent (EC_{50} = 6.26 μM). For all the remaining compounds (**4**, **5**, **6**, **7**, **8**, and **13**) with a lateral tertiary nitrogen part of an alkyl chain, the corresponding EC_{50} values were interesting. All of these compounds are double-substituted with methyl or ethyl groups, but no valid relationships were derived between the methyl/ethyl N-substituents and the potency. On the contrary, structural comparison achieved correspondences between the aliphatic linkers, connecting the nitrogen and the aromatic moiety, and inotropic potency. For compounds **8** and **13** with the same potency (EC_{50} = 0.25 μM), the linker is an ethoxyamine group; compounds **4** and **7** (EC_{50} = 0.35 μM and EC_{50} = 0.30 μM, respectively) have both an ethylenediamine linker, whereas compounds **5** and **6**, with negative inotropic potency greater than 0.8 μM, have different linkers.

For the other five compounds, the nitrogen is part of a six-membered ring. Compounds **15** and **18**, with a morpholine ring, have interesting inotropic potency (EC_{50} = 0.84 μM and EC_{50} = 0.73 μM, respectively) although rather low similarity to **1**. A common feature of compounds **10** and **14** (EC_{50} = 0.34 μM and EC_{50} = 0.45 μM, respectively), with a piperazine ring, and compound **12** (EC_{50} = 0.97 μM), with a piperidine ring, is the

Table 2. Original Codes, Chemical Structures, Similarity Values, Final Scores, and Calculated pK_a Values for the 18 Selected Compounds

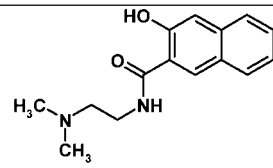
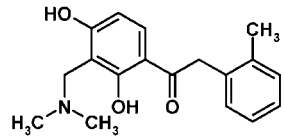
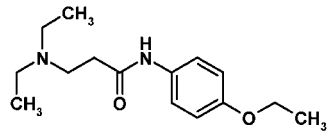
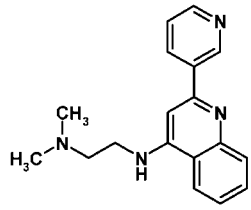
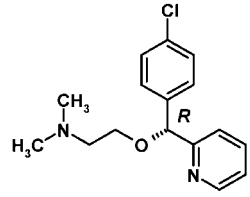
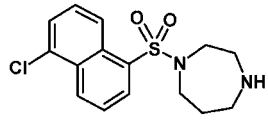
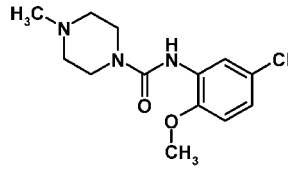
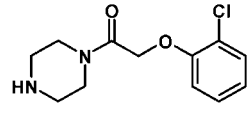
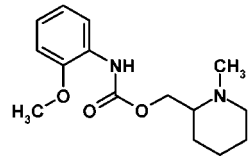
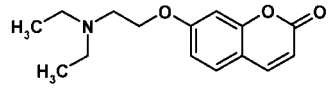
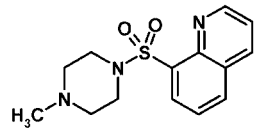
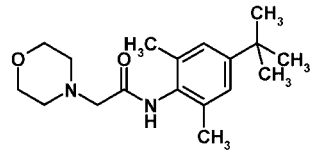
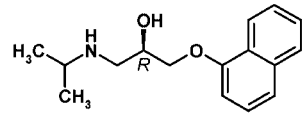
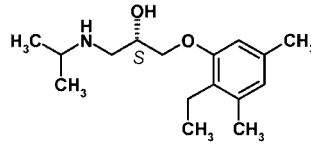
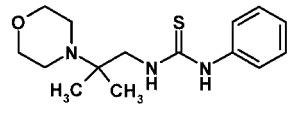
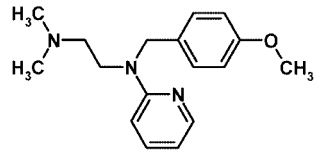
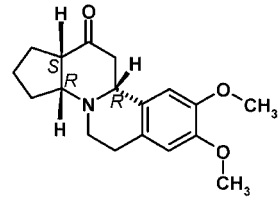
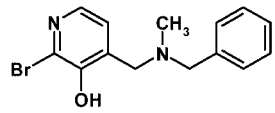
Compound	Code	Structure [a]	DTZ	P1	Score [b]	pK_a [c]
4	R415227		0.897	0.933	0.915	8.57
5	R908940		0.872	0.930	0.901	10.74 [d]
6	S469963		0.886	0.888	0.887	8.74
7	R407348		0.926	0.846	0.886	9.23
8	C8278 (carbinoxamine)		0.828	0.942	0.885	8.67
9	C1172		0.813	0.955	0.884	8.07
10	S794740		0.838	0.922	0.880	7.69
11	R732796		0.819	0.918	0.869	8.30
12	S219061		0.785	0.949	0.867	7.99
13	R735884		0.883	0.846	0.865	8.94

Table 2. Continued

Compound	Code	Structure [a]	DTZ	P1	Score [b]	pK_a [c]
14	R608068		0.788	0.935	0.862	7.88
15	S239534		0.785	0.919	0.852	5.59
16	82065 (*R*-(+)-propranolol)		0.760	0.932	0.846	9.25
17	L129984		0.775	0.895	0.835	9.25
18	S046001		0.746	0.898	0.822	6.45
19	S347108 (pyrilamine)		0.792	0.799	0.796	8.56
20	S761397		0.707	0.799	0.753	8.87
21	R468967		0.678	0.811	0.745	9.70

[a] Stereochemistry: Compound **16** was modeled and purchased as *R*(+). Compounds **8** and **17** were purchased as racemates and compound **20** as a diastereoisomers mixture; however, the computational procedure identified as hits the stereoisomers reported. [b] Good similarity scores were assigned only to compounds with good individual scores obtained toward the two reference compounds. [c] Calculated in silico with the software Moka.[32,33] [d] Measured experimentally.

N-methyl, also present on tetrandrine, a large calcium antagonist molecule that binds to the diltiazem site.[10,50]

Among the structural features discussed above, neither the secondary/tertiary character of the nitrogen nor the chain/cycle type were selective for negative inotropic activity. Compounds containing secondary nitrogen atoms are active only when they are "cyclic" and "lateral". On the contrary, compounds with tertiary nitrogen atoms have medium (**21**) or low (**20**) potency when they are "central" and are mainly active (medium to high potency) when the nitrogen is "lateral", with the only exception of compound **19**. Therefore, the spatial arrangement of the pharmacophore nitrogen atom with respect to the whole molecule, that is, the lateral position of the nitrogen atom within the molecular structure, seems to be related to negative inotropic activity.

Further investigation of molecular characteristics and structures were necessary to find the guidelines to differentiate active from inactive compounds and are described below. To assess

Table 3. Cardiovascular Activity of Tested Compounds

	% decrease (M ± SEM)		EC_{50} of negative inotropic activity		vasorelaxant Activity
compound	negative inotropic activity[a]	negative chronotropic activity[b]	EC_{50}[c] (μM)	95% conf lim ($\times 10^{-6}$)	activity[d] (M ± SEM)
1[e]	78 ± 3.5[f]	94 ± 5.6[g]	0.79	0.70−0.85	88 ± 2.3
2[h]	58 ± 3.4[i]	16 ± 0.8[f]	0.07	0.04−0.08	11 ± 0.7
3[h,j]	76 ± 4.0[k]	64 ± 2.6[k]	0.31	0.20−0.48	19 ± 1.9
4	90 ± 1.4	35 ± 2.1	0.35	0.25−0.49	13 ± 0.9
5	78 ± 0.4[g]	48 ± 0.7[k]	0.84	0.57−1.01	4 ± 0.4*
6	79 ± 1.9	30 ± 2.1	1.05	0.71−1.53	11 ± 1.0
7	76 ± 0.3[g]	4 ± 0.1*	0.30	0.23−0.38	5 ± 0.3*
8	76 ± 0.9[j]	48 ± 1.1[j]	0.25	0.18−0.34	28 ± 1.6
9	98 ± 0.9	46 ± 2.4[k]	0.43	0.32−0.56	43 ± 2.6[k]
10	67 ± 3.0[k]	10 ± 0.9*	0.34	0.27−0.44	7 ± 0.4*
11	60 ± 3.4[k]	3 ± 0.1k[j]*	0.52	0.39−0.69	1 ± 0.03*
12	87 ± 1.9[j]	17 ± 1.3[j]	0.97	0.65−1.36	10 ± 0.9
13	67 ± 0.3[f]	28 ± 1.7[j]	0.25	0.20−0.32	14 ± 0.6
14	84 ± 1.6[f]	19 ± 0.4[k]	0.45	0.27−0.71	18 ± 0.7
15	87 ± 0.2[f]	29 ± 1.3[j]	0.84	0.59−1.19	26 ± 1.7
16	92 ± 2.6[k]	26 ± 0.7[k]	1.54	1.36−1.91	36 ± 2.1
17	76 ± 3.6[k]	40 ± 1.3	3.59	2.31−5.56	30 ± 2.0
18	94 ± 3.5[j]	10 ± 0.9[j]	0.73	0.47−1.02	20 ± 1.8
19	81 ± 2.8[j]	47 ± 1.1	6.26	3.50−9.12	23 ± 1.7
20	64 ± 0.7	4 ± 0.3*	2.17	1.69−2.36	3 ± 0.3*
21	70 ± 2.1	6 ± 0.5*	1.11	0.75−1.63	2 ± 0.1*

[a] Activity: decrease in developed tension in isolated guinea pig left atrium at 10^{-4} M, expressed as percent changes from the control ($n = 4-6$). The left atria were driven at 1 Hz. The 10^{-4} M concentration gave the maximum effect for most compounds. [b] Activity: decrease in atrial rate in guinea pig spontaneously beating isolated right atria at 10^{-4} M, expressed as percent changes from the control ($n = 6-8$). Pretreatment heart rate ranged from 170 to 195 beats/min. The 10^{-4} M concentration gave the maximum effect for most compounds. [c] Calculated from log concentration−response curves (Probit analysis according to Litchfield and Wilcoxon[48] with $n = 6-8$). When the maximum effect was <50%, the EC_{50} inotropic, EC_{30} chronotropic, and IC_{50} values were not calculated. [d] Activity: percent inhibition of calcium-induced contraction on K^+-depolarized guinea pig aortic strip at 10^{-4} M. The 10^{-4} M concentration gave the maximum effect for most compounds. [e] $EC_{30} = 0.07$ (c.l. 0.064−0.075), $IC_{50} = 2.6$ (c.l. 2.2−3.1). [f] At 10^{-5} M. [g] At 5×10^{-6} M. [h] Taken from ref 17. [i] At 10^{-6} M. [j] $EC_{30} = 0.48$ (c.l. 0.32−0.59). [k] At 5×10^{-5} M. * $P < 0.05$.

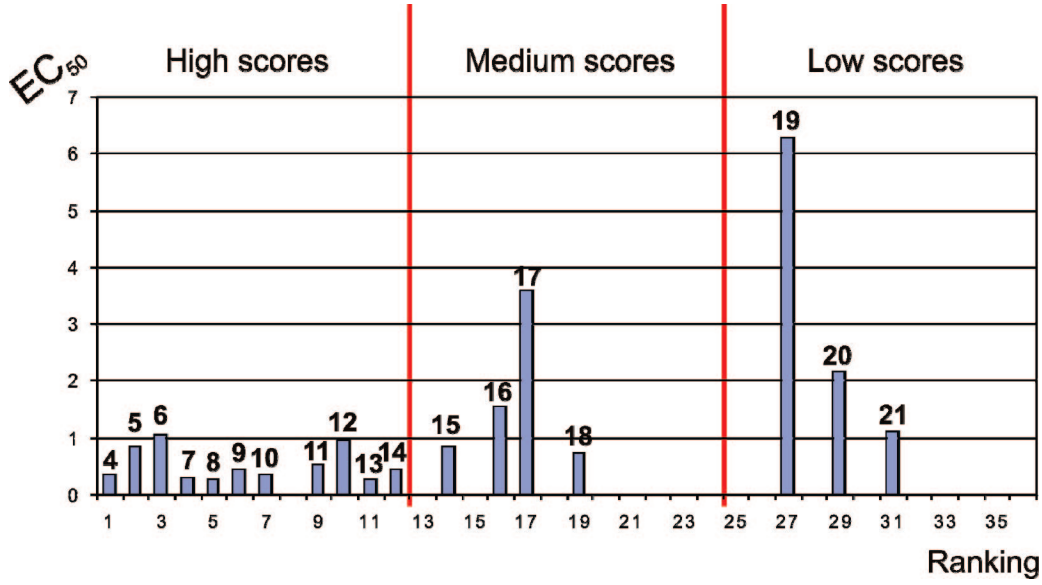


Figure 4. The 36 compounds that passed all filters were ranked according to their scores obtained by SHOP, classified as high, medium, and low. Among them, 18 compounds were selected and the negative inotropic activity was measured. In the *y* axis, their potency is reported as EC_{50} values (μM). Compounds with $EC_{50} < 1.0$ μM are mainly in the region with high scores; two interesting compounds (**15** and **18**) are among the compounds with medium scores, whereas all the compounds with low scores have $EC_{50} > 1.0$ μM.

the basicity of compounds, pK_a values were calculated in silico with the software Moka,[32,33] as already reported in Table 2. Compounds **15** and **18** showed low calculated pK_a values (5.59 and 6.45, respectively), likely due to the morpholine ring, whereas compound **5** showed a high value (10.74), likely due to the simultaneous effect of the two phenolic groups. With the exception of these three cases, for all of the other compounds, the pK_a values range from 7.69 of compound **10** to 9.70 of compound **21**. As highlighted in Figure 6, compounds with low potency have preferentially high calculated pK_a values (more than 8.5), but such high pK_a is not enough to define an inactive molecule because also active compounds have such values (see compounds **4**, **7**, **8**, and **13** in Figure 6). Vice versa, all the potent compounds, with exception of **7**, have pK_a values in the range 7.5−9.0.

Common features for many LTCC blockers are nitro, ethyl carboxylate (COOEt), and *ortho*-dimethoxy groups, for which two oxygen atoms are very close to each other: 2.2, 2.2, and 2.7 Å for nitro and ethyl carboxylate of nifedipine and *ortho*-dimethoxy of verapamil, respectively. In the sulfonyl group,

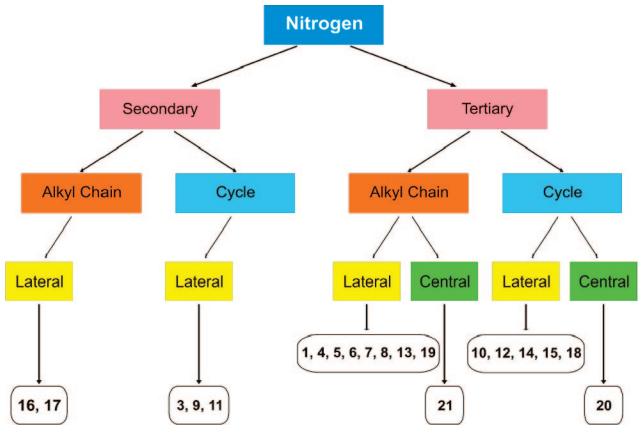


Figure 5. Flowchart summarizing the information concerning nitrogen atoms of selected compounds.

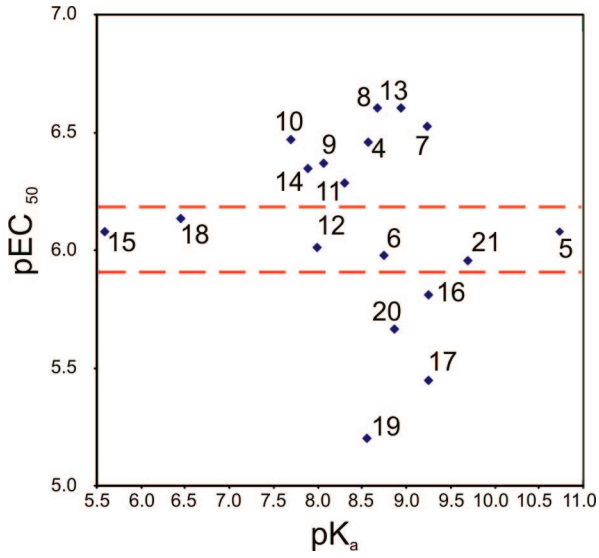


Figure 6. Distribution of selected compounds according to their calculated pK_a (x axis) and pEC_{50} values (y axis). The red dot lines allow classification of the compounds according to their high, medium, and low potency (y axis).

seldom present in LTCC blockers structures,[17,51] the two oxygen atoms are approximately 2.5 Å apart. For LTCC blockers, these groups are supposed to interact with a Ca^{2+} within the channel, chelated by the selectivity filter glutamate residues as suggested by Tikhonov and Zhorov.[52]

Our hypothesis is that two of these atoms likely chelate Ca^{2+}, being highly relevant for the ligand−channel interaction. Therefore, these groups were searched for all the selected compounds, and euclidean distances were measured using the three-dimensional structures given by SHOP alignments. The presence of at least two atoms among carbonyl, ether, and ester oxygen should provide the best interaction, but also alcoholic and sulfonyl oxygen, aromatic nitrogen, and even sulfur atom (thiourea-like) in the least case could be considered. The presence of such groups and the distances are schematized for each molecule in Figure 7, where compounds are reported according to their inotropic potency.

Active compounds have always a short distance, i.e., below 3 Å, between two of the mentioned atoms represented in Figure 7 by red circles. The only exception is compound **7**, for which the distance and the orientation of the two aromatic nitrogen atoms do not allow a simultaneous interaction with the hypothetical Ca^{2+}. On the contrary, for compounds with medium to low potency, no speculation could be derived because they present either short, medium, or long distances between the mentioned groups.

In summary, for the inotropic agents presented, to increase potency, the pK_a could be preferably between 7.5 and 9.0 and the nitrogen atom could be either tertiary or secondary, but in the latter case, only within a cycle and lateral with respect to the molecular structure. Also, the tertiary nitrogen atom should be lateral, whereas both the chain and the cycle features are active. This very general pharmacophore, common between many therapeutic classes, is more selective if coupled with at least two polar atoms, able to chelate Ca^{2+}, about 2−3 Å apart from each other. Altogether, these structural considerations achieved could help in formulating hypotheses for the binding modes of active compounds selected by screening.

Selectivity. As mentioned above, this series of compounds was selective for negative inotropic activity, without negative chronotropic effect or vasorelaxant activity and, for a reduced set of compounds, the characterization was extended also to the relaxant activity on 80 mM K^+-depolarized guinea pig ileum longitudinal smooth muscle (GPILSM). Data are collected in Table 4, together with those of reference compounds.[17]

Interestingly, significant differences exist between aorta and ileal smooth muscle profiles of relaxant activity; while the inhibition of calcium-induced contraction on K^+-depolarized aortic strips at 100 μM was lower than 50% for the entire set of compounds, 7 compounds out of 11 were active at the same concentration on ileal smooth muscle. Nevertheless, their maximum effect was lower than 75% and their potency comprised in the 3−30 μM range; compound **21**, which is the lowest of the series in terms of similarity toward the templates, resulted as the most potent of the series (EC_{50} = 3.28 μM) and compound **8** resulted as slightly less potent (EC_{50} = 9.34 μM), whereas for compounds **4**, **7**, **12**, **16**, and **18**, the activity was greater than 50% but the corresponding EC_{50} was always higher than 10 μM. Finally, compounds **9**, **10**, **13**, and **14** could be classified as inactive on ileal smooth muscle. All the activities are plotted together in Figure 8, whereas all the remaining compounds with low negative inotropic activity were not further investigated.

In general, low affinity of these compounds for ileum longitudinal smooth muscle was assessed. However, specific groups of compounds showed different behavior and three classes could be identified. Compounds **9**, **13**, and **14** were highly selective for negative inotropic activity versus relaxant activity on GPILSM, with significant negative inotropic potency as well (EC_{50} always lower than 0.50 μM); compound **10** was less selective but with interesting potency. Finally, compounds **4**, **7**, **8**, **12**, **16**, **18**, and **21** are negative inotropic agents active on GPILSM as well.

Binding. Some representative compounds, namely **4**, **7**, **8**, **10**, **13**, **14**, and **16**, were selected for binding assay on rat cardiomyocytes to establish whether they interact with the benzothiazepine binding site. [^{3}H]diltiazem (5 nM) was incubated with increasing concentrations (0.1 nM to 100 μM) of the compounds as described in the Experimental Section.

All of the selected compounds affected the binding of [^{3}H]diltiazem in a concentration-dependent manner, showing different efficacy and potency. Compounds **4**, **8**, and **16** caused an almost complete inhibition of [^{3}H]diltiazem binding, whereas **7**, **10**, and **13** displayed a weaker interaction with the benzothiazepine-binding site producing a maximum inhibition of [^{3}H]diltiazem binding of about 50−60% at concentrations between 1 and 100 μM (see Figure 9). Finally, preliminary results

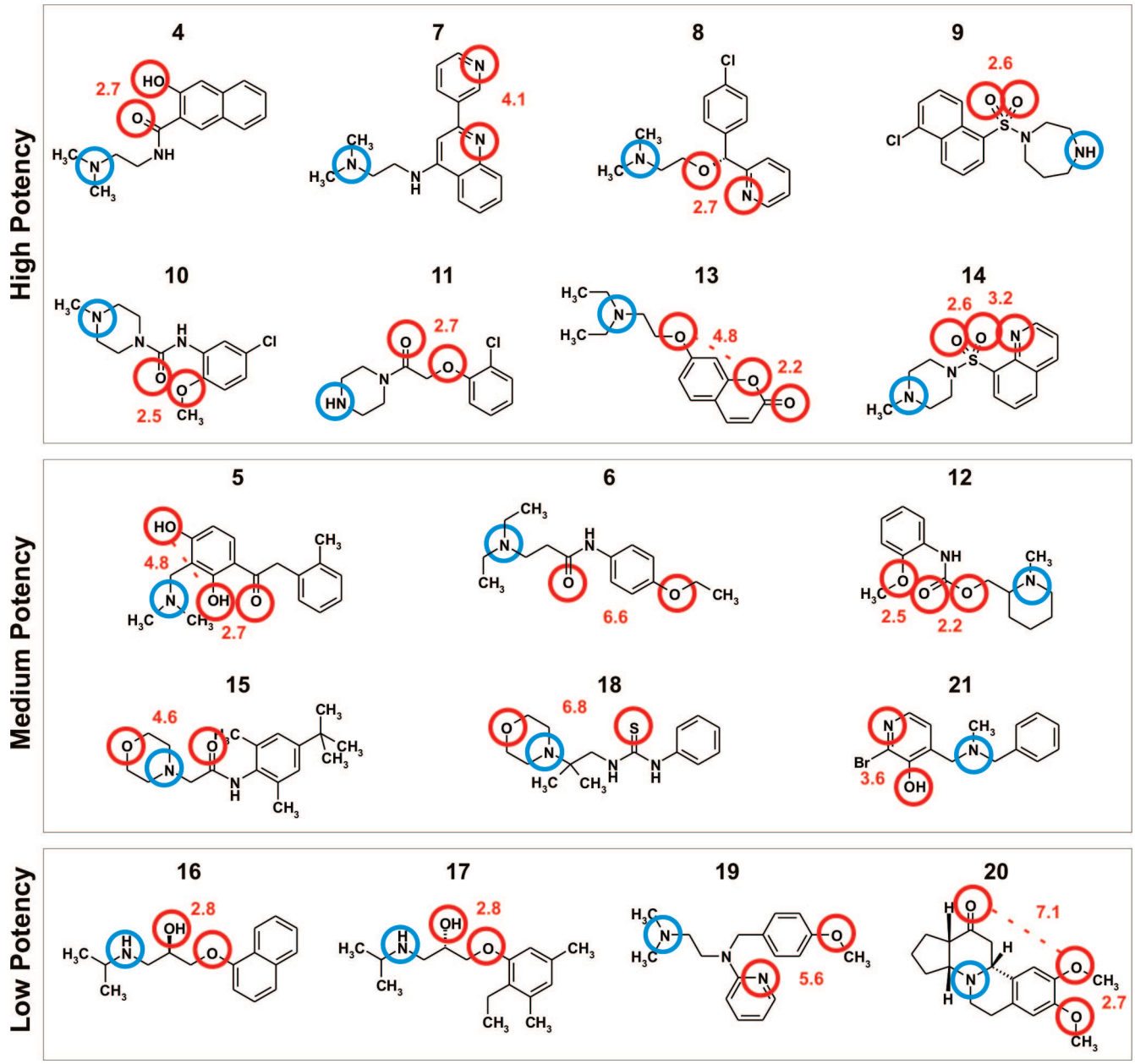


Figure 7. Structural comparison of the tested compounds, reported according to their high, medium, and low potency. The cyan circles mark the nitrogen atoms used as anchor by SHOP, whereas the red circles mark the atoms likely able to chelate Ca^{2+}.

suggested that **14** also interacted with the benzothiazepine-binding site. This compound, in fact, displaced [^{3}H]diltiazem binding although the maximal inhibition observed was about 60% at 10 μM concentration.

The findings presented in this study strongly suggest **8** as a novel LTCC blocker, which can inhibit channel activity by interacting at the benzothiazepine-binding site in the cardiac α_1 subunit of the LTCC. In fact, **8** completely inhibited [^{3}H]diltiazem binding to rat cardiomyocytes with a $K_i = 1.08$ nM, indicating a nonbenzothiazepine structure that can compete directly with diltiazem at its binding site. This is consistent with the classical properties of an LTCC blocker presented above for cardiac and gut smooth muscle preparations. On the contrary, the minor effects displayed on vascular smooth muscle differentiated **8** from diltiazem and highlighted its uniqueness.

Other interesting non-benzothiazepines are compounds **4**, with $K_i = 0.45$ μM, and **16**. To our knowledge, this is the first observation that **16** is able to displace [^{3}H]diltiazem from its binding site in rat cardiomyocytes, with effects evident at 1 μM, a $K_i = 14.5$ μM, and reduction of bound [^{3}H]diltiazem to near 0 at 100 μM. These radioligand binding data concerning the interaction of propranolol with cardiac LTCCs are in close agreement with their reported concentration required to produce a direct vascular relaxing effect in bovine retinal microarteries, which lack adrenergic nerves and β-adrenoceptors, where propranolol has been shown to relax K^+-induced contractions at μM concentrations.[42,53]

Furthermore, this effect is consistent with previous radioligand binding data obtained in rat cortical membranes.[44] However, it must be underlined that 10 μM propranolol did not inhibit Ca^{2+} channel currents recorded in single guinea pig mesenteric artery and portal vein myocytes, although, according to our radioligand binding data, higher concentrations of propranolol would have been required to rule out any inhibitory effect of this drug on Ca^{2+} currents. Noticeably, the antihypertensive effects of propranolol is observed at plasma concentrations of 0.10−0.15

Table 4. Relaxant Activity of Some Compounds in Comparison with those of Reference Compounds on K^+-depolarized Guinea-pig Ileum Longitudinal Smooth Muscle (GPILSM)

compound	activity[a] (M ± SEM)	IC_{50}[b] (μM)	95% conf lim ($\times 10^{-6}$)
1	98 ± 1.5[c]	0.11	0.085–0.13
2[d]	3 ± 0.2*		
3[d]	89 ± 3.2[e]	14.1	10.6–17.9
4	61 ± 4.4	20.2	15.8–23.1
7	72 ± 2.6	20.3	15.1–26.4
8	62 ± 3.4[e]	9.34	7.33–11.9
9	40 ± 2.3[f]		
10	44 ± 0.9		
12	73 ± 3.6	18.4	11.4–23.5
13	22 ± 0.7		
14	28 ± 1.4		
16	74 ± 1.4	27.1	24.2–30.0
18	76 ± 3.1[e]	16.5	12.5–19.8
21	57 ± 3.1	3.28	1.76–5.1

[a] Percent inhibition of calcium-induced contraction on 80 mM K^+-depolarized guinea pig longitudinal smooth muscle at 10^{-4} M. The 10^{-4} M concentration gave the maximum effect for most compounds. [b] Calculated from log concentration−response curves (Probit analysis according to Litchfield and Wilcoxon[48] with $n = 6-8$). When the maximum effect was <50%, the IC_{50} values was not calculated. [c] At 10^{-6} M. [d] Taken from ref 17. [e] At 5×10^{-5} M. [f] At 10^{-5} M. * $P < 0.05$.

μM, but a high interindividual variability exists and serum levels above 1 μM are found in some patients.[54,55]

Although these plasma concentrations appear relatively low as compared with those required to displace [^{3}H]diltiazem binding, it should be noted that, as a result of its high lipophilicity, propranolol may accumulate in the cell membrane when administered over long-term, as other lipophilic drugs do.[56] It has been shown that concentrations of some lipophilic drugs, including propranolol and dihydropyridines, are substantially higher over 3 orders of magnitude in the membrane bilayer than in the extramembrane, aqueous surroundings.[57] Accordingly, propranolol could reach concentrations in the cell membrane high enough to inhibit LTCCs. Further experiments are needed to clarify this point.

Conclusions

The procedure presented herein allowed the identification of novel, structurally diverse LTCC blockers that interfere with the diltiazem binding site. With the combined use of in silico and in vitro techniques, we have studied the cardiac DTZ site. The novel compounds exhibit a selective negative inotropic profile because they do not possess chronotropic activity or vasorelaxant effect. The relaxant activity on ileal smooth muscle was low to moderate as well. These specific binding site interactions in α_1-subunit and tissue selectivity could drive toward the development of drugs to treat specifically cardiovascular diseases.

One of the therapeutic approaches of negative inotropic selective compounds is the treatment of hypertrophic obstructive cardiomyopathy (HOCM), otherwise known as idiopathic hypertrophic subaortic stenosis. It is characterized by an abnormal arrangement of the myocardial cells, which form whorl-like patterns instead of lying in parallel rows. It most commonly affects the interventricular septum but may also involve the entire myocardium or occur in isolated areas undetectable except by detailed histopathologic examination without any obvious cause.[58]

A variety of drugs are used in the treatment of HOCM, and the choice of treatment varies from patient to patient. For the relief of palpitations, breathlessness, and chest pains, β_1-blockers may be used. These are widely indicated for other types of heart disease and to reduce high blood pressure and work by slowing the heart beat, thus reducing its force of contraction. Some years ago, it was proposed a combined therapy using a β_1-adrenoceptor antagonist and β_2-agonist,[59] although cardioselective β-adrenergic therapy is contraindicated for patients with the unusual combination of severe asthma and HOCM. For this reason, compounds possessing selective negative inotropic activity not β_1-adrenoceptor antagonism dependent would be desirable.

The results reported here confirm ligand-based virtual screening as an efficient approach to hit-finding and demonstrates that the program SHOP, although originally designed for scaffold hopping, is well-suited for virtual screening when a specific binding feature can be used as an anchor point and also strongly supports the used sequence of the filters. In particular, the filters used to exclude compounds containing toxic, reactive, or otherwise undesired groups and to retain only drug-like compounds with a basic center were determinant to dramatically reduce the database of starting structures from 49000 to a final 36 compounds. Then, a selection of 18 compounds (15 with moderate to high similarity toward the two templates and 3 with low similarity) has been examined. In vitro screening of this selection revealed that 60% (9 out of 15) of the compounds exhibited the desired pharmacological profile. In particular, *N*-[2-(dimethylamino)ethyl]-3-hydroxy-2-naphthamide, *N*,*N*-dimethyl-*N*′-(2-pyridin-3-ylquinolin-4-yl)ethane-1,2-diamine, 2-[(4-chlorophenyl)(pyridin-2-yl)methoxy]-*N*,*N*-dimethylethanamine (carbinoxamine), and 7-[2-(diethylamino)ethoxy]-2*H*-chromen-2-one revealed interesting activity and bound to the benzothiazepine site. The validity of the procedure was confirmed by including three low-ranked compounds, which all resulted as inactive.

An overview of the hits found reveals the basic nitrogen atom, a 2- or 3-atoms linker, and a large aromatic moiety such as quinoline, naphthamide, and 2*H*-chromen-2-one relevant for inotropic activity. The sulfonamide moiety, already emerged from the first screening experiment,[17] was confirmed as an interesting molecular feature.

Finally, some structural considerations, common to the DTZ-like LTCC blockers found, were achieved; nitrogen atoms could be either tertiary or secondary but, in the latter case, only within a cycle and lateral with respect to the molecular structure. Also tertiary nitrogen atoms should be lateral, whereas both the chain and the cycle features corresponded to active compounds.

This study confirmed the importance of the basic nitrogen atom for negative inotropic activity and binding to diltiazem site as well. This very general pharmacophore, common between many therapeutic classes, should be coupled with at least two polar atoms about 2−3 Å from each other. All of these features together might help to formulate refined SAR hypotheses and to design further and even more selective LTCC antagonists.

Note. During the revision process, a paper was published by Tikhonov and Zhorov that reported a molecular modeling study on the ligand-binding mode of BTZs.[52] Such a paper could contribute significantly to the understanding of the interaction of benzothiazepines with the LTCC: the 3D structure of the channel, optimized by the authors for the BTZs, could drive toward structure-based approaches for virtual screening and modeling studies.

Experimental Section

Virtual Screening. Databases. The Sigma-Aldrich data set used for screening is part of the release 5 of the ZINC archive,[19] downloaded on January 2005 from the ZINC Web site http://zinc.docking.org. The ZINC database represents a great advance for the following reasons: (i) the ZINC collection is properly suited

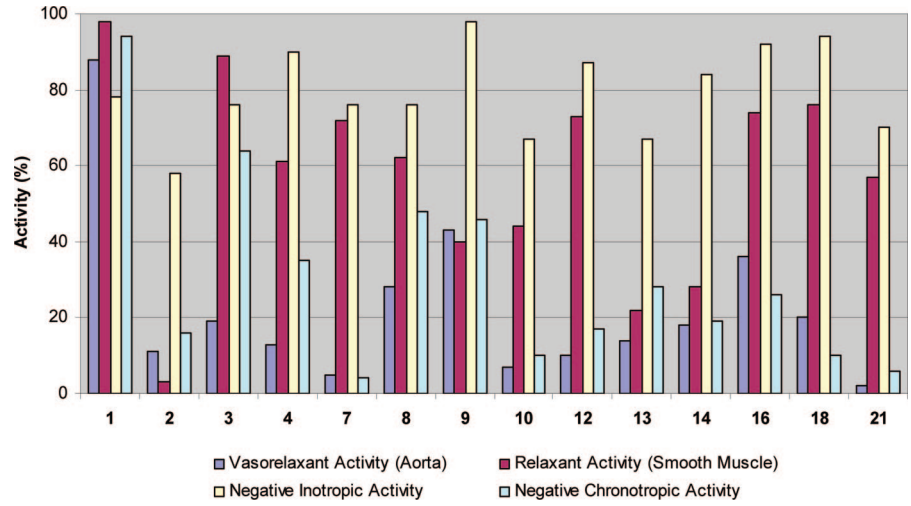


Figure 8. Myorelaxant activity on 80 mM K^+-depolarized guinea pig aortic strips and ileum longitudinal smooth muscle, also compared with negative inotropic activity and negative chronotropic activity, reported for the three reference compounds and for the most interesting hits.

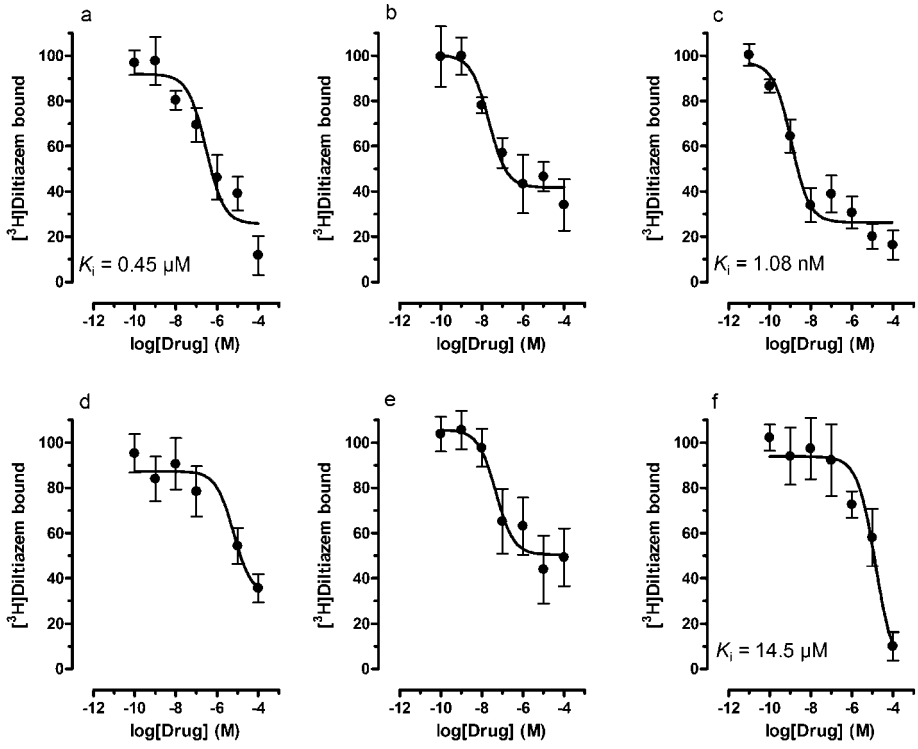


Figure 9. Effects of compounds **4** (a), **7** (b), **8** (c), **10** (d), **13** (e), and **16** (f) on [^{3}H]diltiazem binding.

for virtual screening and docking applications, (ii) the library is available in several modes and in several file formats such as SDF, mol2, and SMILES, (iii) each compound is assigned a unique code and is easily reconducted to the vendor(s), and (iv) each compound is provided in several modes when necessary. The same molecule could be screened with multiple structures in case of acid and/or basic centers, chiral centers, and tautomeric forms.

VolSurf. VolSurf is a program to transform 3D energy maps into molecular descriptors. In the VolSurf procedure, first the GRID[60,61] force field is applied to characterize potential polar and hydrophobic interaction sites around target molecules by the water (OH2), the hydrophobic (DRY), and the hydrogen bonding acceptor carbonyl oxygen (O) probe. Afterward, molecular descriptors are calculated from these 3D maps. The used version 4.1 comprises 94 descriptors covering biologically relevant properties such as shape, surface and volume, electrostatic forces, hydrogen bonding, and lipophilicity. Chemometric tools can be used to relate the VolSurf descriptor matrix with ADME properties: in the models used to determine the PK-profile of data set compounds, PLS was used to quantitatively correlate with aqueous solubility or qualitatively (PLS-discriminant analysis) to classify compounds according to high or low intestinal absorption, metabolic stability, or brain membrane permeability.

SHOP. The calculations were run using the version 2.0 of the program SHOP, which is based on molecular interaction fields (MIF) obtained with the program GRID.[60] MIF are widely used in the field of computer-aided drug design because they well simulate the interaction between a ligand and the cavity of a protein.[57] Here MIF are encoded in correlogram-like descriptors for fast molecular comparisons. In fact, in the program SHOP, the similarity between two molecules is calculated, according to the Hodgkin index,[62] as the sum of similarity indices obtained along single sets of descriptors used. These are geometrical features such as distances, molecular shape, and pharmacophore regions energetically relevant for the interaction with the biological target, encoded as anchor-GRIND descriptors derived from the hydrophobic (DRY), donor (N1),

acceptor (O), negative (O::), and positive (N1+) charged probes. In the search process, different weights for similarity index can be set. In this case, the molecular comparison was based only on the hydrophobic moieties of the molecules and on their hydrogen bond acceptor atoms: a weight of 1 was assigned to the descriptors obtained with the fields of the hydrophobic probe DRY and hydrogen-bonding donor probes N1+ and N1, whereas a weight of 0 was assigned to all the other descriptors. A conformational analysis using an internal MM3 parametrization was carried out on-the-fly and for each molecule $2n_i$ conformations were generated, where n_i is the number of rotatable bonds of the molecule i. The conformation giving the best score, i.e., that represents the best superposition to both the templates, was stored for each data set compound.

Potential Toxicity and Reactivity Filter. Potentially toxic functional groups as well as reactive ones were removed following the suggestions of several authors.[34,35] Therefore, the presence of the following chemical moieties served as criterion for removal: (a) five or more halogen atoms, (b) two or more cyano or nitro groups, (c) epoxide or aziridine, (d) two or more aliphatic esters, (e) 1,2-dicarbonyl groups, (f) sulfonate and phosphonate esters, (g) aldehydes, (h) acyl or sulfonyl halides, (i) anhydrides, and (j) heteroatom−heteroatom single bonds, when not in a cycle or linked to aromatic/olefinic systems.

In Vitro Screening. Isolated Guinea Pig Left and Right Atria Preparations. Guinea pigs (300−400 g female) were sacrificed by cervical dislocation. After thoracotomy, the heart was immediately removed and washed by perfusion through the aorta with oxygenated Tyrode solution of the following composition (mM): NaCl, 136.9; KCl, 5.4; $CaCl_2$, 2.5; $MgCl_2$, 1.0; $NaH_2PO_4 \cdot H_2O$, 0.4; $NaHCO_3$, 11.9; glucose, 5.5. The physiological salt solution (PSS) was buffered to pH 7.4 by saturation with 95% O_2/5% CO_2 gas, and the temperature was maintained at 37 °C. Isolated guinea pig heart preparations were used, spontaneously breathing right atria and left atria driven at 1 Hz. For each preparation, the entire left and right atria were dissected from the ventricles, cleaned of excess tissue, and hung vertically in a 15 mL organ bath containing the PSS continuously bubbled with 95% O_2/5% CO_2 gas at 35 °C, pH 7.4. The contractile activity was recorded isometrically by means of force transducer (FT 0.3, Grass Instruments, Quincy, MA) using Power Laboratory software (ADInstruments Pty Ltd., Castle Hill, Australia). The left atria were stimulated by rectangular pulses of 0.6−0.8 ms duration and about 50% threshold voltage through two platinum contact electrodes in the lower holding clamp (Grass S88 stimulator, Quincy, MA). The right atrium was in spontaneously activity. After the tissue was beating for several minutes, a length−tension curve was determined and the muscle length was maintained at which elicited 90% of maximum contractile force was observed at the optimal length. A stabilization period of 45−60 min was allowed before the atria were used to test compounds. During the equilibration period, the bathing solution was changed every 15 min and the threshold voltage was ascertained for the left atria. Atrial muscle preparations were used to examine the inotropic and chronotropic activity of the compounds (0.1, 0.5, 1, 5, 10, 50, and 100 μM) first dissolved in DMSO and then diluted with PSS. According to this procedure, the concentration of DMSO in the bath solution never exceeded 0.3%, a concentration that did not produce appreciable inotropic and chronotropic effects. During the construction of cumulative dose−response curves, the next higher concentration of the compounds was added only after the preparation reached a steady state.

Guinea Pig Aortic Strips and Ileum Longitudinal Smooth Muscle (GPILSM). The thoracic aorta and ileum were removed and placed in Tyrode solution of the following composition (mM): NaCl, 118; KCl, 4.75; $CaCl_2$, 2.54; $MgSO_4$, 1.20; KH_2PO_4, 1.19; $NaHCO_3$, 25; glucose, 11 equilibrated with 95% O_2/5% CO_2 gas at pH 7.4. The vessel was cleaned of extraneous connective tissue. Two helicoidal strips (10 mm × 1 mm) were cut from each aorta beginning from the end most proximal to the heart. Vascular strips were then tied with surgical thread (6−0) and suspended in a jacketed tissue bath (15 mL) containing aerated PSS at 35 °C. Aortic strips were secured at one end to plexiglass hooks and connected via the surgical thread to a force displacement transducer (FT 0.3, Grass Instruments Corporation) for monitoring changes in isometric contraction. Aortic strips were subjected to a resting force of 1 g. The intestine was removed above the ileocecal junction. GPILSM segments of 2 cm length were mounted under a resting tension of 300−400 mg. Strips were secured at one end to a force displacement transducer (FT 0.3, Grass Instruments Corporation) for monitoring changes in isometric contraction and washed every 20 min with fresh PSS for 1 h. After the equilibration period, guinea pig aortic strips were contracted by washing in PSS containing 80 mM KCl (equimolar substitution of K^+ for Na^+). When the contraction reached a plateau (about 45 min) various concentrations of the compounds (0.1, 0.5, 1, 5, 10, 50, 100, and 500 μM) were added cumulatively to the bath allowing for any relaxation to obtain an equilibrated level of force. Addition of the drug vehicle had no appreciable effect on K^+-induced contraction (DMSO for all compounds).

Statistical Analysis. Data were analyzed using Student's t test and are presented as mean ± SEM.[48] Because the drugs were added in cumulative manner, only the not significant difference ($P < 0.05$) between control and experimental values at each concentration was indicated by asterisks. The potency of drugs defined as EC_{50}, EC_{30}, and IC_{50} was evaluated from log concentration−response curves (Probit analysis using Litchfield and Wilcoxon[48] or GraphPad Prism software)[63] in the appropriate pharmacological preparations.

Binding Experiments. Cardiomyocytes Isolation. Single cardiac myocytes were isolated from male Sprague−Dawley rats (body weight 376 ± 12 g, n = 19, Charles River Italia, Como, Italy), injected with 500 U/100 g by weight heparin ip, anaesthetized with a mixture of Ketavet (Gellini, Italy) and Rompum (Bayer, Germany), decapitated, and bled.[64] After thoracotomy, the heart was rapidly removed, mounted on a micro-Langendorff apparatus, and perfused for 20 min at 37 °C with a nominally Ca^{2+}-free solution (low Ca^{2+} solution, LCS) of the following composition (mM): NaCl, 120; KCl, 10; HEPES, 10; glucose, 10; $MgCl_2$, 1.2; KH_2PO_4, 1.2; Na-pyruvate, 5; taurine, 20; pH 7.2, equilibrated with 95% O_2/5% CO_2. The solution was then quickly changed to LCS complemented with 0.9 mg/mL collagenase type I (Sigma Chimica, Milan, Italy), 0.05 mg/mL Dispase I (Roche Gmbh, Penzbeg, Germany), and 1.5 mg/mL bovine serum albumin acid-free (Sigma Chimica, Milan, Italy) for 10 −15 min. When the heart was soft, perfusion was stopped and the tissue was chopped into small pieces and gently stirred in fresh LCS at room temperature. The cardiomyocytes that appeared in the supernatant were purified by centrifugation (5 min at 800g), and frozen at −80 °C until use. Pooled cells derived from at least two animals have been used for each binding experiment. Protein concentrations were estimated by using the method of Bradford[65] with bovine serum albumin as standard.

[^{3}H]Diltiazem Binding Assay. [^{3}H]Diltiazem binding assay was performed according to earlier methods[6,17,66] with some modifications. Aliquots of defrozen rat cardiac myocytes (200 μg) were incubated with ligands in 50 mM Tris buffer (pH 7.4) at 25 °C for 90 min in a final volume of 0.2 mL. In homologous competition curves [^{3}H]diltiazem (specific activity 85.5 Ci/mmol) was present at 20 nM in tubes containing increased concentrations of unlabeled diltiazem (5 nM to 100 μM) and at 0.1−20 nM in tubes without unlabeled ligand. In heterologous competition curves, fixed amounts of the tracer (5 nM) were displaced by increasing concentrations of several unlabeled ligands (0.1 nM to 100 μM). The incubation was terminated by a rapid filtration on Whatman GF/B glass fiber filters (presoaked for at least 1 h in polyethyleneimmine 0.5%) and washed three times with 3 mL of ice-cold wash buffer. The filters were then placed in scintillation vials, 5 mL of liquid scintillation added, and the radioactivity measured in a LS5000CE β-counter (Beckman Instruments, Palo Alto, CA). Nonspecific binding was defined by means of 100 μM unlabeled diltiazem. All the experiments were run in triplicate.

Data Analysis. All the experiments were performed by using rat cardiomyocytes, and all the data are reported as mean ± SEM. Saturable binding constants relative to the binding of [^{3}H]diltiazem were calculated by using LIGAND Program,[67,68] while the log of the half-maximal concentration (i.e., the pIC_{50} value) for the inhibition afforded by the derivatives was obtained by plotting specific binding (% of control) versus log of the inhibitor concentration (M) and fitted with a nonlinear (sigmoidal) analysis.[69,70] The K_i was calculated according to the method of Cheng and Prusoff[71] by considering a dissociation constant (K_d) of 108 nM.[17]

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Supporting Information Available: Detail of PK-profile, profile of commercial databases screened, Sigma-Aldrich codes and IUPAC names for the selected compounds, superposition proposed by SHOP for the selected compounds over the templates **1** and **3**. This material is available free of charge via the Internet at http://pubs.acs.org.

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