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1,4-Benzothiazine ATP-Sensitive Potassium Channel Openers: Modifications at the C-2 and C-6 Positions

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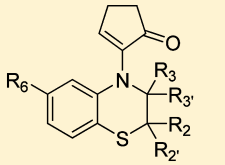
S *Supporting Information*

ABSTRACT: ATP-sensitive potassium (K_{ATP}) channels play a prominent role in controlling cardiovascular function. In this paper, a novel series of 4-(1-oxo-2-cyclopentenyl)-1,4-benzothiazine derivatives modified at the C-2, and C-6 positions were synthesized as openers of vascular K_{ATP} channels. Most of the tested compounds evoked vasorelaxing effects on rat aortic rings and membrane hyperpolarization in human vascular smooth muscle cells, with potency similar or superior to that of the reference levcromakalim (LCRK). The selective K_{ATP} blocker glibenclamide antagonized the above vascular effects, confirming that K_{ATP} channels are closely involved in the mechanism of action. The experimental results confirmed the 1,4-benzothiazine nucleus as an optimal scaffold for activators of vascular K_{ATP} channels; moreover, the high level of potency exhibited by the 6-acetyl substituted benzothiazine **8**, along with the lack of any significant interference with insulin secretion from pancreatic β-cells, paves the way to further develop a new series of potent activators of vascular K_{ATP} channels.



R_2, $R_{2'}$, R_3, $R_{3'}$ = H, Me

R_6 = H, Me, $SO_2N(i\text{-}Bu)_2$, Ac, Et, NH_2, NHAc

INTRODUCTION

The ATP-sensitive potassium (K_{ATP}) channels represent an efficient biological mechanism able to link the metabolic status of cells with their excitability.[1] In fact, they are inward rectifier potassium channels showing a sensitivity to intracellular ATP concentration.[2] In particular, under conditions of satisfactory phosphorylation potential, high intracellular levels of ATP are the primary inhibitory factor on K_{ATP} channels. By contrast, under conditions of reduced energetic metabolism, a decrease in the ATP/ADP ratio and an increase in the ADP level determine the activation of the K_{ATP} channel and thus an outward flow of potassium ions with consequent membrane hyperpolarization.[1]

From a structural point of view, K_{ATP} channels are heteroctameric complexes formed by four pore-forming subunits (Kir6) of the inward rectifier family of potassium channels and by four larger regulatory proteins, the sulfonylurea receptor subunits (SUR) that function as the sensor of the ATP/ADP ratio.[3] Different subtypes of Kir6 proteins (Kir6.1 or Kir6.2) and SUR proteins (SUR1, SUR2A, or SUR2B) form K_{ATP} channels in different tissues.[4] In particular, vascular smooth muscle expresses K_{ATP} channels mainly formed by Kir6.1 and SUR2B.[5] The K_{ATP} channels expressed in vascular smooth muscle cells are of particular interest, as they contribute to the control of membrane potential, arterial diameter, and blood flow regulation.[6] Vascular K_{ATP} channels are able to control the vascular tone through the action of many endogenous modulators such as levels of ATP/ADP,[7] hypercapnic acidosis,[8] many hormones and neurotransmitters,[9] caveolin-1,[10] and even the gaseous modulator hydrogen sulfide.[11] K_{ATP} channels are inhibited by sulfonylurea agents such as glibenclamide and tolbutamide, which are able to block the channel at concentrations spanning from the low nanomolar to the low micromolar range.[7] In the past few decades, the therapeutic potential of K_{ATP} channel opening in cardiovascular diseases like hypertension led to the design of several chemical entities able to activate these channels such as benzopyrans like cromakalim,[12] cyanoguanidines like pinacidil,[13] pyridyl nitrates like nicorandil,[14] thioformamides like aprikalim,[15] benzothiadiazines like diazoxide,[16] and recently propylamines like iptakalim.[17] These potassium channel openers (KCOs) evoke membrane hyperpolarization with consequent inactivation of voltage-operated calcium channels, leading in turn to a reduction of intracellular calcium and vasodilation. This hyperpolarization has been observed in vascular smooth muscle cells[18,19] and is antagonized by glibenclamide but not by blockers of other potassium channels.[16] In coronary arteries of canine and rabbit hearts[20] and in hamster microcirculation, K_{ATP} channels have been found to play an important role in controlling the resting vascular tone. In fact, in both these vascular beds, the administration of glibenclamide evokes vasoconstriction, indicating that K_{ATP} channels are activated even at resting

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conditions.[21] Finally, a new K_{ATP} opener, iptakalim, has been shown to induce arteriolar vasodilation and reduce the blood pressure of hypertensive rodents and humans.[17] Moreover, iptakalim exerted protective effects against hypertensive damage to target organs in rats and improved endothelial dysfunction by selective activation of the Kir6.1/SUR2B subtype of K_{ATP} channels expressed in the vascular endothelium.[17] Presently, its clinical trials for the treatment of mild−moderate hypertension have been completed in China,[22] providing an exciting base to develop additional series of selective K_{ATP} channels openers able to modulate the vascular tone and to be employed in several cardiovascular diseases.

In our previous work on KCOs, we have found that the 1,4-benzothiazine scaffold with a cyclopentenone moiety at the N-4 position, a *gem*-dimethyl group at the C-2 position, and an electron-withdrawing substituent at the C-6 position led to KCOs with the highest potencies.[23] The lead compound of this series is represented by derivative **1** bearing a CF_3 group at the C-6 position (Figure 1).

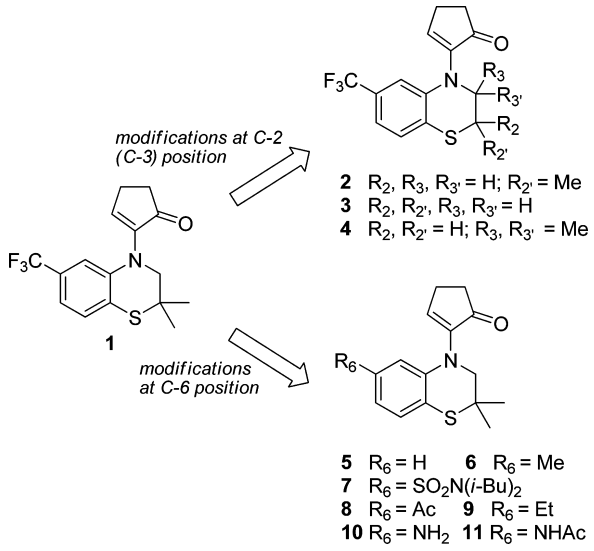


Figure 1. Chemical structure of 1,4-benzothiazine derivative **1** and structural modifications at the C-2/(C-3) and C-6 positions.

The structure−activity relationship (SAR) of this class of KCOs has now been extended by exploring the C-2, C-3, and C-6 positions, thus obtaining derivatives **2**−**11** (Figure 1). In particular, taking the 1,4-benzothiazine derivative **1**[23] as the reference compound, the influence of the *gem*-dimethyl at the C-2 position was examined by deleting one (compound **2**) or both methyl groups (compound **3**). In addition, the *gem*-dimethyl group was shifted from the C-2 to the C-3 position (compound **4**).

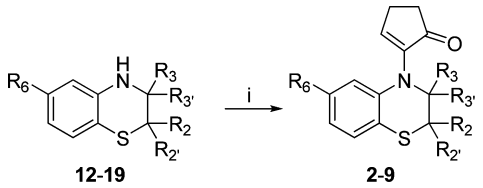
Regarding the C-6 position, it is known that the substituent at this site has a great impact on modulating the biological activity in the benzopyran KCOs.[24] In particular, it has been postulated that the C-6 substituent could contribute to receptor affinity either by a direct interaction with the receptor site or indirectly by withdrawing electrons from the benzene moiety of the benzopyran nucleus, thus increasing charge transfer interactions of the aromatic moiety with the receptor.[25] In early investigations, optimum substitution was attributed to small, electron-withdrawing substituents such as NO_2, CF_3, and CN.[26] Later, it was found that even larger substituents such as phenylsulfonyl[27] or arylaminosulfonyl[28] confered high potency. Further variations of 6-substitution surprisingly showed that alkyl groups, lacking any electron-withdrawing effects, could be incorporated conferring approximately one-third of the activity of the nitrile group.[29]

To date, the C-6 position has been marginally explored in the benzothiazine KCOs.[23] For this reason, we now report the synthesis of new C-6 modified derivatives **5**−**11**. In particular, the CF_3 group of the reference benzothiazine **1** was replaced by groups having different electronic, steric, and hydrogen bond acceptor/donor properties (Figure 1).

CHEMISTRY

The synthetic approach used to obtain the target compounds **2**−**9** involved the preparation of 1,4-benzothiazine intermediates **12**−**19**, appropriately substituted at C-2 and C-6 position. These were then functionalized at the N-4 position with a cyclopentenone moiety by treatment with the crude product obtained from reacting cyclopentanone and NBS in the presence of a catalytic amount of dibenzoyl peroxide (Scheme 1).

Scheme 1[a]

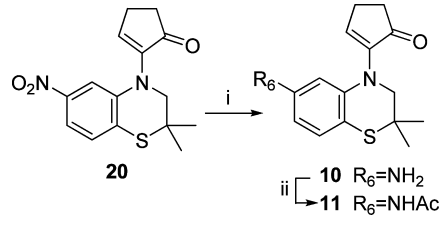


12, 2	R_2=H $R_{2'}$=Me	R_3, $R_{3'}$=H	R_6 = CF_3
13, 3	R_2, $R_{2'}$=H	R_3, $R_{3'}$=H	R_6 = CF_3
14, 4	R_2, $R_{2'}$=H	R_3, $R_{3'}$=Me	R_6 = CF_3
15, 5	R_2, $R_{2'}$=Me	R_3, $R_{3'}$=H	R_6 = H
16, 6	R_2, $R_{2'}$=Me	R_3, $R_{3'}$=H	R_6 = Me
17, 7	R_2, $R_{2'}$=Me	R_3, $R_{3'}$=H	R_6 = $SO_2N(i\text{-}Bu)_2$
18, 8	R_2, $R_{2'}$=Me	R_3, $R_{3'}$=H	R_6 = Ac
19, 9	R_2, $R_{2'}$=Me	R_3, $R_{3'}$=H	R_6 = Et

[a]Reagents and conditions: (i) [cyclopentanone, NBS, DBP, CCl_4], DIPEA, THF, reflux.

The target 6-amino-4-(1-oxo-2-cyclopentenyl)benzothiazine derivative **10** was obtained from the corresponding 6-nitro derivative **20**,[23] previously reported by us, through selective reduction of nitro group with Zn/AcOH. Subsequent acylation of **10** with AcCl in CH_2Cl_2 furnished the target 6-acetamido-4-(1-oxo-2-cyclopentenyl)benzothiazine derivative **11** (Scheme 2).

Scheme 2[a]



[a]Reagents and conditions: (i) Zn, AcOH; (ii) AcCl, DIPEA, CH_2Cl_2.

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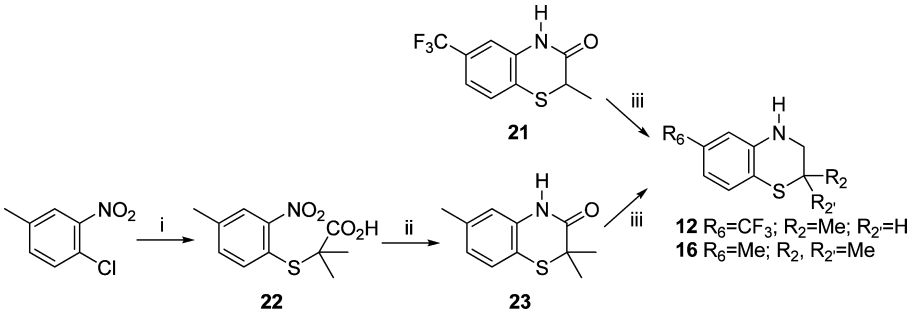
Scheme 3[a]



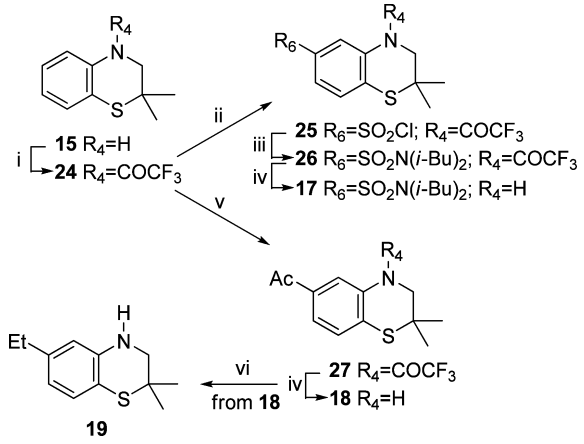
[a]Reagents and conditions: (i) $HSC(Me)_2CO_2H$, TBAB, Cs_2CO_3, DMSO; (ii) Fe, AcOH, H_2O; (iii) $LiAlH_4$, THF.

1,4-Benzothiazine intermediate **12** was prepared from benzothiazinone **21**[30] by reduction with $LiAlH_4$ (Scheme 3), whereas benzothiazines **13**,[31] **14**,[32] and **15**,[33] were prepared following literature procedures.

6-Methylbenzothiazine **16** was obtained by reacting the 1-chloro-4-methyl-2-nitrobenzene with 2-mercapto-2-methypropionic acid in DMSO at 100 °C using Cs_2CO_3 as base and a catalytic amount of tetra-*n*-butylammonium bromide (TBAB). Reductive ring closure of the obtained nitroacid **22** gave benzothiazinone **23**, which was then reduced with $LiAlH_4$ (Scheme 3).

A different synthetic procedure was used to prepare 6-diisobutylsulfonylbenzothiazine **17**, which entails the introduction of the C-6 substituent by electrophilic aromatic substitution on the preformed 1,4-benzothiazine nucleus (Scheme 4), where the C-6 position is the most favored.[34]

Scheme 4[a]



[a]Reagents and conditions: (i) $(CF_3CO)_2O$, Pyr, CH_2Cl_2; (ii) HSO_3Cl; (iii) Et_3N, $NH(i\text{-}Bu)_2$, THF; (iv) MeOH, K_2CO_3; (v) AcCl, $AlCl_3$; (vi) $LiAlH_4$, THF.

Thus, benzothiazine **15**[33] was initially protected at the N-4 position by using $(CF_3CO)_2O$ and then reacted with chlorosulfonic acid/$AlCl_3$, affording the intermediate **25** regioselectively. The structure of **25** and therefore the reaction regioselectivity have been confirmed by X-ray structural analysis (Figure 2). Intermediate **25** was then elaborated to 6-diisobutylsulfonylbenzothiazine **17** by reaction with *N*,*N*-diisobutylamine in THF and in the presence of Et_3N, followed by the removal of the amine protecting group at the N-4 position by treatment with K_2CO_3 in MeOH.

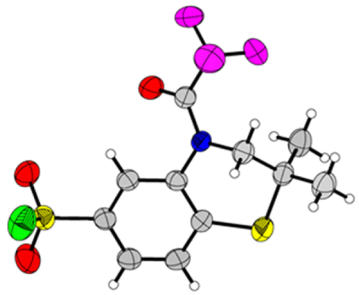


Figure 2. ORTEP drawing of compound **25**.

Following a similar procedure and employing AcCl, 4-trifluoroacetyl intermediate **24** was converted into derivative **27** whose structure was confirmed by ^1H NOESY NMR data (Figure 3). Subsequent deprotection at C-4 position gave the desired 6-acetylbenzothiazine **18**[35] in 71% overall yield (lit. 32%).

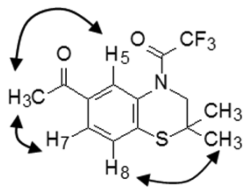


Figure 3. NOESY experiment for **27** showed three main interactions: CH_3CO → H-5; CH_3 → H-7; *gem*-CH_3 → H-8.

Further modification of 6-acetylbenzothiazine **18** by reduction with $LiAlH_4$ in THF provided 6-ethylbenzothiazine **19** (Scheme 4).

RESULTS AND DISCUSSION

The K_{ATP}-opening activity of the synthesized compounds was first tested by evaluating their vasorelaxant effect on endothelium-denuded rat aortic rings precontracted with KCl (25 mM) according to the protocol described in the Experimental Section. The vasorelaxing activity data, expressed as efficacy (%) and potency (pIC_{50}), are reported in Table 1 along with those of reference compounds **1** and levcromakalim (LCRK), a well-known KCO belonging to the benzopyran class.

All of the synthesized compounds exhibited full levels of vasorelaxing efficacy, while a great variation in the potency index could be observed among the compounds, thus

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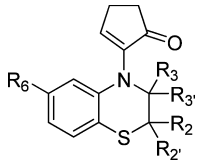
Table 1. Vasorelaxant Activity of the 1,4-Benzothiazine Derivatives 2-11 Modified at the C-2, C-3, and C-6 Positions[a]



compd	R_2	$R_{2'}$	R_3, $R_{3'}$	R_6	E_{max},[b,d] %	pIC_{50}[c,d,e]
2	H	Me	H	CF_3	94 ± 2	8.08 ± 0.07
					(63 ± 6)	(6.15 ± 0.12)
3	H	H	H	CF_3	94 ± 3	6.27 ± 0.09
					(64 ± 4)	(4.63 ± 0.21)
4	H	H	Me	CF_3	89 ± 1	7.18 ± 0.03
					(NT)[f]	(NT)[f]
5	Me	Me	H	H	90 ± 8	6.14 ± 0.12
					(61 ± 7)	(4.68 ± 0.22)
6	Me	Me	H	Me	89 ± 2	6.03 ± 0.05
					(78 ± 3)	(4.71 ± 0.09)
7	Me	Me	H	$SO_2N(i\text{-}Bu)_2$	85 ± 2	6.10 ± 0.05
					(NT)[f]	(NT)[f]
8	Me	Me	H	Ac	88 ± 3	8.84 ± 0.18
					(77 ± 2)	(7.25 ± 0.26)
9	Me	Me	H	Et	97 ± 2	6.81 ± 0.02
					(69 ± 8)	(5.19 ± 0.09)
10	Me	Me	H	NH_2	96 ± 5	5.40 ± 0.13
					(NT)[f]	(NT)[f]
11	Me	Me	H	NHAc	97 ± 10	5.18 ± 0.08
					(NT)[f]	(NT)[f]
1	Me	Me	H	CF_3	92 ± 5	10.38 ± 0.14
					(96 ± 4)	(8.08 ± 0.07)
LCRK					99 ± 5	6.49 ± 0.07
					(93 ± 3)	(5.60 ± 0.13)

[a]Vasorelaxant potency was evaluated on endothelium denuded isolated aortic ring of male normotensive Wistar rats. [b]Maximal vasorelaxing response expressed as percentage of contractile tension. [c]Values are the mean of 5−10 separate experiments ± standard error. [d]In parentheses, the corresponding values obtained in the presence of glibenclamide are reported. [e]Vasorelaxant potency expressed as negative log of the concentration evoking a half-reduction of the contractile tone. [f]NT = not tested in the presence of glibenclamide.

confirming the importance of the C-2 and C-6 substituents in modulating the biological activity.

Although all the synthesized compounds showed a reduction in potency when compared with the benzothiazine progenitor **1**, it is noteworthy that the potency reached nanomolar levels for the 2-monomethyl derivative **2** and the 6-acetyl derivative **8**. In particular, the potency of the latter was nearly 100-fold higher than that exhibited by LCRK (Table 1). Furthermore, derivatives **3**−**7** and **9** exhibited potency parameters comparable with that of LCRK. The 6-amino derivative **10** and the 6-acetamido derivative **11** had the lowest potency, more than 10-fold lower than that of LCRK.

In the modifications carried out at the C-2 position, the removal of one or both methyl groups (compounds **2** and **3**, respectively) caused a progressive reduction of the vasorelaxant potency. These findings are in agreement with those already reported for the KCO benzopyran class,[24c,36] confirming a primary role played by the C-2 *gem*-dimethyl group in obtaining highly active compounds. A reduction in potency was also observed when the *gem*-dimethyl group was shifted from the C-2 to the C-3 position (compound **4**). However, it is worth noting that the C-3 *gem*-dimethyl derivative **4** is as active as LCRK while 2-monomethyl derivative **2** is about 10 times more active than LCRK. The stereochemistry of the methyl group in derivative **2** remains unassigned.

Among the substituents at the C-6 position, the acetyl-substituted derivative **8** elicited the greatest potency, reaching a vasorelaxant activity in the nanomolar range (Table 1). Since the acetyl is a small and electron-withdrawing group, the result is in agreement with the known SAR of the KCO benzopyran class.[24a] Replacement of the acetyl with an acetamide (derivative **11**) resulted in a dramatic decrease in the activity. This suggests (a) the importance of the electron-withdrawing effect, which may occur only if the acetyl group is directly linked to the aromatic ring and/or (b) the need for a precise spatial arrangement to allow the substituent at C-6 to set up hydrogen bond interactions with defined areas of the receptor. Low activity was also observed with derivative **10** bearing a C-6 amino group, which is able to establish hydrogen bond interactions but has electron donor properties. Despite the presence of an electron-withdrawing group, the 6-diisobutyl-sulfonamide derivative **7** did not show high vasorelaxant potency. This result could be explained by considering the steric hindrance of the ramified alkyl chains, supporting the conclusion that the dimension of the C-6 group has some influence on the potency. The replacement of the C-6 trifluomethyl group of compound **1** with a methyl (derivate **6**), which is similar in shape but not in electronic effect, reduced the activity by over 4 orders of magnitude. The same behavior was also observed for the C-6 unsubstituted derivative

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5. Irrespective of the requirements for electron-withdrawing capability and/or hydrogen bond interaction, the 6-ethyl substituted analogue (compound **9**) showed an unexpected increase of potency, which was almost equivalent to that of LCRK. However, the same behavior was also reported in the early SAR studies on the KCO benzopyran class.[29] In an attempt to explain the singular activity of 6-ethyl derivative **9**, we have hypothesized that the 6-ethyl group could be at least partially metabolized in tissue into active 6-acetyl derivative **8**. To support this hypothesis, the software MetaSite[37] was used to predict metabolic transformation of **9** related to cytochrome-mediated reactions in phase I metabolism. The in silico results indicated the 6-ethyl group as the primary site of metabolism (computed score of 7) in all the considered tissues. In addition, the metabolic pathway analysis underlined that the oxidation of the ethyl group into the acetyl is predominant. On the contrary, the MetaSite procedure applied to 6-methyl derivative **6** predicted the corresponding secondary alcohol, which does not have electronic withdrawing properties, as the principal metabolite.

In order to evaluate the involvement of the activation of K_{ATP} channel in the mechanism of action, the vasorelaxing effects of some selected benzothiazines were also tested in the presence of glibenclamide, a well-established selective K_{ATP}-blocker able to antagonize the effects of compound **1** and LCRK. This sulfonylurea significantly antagonized the effects of all the tested compounds, causing a marked and significant rightward shift of the concentration−response curves of compounds **2**, **3**, **5**, **6**, **8**, and **9** (Table 1, Figure 4). These pharmacological results indicate that the activation of vascular K_{ATP} channels is likely to account for the vasorelaxing properties of these compounds.

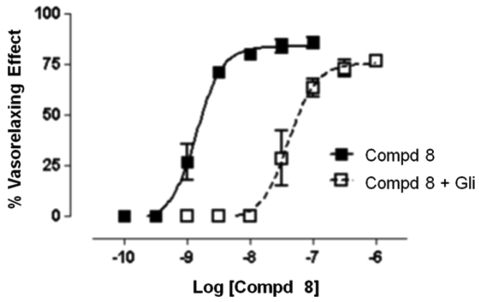

Figure 4. Concentration−vasorelaxing response curve for compound **8**. Increasing concentrations of compound **8** were cumulatively added to endothelium-denuded rat aortic rings KCl in 25 mM, in the absence (black squares) or in the presence (white squares) of 1 μM glibenclamide (Gli). The vertical bars represent the SEM.

An additional experimental approach was aimed to reach a more complete pharmacodynamic characterization and to test the pharmacological profile on human vascular K_{ATP} channels. In particular, the effects of three benzothiazines and LCRK on the membrane potential of cultured human aortic smooth muscle cells (HASMCs) were tested by means of the membrane-potential-sensitive dye bis(1,3-dibutylbarbituric acid)trimethine oxonol (DiBAC4(3)), allowing an indirect electrophysiological recording. Compounds **2** and **8** were selected because of the high level of potency exhibited in the functional tests on rat aortic rings, while compound **9** was selected for its vasorelaxing potency almost comparable to that of the reference drug LCRK (Table 1). The three selected benzothiazines caused a concentration-dependent decrease of the fluorescence of HASMCs preincubated with DiBAC4(3), indicating their hyperpolarizing effects on the membrane potential (Figure 5).

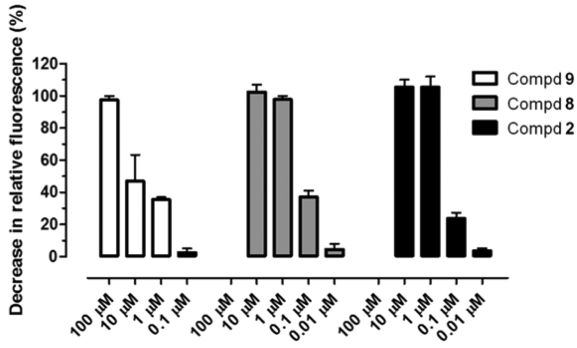

Figure 5. Concentration dependent hyperpolarizing effects induced by compounds **2**, **8**, and **9** in HASMCs. The effects represent the decrease of relative fluorescence, expressed as a % of the maximal effect induced by 100 μM LCRK. Each bar represents the mean value of six independent measurements. Vertical bars represent the SEM.

Noteworthy, the effects of the three benzothiazines and of the reference drug LCRK were strongly antagonized by glibenclamide (Figure 6), indicating that such a membrane

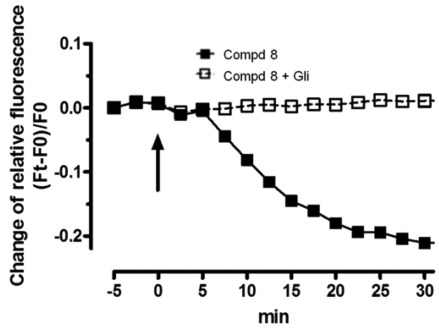

Figure 6. Representative experiment of spectrofluorimetric recording of change in membrane potential of HASMCs, induced by the tested compounds. The addition of compound **8** (10 μM, arrow) caused a significant decrease of relative fluorescence (black squares), indicating a hyperpolarizing effect. This effect was completely prevented when compound **8** was added in the presence of 1 μM glibenclamide (Gli, white squares).

hyperpolarization is almost completely due to the activation of K_{ATP} channels expressed on the sarcolemmal membranes of HASMCs. The maximal effect produced by all these compounds was equivalent to that produced by LCRK (Table 2). The submicromolar potency of compound **9** was comparable with that of LCRK, while the potencies of compounds **2** and **8** were improved by about 1.5 orders of magnitude (Table 2). These results are consistent with the experimental data obtained in the functional studies on rat aortic rings (Table 1), where the potency of compound **9** was equivalent to that of LCRK, while it was notably improved for compounds **2** and **8**. Nevertheless, the potency parameters recorded on HASMCs were always lower than those observed on rat aortic rings. This difference may reflect mechanistic aspects (full vasorelaxing effect does not necessarily require full

Table 2. Hyperpolarizing Effects of Compounds 2, 8, and 9[a]

compd	E_{max},[b,c] %	pEC_{50} [d,c]	Gli[e]
2	106 ± 5	6.87 ± 0.06	+
8	103 ± 5	6.93 ± 0.02	+
9	98 ± 3	5.13 ± 0.03	+
LCRK	100	5.27 ± 0.08	+

[a]Hyperpolarizing effects were spectrofluorimetrically evaluated by means of the membrane-potential-sensitive dye DiBAC4(3) on HASMCs. [b]Maximal response expressed as percentage of the maximal hyperpolarization evoked by the reference drug LCRK (100 μM). [c]Values are the mean of six replicates ± standard error. [d]Potency value expressed as negative log of the concentration evoking a half-maximal response. [e]+ indicates that the hyperpolarizing effects were significantly antagonized by glibenclamide (Gli).

hyperpolarization) or may be due to difference of species and/or methodological approaches.

Since the activation of pancreatic β-cell K_{ATP} channels, with consequent inhibition of insulin release and possible hyperglycaemia, is one of the most usual and worrying adverse effects of poorly selective K_{ATP} activators, a final experimental approach was aimed at evaluating the influence of two selected compounds, **8** and **9**, on the release of insulin in isolated rat pancreatic islets, according to standard procedures.[38] The results are shown in Figure 7. When exposed to low levels of glucose (LG, 2.8 mM), the isolated islets exhibited a basal insulin secretion (3.23 ± 0.40 ng/mL). Stimulation with high levels of glucose (HG, 16.7 mM) led to a significant 3-fold increase in insulin release (9.36 ± 0.68 ng/mL). Such a secretagogue effect was further increased by the K_{ATP}-blocker glibenclamide. Indeed, in the presence of HG and 1 μM glibenclamide the insulin release achieved 12.89 ± 0.98 ng/mL.

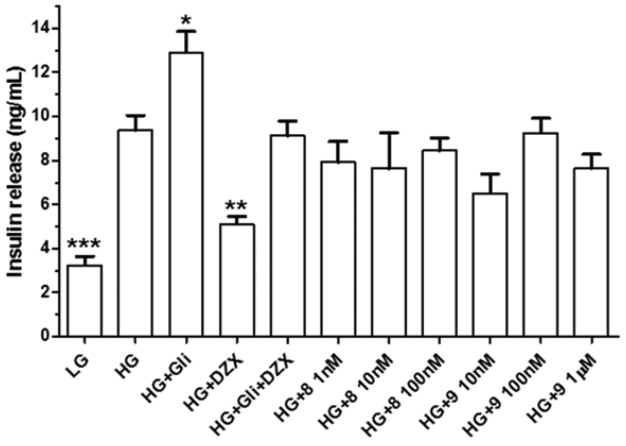

Figure 7. Histograms reflect the insulin release detected after 1 h of incubation in KRH buffer of batches of isolated rat pancreatic islets exposed to low glucose (LG) (2.8 mM) or high glucose (HG) (16.7 mM) concentrations. Moreover, the effects of the K_{ATP} blocker glibenclamide 1 μM (HG + Gli), the K_{ATP} activator diazoxide 80 μM (HG + DZX), and their combination (HG + Gli + DZX) on the HG-induced insulin release are reported. The histograms show the effects of different concentrations of compounds **8** (HG + 8) and **9** (HG + 9) on the HG-induced insulin release. The total islet insulin content, as determined at the end of the incubation, was homogeneous among the islet batches. Data are expressed as mean ± SEM (vertical bars) of at least five replicas for each treatment. The asterisks indicate significant differences, with respect to HG (*, $P < 0.05$; **, $P < 0.01$; ***, $P \leq 0.001$).

In order to evaluate the effects of the activation of K_{ATP} channels, diazoxide (80 μM) was selected as reference drug, since it is known to produce inhibition of insulin release (and even vasorelaxing effects).[39] As expected, the HG-induced secretion of insulin was almost half-reduced by diazoxide. Glibenclamide antagonized the effects of diazoxide, indicating that the diazoxide-mediated decrease of insulin release is actually due to activation of K_{ATP} channels. Noteworthy, neither compound **8** nor compound **9** (at those concentrations evoking strong vasorelaxing effects) was able to significantly influence the HG-induced release of insulin. Such experimental evidence indicates that these new K_{ATP} activators are highly vasoselective and that their vasorelaxing effects are unlikely to be associated with significant metabolic adverse effects due to impairment of insulin release and consequent hyperglycaemia.

In summary, SAR analysis of the 1,4-benzothiazine series clearly shows a good overlapping with that of the benzopyran KCOs. Most of the benzothiazine derivatives synthesized in this study showed a potency similar or superior to that of LCRK. These findings confirm the 1,4-benzothiazine nucleus as an excellent scaffold to warrant good vasorelaxant activity mediated by the activation of K_{ATP} channels. Moreover, the high level of potency exhibited by the 6-acetyl substituted benzothiazine **8**, associated with the lack of any significant interference with insulin secretion from pancreatic β-cells, paves the way to the development of new series of powerful and safe activators of vascular K_{ATP} channels.

EXPERIMENTAL SECTION

Chemistry. All starting materials were commercially available, unless otherwise indicated. Reagents and solvents were purchased from common commercial suppliers and were used as such. Organic solutions were dried over anhydrous Na_2SO_4 and concentrated with a rotary evaporator at low pressure. All reactions were routinely checked by thin-layer chromatography (TLC) on silica gel $60F_{254}$ (Merck) and visualized by using UV or iodine. Column chromatography separations were carried out on Merck silica gel 60 (mesh 70−230), and flash chromatography separations were carried out on Merck silica gel 60 (mesh 230−400). Melting points were determined in capillary tubes (Büchi Electrothermal model 9100) and are uncorrected. Yields were of purified products and were not optimized. ^{1}H NMR spectra were recorded at 200 or 400 MHz (BrukerAvance DRX-200 or 400, respectively), and 2D ^{1}H NMR NOESY was run in face sensitive mode. Spectra were acquired at 298 K. Deuterochloroform was used as solvent unless otherwise indicated. Chemical shifts (δ) are given in ppm relative to TMS and calibrated using residual undeuterated solvent as internal reference. Coupling constants (*J*) are reported in Hz. Data processing was performed with standard Bruker software XwinNMR, and the spectral data are consistent with the assigned structures. GC−MS analyses were carried out with an HP 6890 gas chromatograph (25 m dimethylsilicone capillary column) equipped with an HP 5973 mass selective detector. The purity of the compounds was determined by combustion analysis employing a Fisons elemental analyzer, model EA1108CHN, and data for C, H, and N are within 0.4% of the theoretical values (≥96%).

General Procedure for the Preparation of the Target Derivatives 2−9. A mixture of freshly distilled cyclopentanone (16.88 g, 17.75 mL, 30 mmol), NBS (35.78 g, 30 mmol), and a catalytic amount of dibenzoyl peroxide in dry CCl_4 (20 mL) was refluxed for 3 h under nitrogen atmosphere, cooled, filtered, and evaporated to dryness. The obtained dark oil was quickly poured into a solution benzothiazine intermediate **12−19** (1 mmol) in dry THF (15 mL), and then freshly distilled DIPEA (3.86 g, 5.21 mL, 24 mmol) was added dropwise. The resulting mixture was stirred at room temperature for 10−15 h, and then Et_2O was added. The dark precipitate was filtered off, washing with Et_2O. The filtrate was sequentially washed with 1 N HCl, brine, dried, and evaporated to

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dryness. The obtained residue was purified by flash column chromatography, eluting with cyclohexane/EtOAc (95:5) to cyclohexane/EtOAc (85:15). Additional purification method, yield, melting point, and spectral data of each compound are given below.

2-Methyl-4-(1-oxo-2-cyclopenten-2-yl)-6-trifluoromethyl-3,4-dihydro-2*H*-1,4-benzothiazine (2). Recrystallized from petroleum ether/Et_2O, 14% yield, white solid, mp 94−95 °C. ^{1}H NMR (400 MHz) δ 1.35 (3H, d, J = 6.6 Hz, CH_3), 2.50−2.60 and 2.60−2.70 (each 2H, m, CH_2CH_2CO), 3.25 (1H, dd, J = 8.8, 13.2 Hz, CH_2), 3.30−3.40 (1H, m, SCH), 4.20 (1H, dd, J = 2.7, 13.2 Hz, CH_2), 6.95 (1H, t, J = 3.1 Hz, vinyl-H), 7.00−7.10 (2H, m, H-5 and H-7), 7.15 (1H, d, J = 8.1 Hz, H-8). GC−MS: m/z = 314 (15) [M^+ + 1], 313 (68) [M^+], 299 (10), 298 (53), 285 (23), 284 (100), 256 (12), 242 (20), 241 (10), 230 (11), 217 (10). Anal. ($C_{15}H_{14}F_3NOS$) C, H, N.

4-(1-Oxo-2-cyclopenten-2-yl)-6-trifluoromethyl-3,4-dihydro-2*H*-1,4-benzothiazine (3). Recrystallized from EtOH, 31% yield, white solid, mp 120−121 °C. ^{1}H NMR (200 Hz) δ 2.55−2.75 (4H, m, CH_2CH_2CO), 3.05−3.15 (2H, m, SCH_2), 3.95−4.00 (2H, m, CH_2), 6.95 (1H, t, J = 2.9 Hz, vinyl-H), 7.05−7.15 (2H, m, H-5 and H-7), 7.20−7.30 (1H, m, H-8). GC−MS: m/z = 299 (63) [M^+], 285 (10), 284 (100), 243 (11), 242 (15). Anal. ($C_{14}H_{12}F_3NOS$) C, H, N.

3,3-Dimethyl-4-(1-oxo-2-cyclopenten-2-yl)-6-trifluoromethyl-3,4-dihydro-2*H*-1,4-benzothiazine (4). Recrystallized from Et_2O/petroleum ether, 20% yield, whitish solid, mp 87−88 °C. ^{1}H NMR (200 Hz) δ 1.30 (6H, s, CH_3), 2.50−2.60 and 2.75−2.85 (each 2H, m, CH_2CH_2CO), 2.95 (2H, s, SCH_2), 6.50 (1H, bs, H-5), 6.85−6.95 (1H, m, H-7), 7.15 (1H, d, J = 8.3 Hz, H-8), 7.60 (1H, t, J = 2.8 Hz, vinyl-H). GC−MS: m/z = 327 (100) [M^+], 312 (77), 284 (14), 271 (49), 230 (56), 217 (20). Anal. ($C_{16}H_{16}F_3NOS$) C, H, N.

2,2-Dimethyl-4-(1-oxo-2-cyclopenten-2-yl)-3,4-dihydro-2*H*-1,4-benzothiazine (5). 79% yield, yellow oil. ^{1}H NMR (MeOH-d_4) (200 MHz) δ 1.30 (6H, s, CH_3), 2.25−2.45 and 2.55−2.65 (each 2H, m, CH_2CH_2CO), 3.45 (2H, s, CH_2), 6.55 (1H, dt, J = 1.4, 8.1 Hz, H-6), 6.70 (1H, dd, J = 1.5, 7.6 Hz, H-8), 6.75−6.85 (1H, m, H-7), 6.90 (1H, dd, J = 1.7, 7.6 Hz, H-5), 7.19 (1H, t, J = 3.1 Hz, vinyl-H). GC−MS: m/z = 260 (14) [M^+ + 1], 259 (79) [M^+], 217 (15), 216 (100), 203 (12), 188 (18), 162 (14), 161 (13). Anal. ($C_{15}H_{17}NOS$) C, H, N

4-(1-Oxo-2-cyclopenten-2-yl)-2,2,6-trimethyl-3,4-dihydro-2*H*-1,4-benzothiazine (6). Recrystallized from petroleum ether, 15% yield, orange solid, mp 63−65 °C. ^{1}H NMR (200 MHz) δ 1.40 (6H, s, CH_3), 2.20 (3H, s, $ArCH_3$), 2.50−2.60 and 2.60−2.70 (each 2H, m, CH_2CH_2CO), 3.60 (2H, s, CH_2), 6.60−6.75 (2H, m, H-5 and H-7), 6.95 (1H, d, J = 8.0 Hz, H-8), 7.10 (1H, t, J = 3.0 Hz, vinyl-H). GC−MS: m/z = 274 (26) [M^+ + 1], 273 (100) [M^+], 231 (26), 230 (100), 216 (20), 202 (30), 176 (23), 175 (20). Anal. ($C_{16}H_{19}NOS$) C, H, N.

***N*,*N*-Diisobutyl-2,2-dimethyl-4-(5-oxo-1-cyclopenten-1-yl)-3,4-dihydro-2*H*-1,4-benzothiazine-6-sulfonamide (7).** Recrystallized from petroleum ether/Et_2O, 15% yield, white solid, mp 115−116 °C. ^{1}H NMR (200 MHz) δ 0.95 (12H, d, J = 6.6 Hz, $CH_2CH(CH_3)_2$), 1.45 (6H, s, CH_3), 1.85−2.00 (2H, m, $CH_2CH(CH_3)_2$), 2.50−2.55 and 2.60−2.65 (each 2H, m, CH_2CH_2CO), 2.70 (4H, d, J = 7.5 Hz, $CH_2CH(CH_3)_2$), 3.60 (2H, s, CH_2), 7.00−7.05 (1H, m, H-5), 7.10−7.20 (3H, m, H-7, H-8 and vinyl-H). GC−MS: m/z = 450(29) [M^+], 407 (35), 258 (100), 207 (10). Anal. ($C_{23}H_{34}N_2O_3S_2$) C, H, N.

6-Acetyl-2,2-dimethyl-4-(1-oxo-2-cyclopenten-2-yl)-3,4-dihydro-2*H*-1,4-benzothiazine (8). Recrystallized from Et_2O, 51% yield, yellow solid, mp 131−132 °C. ^{1}H NMR (200 MHz) δ 1.40 (6H, s, CH_3), 2.45 (3H, s, CH_3CO), 2.50−2.55 and 2.55−2.60 (each 2H, m, CH_2CH_2CO), 3.55 (2H, s, CH_2), 7.05−7.15 (2H, m, H-8 and vinyl-H), 7.30−7.40 (2H, m, H-5 and H-7). GC−MS: m/z = 301 (83) [M^+], 258 (100), 245 (16), 230 (19), 204 (14), 188 (12). Anal. ($C_{17}H_{19}NO_2S$) C, H, N.

2,2-Dimehyl-6-ethyl-4-(1-oxo-2-cyclopenten-2-yl)-3,4-dihydro-2*H*-1,4-benzothiazine (9). Recrystallized from petroleum ether, 34% yield, white solid, mp 98−99 °C. ^{1}H NMR (200 MHz) δ 1.15 (3H, t, J = 7.6 Hz, CH_2CH_3), 1.35 (6H, s, CH_3), 2.30−2.65 (6H, m, CH_2CH_2CO and CH_2CH_3), 3.60 (2H, s, CH_2), 6.65−6.70 (2H, m, H-5 and H-7), 6.95 (1H, d, J = 8.6 Hz, H-8), 7.05 (1H, t, J = 3.0 Hz, vinyl-H). GC−MS: m/z = 288 (21) [M^+ + 1], 287 (100) [M^+], 245 (18), 244 (99), 231 (18), 216 (20), 190 (10). Anal. ($C_{17}H_{21}NOS$) C, H, N.

6-Amino-2,2-dimethyl-4-(1-oxo-2-cyclopenten-2-yl)-3,4-dihydro-2*H*-1,4-benzothiazine (10). A solution of 6-nitro-4-cyclopentenyl-2*H*-1,4-benzothiazine **20**[23] (0.1 g, 0.31 mmol) in AcOH (5 mL) and MeOH (5 mL) was added dropwise to a suspension of Zn powder (0.11 g, 1.68 mmol) in water (1 mL) cooled to 0 °C. After the addition was complete, the mixture was stirred at room temperature for 30 min and then filtered over Celite. The filtrate was diluted with water, basified with concentrated NH_4OH, and extracted with EtOAc. The organic layer was washed with brine, dried, and evaporated to dryness. The crude dark residue was purified by flash column chromatography, eluting with cyclohexane/EtOAc (8:2 to 1:1) to give **10** as a yellow oil (0.027 g, 30%). ^{1}H NMR (200 MHz) δ 1.40 (6H, s, CH_3), 2.45−2.50 and 2.60−2.65 (each 2H, m, CH_2CH_2CO), 3.60 (2H, s, CH_2), 6.20−6.30 (2H, m, H-5 and H-7), 6.85 (1H, d, J = 8.2 Hz, H-8), 7.15 (1H, t, J = 3.0 Hz, vinyl-H). GC−MS: m/z = 275 (19) [M^+ + 1], 274 (100) [M^+], 232 (11), 231 (69), 203 (15), 177 (13). Anal. ($C_{15}H_{18}N_2OS$) C, H, N.

6-Acetamido-2,2-dimethyl-4-(1-oxo-2-cyclopenten-2-yl)-3,4-dihydro-2*H*-1,4-benzothiazine (11). A solution of acetyl chloride (0.086 g, 0.078 mL, 0.99 mmol) in dry CH_2Cl_2 (3 mL) was added dropwise and under nitrogen atmosphere to a solution of cyclopentenyl derivative **10** (0.25 g, 0.87 mmol) and dry DIPEA (1.17 g, 1.58 mL, 9.05 mmol) in dry CH_2Cl_2 (10 mL) cooled to −10 °C. After 10 min the mixture was poured into ice−water and acidified to pH 5 with 0.2 N HCl. The organic layer was separated, washed with water, dried, and evaporated to dryness. The crude brown residue was crystallized from EtOH, giving the cyclopentenyl derivative **11** (0.19 g, 65%) as a yellow solid, mp 182−183 °C. ^{1}H NMR (200 MHz) δ 1.35 (6H, s, CH_3), 2.05 (3H, s, $COCH_3$), 2.50−2.60 and 2.60−2.70 (each 2H, m, CH_2CH_2CO), 3.55 (2H, s, CH_2), 6.65 (1H, dd, J = 2.2, 8.4 Hz, H-7), 6.90 (1H, d, J = 8.4 Hz, H-8), 7.15 (1H, t, J = 3.0 Hz, vinyl-H), 7.25 (1H, d, J = 2.2 Hz, H-5), 7.35 (1H, bs, NH). GC−MS: m/z = 318 (19) [M^+ + 2], 317 (55) [M^+ + 1], 316 (95) [M^+], 275 (19), 274 (53), 273 (100), 256 (19), 253 (10), 252 (15), 245 (18), 231 (11), 219 (18), 218 (11), 207 (20), 176 (11). Anal. ($C_{17}H_{20}N_2O_2S$) C, H, N.

2-Methyl-6-trifluoromethyl-3,4-dihydro-2*H*-1,4-benzothiazine (12). A solution of **21**[30] (0.80 g, 3.24 mmol) in dry THF (50 mL) was added dropwise and under nitrogen atmosphere to a suspension of $LiAlH_4$ (0.31 g, 8.17 mmol) in THF (20 mL) cooled to 0 °C. After the addition was complete, the mixture was refluxed for 3 h and then cooled, and EtOAc was carefully added to destroy the excess $LiAlH_4$. The mixture was diluted with water and extracted with EtOAc. The combined organic layers were washed with HCl, 2 N, brine, and then dried and evaporated to dryness, yielding the title compound **12** as a whitish solid (0.49 g, 65%), which was used in the next step without further purification, mp 106−107 °C. ^{1}H NMR (400 MHz) δ 1.50 (3H, d, J = 6.7 Hz, CH_3), 3.35 (1H, dd, J = 7.9, 11.8 Hz, CH_2), 3.45−3.55 (1H, m, SCH), 3.75 (1H, dd, J = 2.8, 11.8 Hz, CH_2), 4.30 (1H, bs, NH), 6.80 (1H, d, J = 1.7 Hz, H-5), 6.95 (1H, dd, J = 1.7, 8.1 Hz, H-7). 7.20 (1H, d, J = 8.1 Hz, H-8). GC−MS: m/z = 233 (100) [M^+], 232 (16), 218 (34), 217 (14), 214 (16), 205 (16), 204 (96), 200 (10), 198 (14), 185 (17), 184 (25), 172 (21), 157 (12), 145 (10).

2,2,6-Trimethyl-3,4-dihydro-2*H*-1,4-benzothiazine (16). The title compound was prepared according to the procedure used for compound **12** starting from 2,2,6-trimethylbenzothiazinone **23**. Compound **16** was obtained in 94% yield as a whitish solid, mp 67−68 °C. ^{1}H NMR (200 MHz) δ 1.40 (6H, s, CH_3), 2.20 (3H, s, $ArCH_3$), 3.25 (2H, s, CH_2), 4.05 (1H, bs, NH), 6.35 (1H, s, H-5), 6.45 (1H, dd, J = 1.2, 7.8 Hz, H-7), 6.85 (1H, d, J = 7.8 Hz, H-8).

***N*,*N*-Diisobutyl-2,2-dimethyl-3,4-dihydro-2*H*-1,4-benzothiazine-6-sulfonamide (17).** A mixture of **26** (2.07 g, 4.31 mmol) and dry K_2CO_3 (1.24 g, 8.97 mmol) in dry MeOH (20 mL) was stirred for 20 min. The solvent was evaporated to dryness, and the obtained residue was treated with water. The formed precipitate was filtered off and dried to give **17** (1.66 g, 98%) as a whitish solid which was used in the next step without further purification, mp 119−120 °C. ^{1}H NMR (400 MHz) δ 0.90 (12H, d, J = 6.6 Hz, $CH_2CH(CH_3)_2$), 1.40 (6H, s, CH_3), 1.85−1.95 (2H, m, $CH_2CH(CH_3)_2$), 2.85 (4H, d, J = 7.5 $CH_2CH(CH_3)_2$), 3.25 (2H, s, CH_2), 6.90 (1H, d, J = 1.7 Hz, H-5),

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6.95 (1H, dd, J = 1.7, 8.1 Hz, H-7), 7.05 (1H, d, J = 8.1 Hz, H-8). GC−MS: m/z = 370 (50) [M+], 327 (63), 242 (41), 178 (100), 122 (13).

6-Acetyl-2,2-dimethyl-3,4-dihydro-2*H*-1,4-benzothiazine (18).[32] The title compound was prepared according to the procedure used for compound **17** starting from 4-trifluoroacetyl derivative **27**. The crude dark product was purified by flash column chromatography, eluting with cyclohexane/EtOAc, 9:1, followed by crystallization from petroleum ether/Et_2O (9:1) to give **18** (75%) as colorless solid, mp 108−110 °C (lit., mp 109−110 °C).[32] ^{1}H NMR (200 MHz) δ 1.45 (6H, s, CH_3), 2.50 (3H, s, CH_3CO), 3.25 (2H, d, J = 2.9 Hz, CH_2), 4.25 (1H, bs, NH), 7.00 (1H, d, J = 8.1 Hz, H-8), 7.15 (1H, d, J = 1.8 Hz, H-5), 7.20 (1H, dd, J = 1.8, 8.1 Hz, H-7). GC−MS: m/z = 222(13) [M^+ + 1], 221 (99) [M^+], 179 (14), 178 (100), 166 (10), 136 (10), 135 (12).

6-Ethyl-2,2-dimethyl-3,4-dihydro-2*H*-1,4-benzothiazine (19). The title compound was prepared according to the procedure used for compound **12** starting from 6-acetylbenzothiazine **18**. The crude reaction product was purified by flash column chromatography, eluting with cyclohexane/EtOAc, 9:1, to yield **19** as a brown oil (52%). ^{1}H NMR (200 MHz) δ 1.25 (3H, t, J = 7.6 Hz, CH_2CH_3), 1.45 (6H, s, CH_3), 2.55 (2H, q, J = 7.6 Hz, CH_2CH_3),), 3.25 (2H, s, CH_2), 3.95 (1H, bs, NH), 6.40 (1H, d, J = 1.8 Hz, H-5), 6.55 (1H, dd, J = 1.8, 8.0 Hz, H-7), 6.90 (1H, d, J = 8.0 Hz, H-8). GC−MS: m/z = 208 (25) [M^+ + 1], 207 (100) [M^+], 192 (40), 165 (18), 164 (95), 152 (21), 149 (21), 136 (15).

2-Methyl-2-[(4-methyl-2-nitrophenyl)thio]propanoic Acid (22). A catalytic amount of TBAB (5−10% w/w) was added to a stirred solution of 4-chloro-3-nitrotoluene (3.00 g, 2.31 mL, 17.48 mmol) in dry DMSO (10 mL) heated at 100 °C. After 5 min, Cs_2CO_3 (17.10 g, 52.48 mmol) and 2-mercapto-2-methylpropanoic acid[40] (4.19 g, 34.87 mmol) were added. After 2 h, the mixture was cooled, poured into ice−water, washed with EtOAc, acidified to pH 2 with 12 N HCl, and extracted with EtOAc. The combined organic layers were washed with brine, dried, and evaporated to dryness. The residue was treated with water, filtered off, and dried, yielding **22** (4.03 g, 90%) as a yellow ochre solid which was used in the next step without further purification, mp 100−102 °C. ^{1}H NMR (200 MHz) δ 1.50 (6H, s, CH_3), 2.45 (3H, s, $ArCH_3$), 7.35 (1H, ddd, J = 0.7, 1.9, 8.0 Hz, H-5), 7.55 (1H, d, J = 8.0, H-6), 7.60 (1H, dd, J = 0.7, 1.9 Hz, H-3).

2,2,6-Trimethyl-2*H*-1,4-benzothiazin-3(4*H*)-one (23). Activated Fe powder (3.94 g, 70.56 mmol) was added portionwise to a suspension of acid **22** (4.00 g, 15.67 mmol) in a mixture of AcOH (30 mL) and water (10 mL). After 1 h the reaction mixture was filtered over Celite, washing with AcOH, and the filtrate was diluted with water. The obtained precipitate was filtered off and washed with a saturated solution of $NaHCO_3$ and dried to give **23** (2.08 g, 64%) as whitish solid which was used in the next step without further purification, mp 176−177 °C. ^{1}H NMR (200 MHz) δ 1.50 (6H, s, CH_3), 2.35 (3H, s, $ArCH_3$), 6.70 (1H, s, H-5), 6.85 (1H, d, J = 8.0 Hz, H-7), 7.20 (1H, d, J = 8.0 Hz, H-8), 8.65 (1H, bs, NH).

2,2-Dimethyl-4-trifluoroacetyl-3,4-dihydro-2*H*-1,4-benzothiazine (24). A solution of trifluoroacetic anhydride (18.75 g, 12.58 mL, 183.66 mmol) in dry CH_2Cl_2 (10 mL) was added dropwise to a solution of 1,4-benzothiazine **1S**[33] (10 g, 55.86 mmol) in dry CH_2Cl_2 (30 mL) and dry pyridine (8.81 g, 8.99 mL, 111.58 mmol) cooled to 0 °C. The mixture was stirred for 10 min and then poured into ice−water. The organic layer was separated and evaporated to dryness. The residue was dissolved in EtOAc and sequentially washed with 2 N HCl, water, and brine, then dried and evaporated to dryness, giving **24** as a red oil (14.6 g, 97%) which was used in the next step without further purification. ^{1}H NMR (200 MHz) δ 1.50 (6H, s, CH_3), 3.80 (2H, bs, CH_2), 7.00−7.25 (4H, m, ArH). GC−MS: m/z = 276 (17) [M^+ + 1], 275 (100) [M^+], 232 (11), 206 (50), 178 (11), 163 (23), 144 (13), 136 (24).

2,2-Dimethyl-4-trifluoroacetyl-3,4-dihydro-2*H*-1,4-benzothiazine-6-sulfonyl Chloride (25). Chlorosulfonic acid (21.10 g, 12.04 mL, 181.1 mmol) was added dropwise under nitrogen atmosphere to 4-trifluoroacetylbenzothiazine **24** (10.0 g, 36.30 mmol) cooled to 0 °C. The reaction mixture was stirred at room temperature for 2 h, then poured into ice−water and extracted with EtOAc. The combined organic layers were washed with water, brine, then dried and evaporated to dryness. The obtained solid residue was purified by flash column chromatography, eluting with cyclohexane/EtOAc, 9:1, to give **25** (4.48 g, 33%) as a yellowish solid, mp 88−89 °C. ^{1}H NMR (400 MHz) δ 1.60 (6H, s, CH_3), 4.00 (2H, bs, CH_2), 7.45 (1H, d, J = 8.5 Hz, H-8), 7.85 (1H, d, J = 8.5 Hz, H-7), 8.30 (1H, bs, H-5).

***N*,*N*-Diisobutyl-2,2-dimethyl-4-trifluoroacetyl-3,4-dihydro-2*H*-1,4-benzothiazine-6-sulfonamide (26).** A mixture of *N*,*N*-diisobutylamine (0.69 g, 0.93 mL, 5.35 mmol) and Et_3N (0.54 g, 0.74 mL, 5.35 mmol) in dry THF (10 mL) was added dropwise to a solution of sulfonyl chloride derivative **25** (2.00 g, 5.35 mmol) in dry THF (10 mL). The mixture was stirred at 0 °C for 1 h and at room temperature for 3 h, then poured into ice−water and extracted with EtOAc. The combined organic layers were washed with brine, dried, and evaporated to dryness to give derivative **26** (2.49 g, 99%) as a light yellow solid which was used in the next step without further purification, mp 84−85 °C. ^{1}H NMR (400 MHz) δ 0.90 (12H, d, J = 6.6 Hz, $CH_2CH(CH_3)_2$), 1.50 (6H, s, CH_3), 1.85−1.95 (2H, m, $CH_2CH(CH_3)_2$), 2.85 (4H, d, J = 7.5 Hz, $CH_2CH(CH_3)_2$), 3.85 (2H, bs, CH_2), 7.20−7.35 (1H, m, H-8), 7.50−8.10 (2H, m, H-5 and H-7). GC−MS: m/z = 466 (8) [M+], 425 (24), 424 (42), 423 (100), 340 (11), 339 (16), 338 (74), 290 (21), 275 (17), 274 (79), 218 (10), 190 (14).

6-Acetyl-2,2-dimethyl-3,4-dihydro-4-trifluoroacetyl-2*H*-1,4-benzothiazine (27). Under strictly dry conditions, freshly distilled acetyl chloride (6.83 g, 6.19 mL, 87.01 mmol) was added dropwise to a mechanically stirred mixture of 4-trifluoroacetylbenzothiazine **24** (8.00 g, 29.10 mmol) and $AlCl_3$ (7.77 g, 58.27 mmol). After 1 h, an additional amount of acetyl chloride (4.55 g, 4.12 mL, 57.96 mmol) was added. The mixture was stirred for an additional 4 h and then poured into ice−water. The obtained pink solid was filtered off, washed with petroleum ether, and dried to afford **27** (5.72 g, 62%) as a white solid which was used in the next step without further purification, mp 66−68 °C. ^{1}H NMR (200 MHz) δ 1.50 (6H, s, CH_3), 2.55 (3H, s, CH_3CO), 3.85 (2H, bs, CH_2), 7.15 (1H, d, J = 8.2 Hz, H-8), 7.75 (1H, dd, J = 2.1, 8.2 Hz, H-7), 7.95 (1H, d, J = 2.1 Hz, H-5). 2D ^{1}H NMR NOESY showed three relevant NOE cross-peaks: CH_3CO → H-5; CH_3 → H-7; *gem*-CH_3 → H-8. GC−MS: m/z = 318 (22) [M^+ + 1], 317 (100) [M^+], 302 (45), 274 (12), 248 (49), 220 (11), 206 (12), 178 (15).

Pharmacology. All the animal experimental procedures were carried out following the guidelines of the European Community Council Directive 86-609 and were approved by the Ethical Committee of the University of Pisa, Italy, for animal experimentation. Reagents and reference compounds are purchased from Sigma-Aldrich unless otherwise indicated. ANOVA and Student t test were selected as statistical analysis; $P < 0.05$ was considered a significant statistical difference. Experimental data were analyzed by a computer fitting procedure (software GraphPad Prism 4.0).

Vasorelaxant Activity. To determine a possible vasodilator mechanism of action, the compounds were tested on isolated thoracic aortic rings of male normotensive Wistar rats (250−350 g). The rats were sacrificed by cervical dislocation under light ether anesthesia and bled. The aorta were immediately excised and freed of extraneous tissues. The endothelial layer was removed by gently rubbing the intimae surface of the vessels with a hypodermic needle. Then 5 mm wide aortic rings were suspended under a preload of 2 g in 10 mL of organ baths containing Tyrode solution (composition of saline in mM: NaCl 136.8; KCl 2.95; $CaCl_2$ 1.80; $MgSO_4{\cdot}7H_2O$ 1.05; NaH_2PO_4 0.41; $NaHCO_3$ 11.9; glucose 5.5) thermostated at 37 °C and continuously gassed with a mixture of O_2 (95%) and CO_2 (5%). Changes in tension were recorded by means of an isometric transducer (Basile model 7005) connected to a unirecord microdynamometer (Basile model 7050). After an equilibration period of 60 min, the endothelial removal was confirmed by administrating acetylcholine (ACh) (10 μM) to KCl (25 mM) precontracted vascular rings. A <10% relaxation of the KCl-induced contraction was indicative of an acceptable lack of the endothelial layer, while the organs, showing a

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10% relaxation (i.e., significant presence of the endothelium), were discarded. At 30−40 min after the confirmation of the endothelium removal, the aortic preparations were contracted by treatment with a single concentration of KCl (25 mM). When the contraction reached a stable plateau, 3 times increasing concentrations of the tested compounds or of the reference compound LCRK were added cumulatively. Preliminary experiments showed that KCl-induced contractions remained in a stable tonic state for at least 1 h. In other sets of experiments, the potassium channel blocker glibenclamide (1 μM) was added before the KCl (25 mM) induced contraction, and then selected compounds were administered. KCl and ACh hychloride were dissolved in bidistilled water. Glibenclamide, LCRK, and all the other synthesized derivatives were dissolved (10 mM) in DMSO and further diluted in bidistilled water. All the solutions were freshly prepared immediately before the pharmacological experimental procedures. Previous experiments showed a complete ineffectiveness of the administration of the vehicle. The vasorelaxing efficacy was evaluated as the maximal vasorelaxing response, expressed as the percentage (%) of the contractile tone induced by KCl, 25 mM. The potency parameter was expressed as pIC_{50}, which was calculated as the negative logarithm of the molar concentration of the test compounds, evoking a half reduction of the contractile tone induced by 25 mM KCl. The efficacy and potency parameters were expressed as the mean ± standard error, for 5−10 experiments.

Vascular Smooth Muscle Hyperpolarizing Activity. Human aortic smooth muscle cell (HASMC, Invitrogen) were cultured in medium 231 (Invitrogen) supplemented with smooth muscle growth supplement (SMGS, Invitrogen) and 1% of 100 units/mL penicillin and 100 mg/mL streptomycin in tissue culture flasks at 37 °C in a humidified atmosphere of 5% CO_2.

HASMCs were cultured up to about 90% confluence, and at 24 h before the experiment cells were seeded onto a 96-well black plate, clear bottom precoated with 1% gelatin (from porcine skin) at density of 72×10^3 per well. After 24 h to allow cell attachment, the medium was replaced and cells were incubated for 1 h with an appropriate buffer (HEPES 20 mM, NaCl 120 mM, KCl 2 mM, $CaCl_2{\cdot}2H_2O$ 2 mM, $MgCl_2{\cdot}6H_2O$, glucose 5 mM, pH 7.4, at room temperature) containing the anionic DiBAC4(3) 2.5 μM. This probe spreads between cellular and extracellular fluids in a membrane-potential-dependent manner (following the Nernst rule), thus allowing assessment of changes in membrane potential by means of spectrofluorimetric recording at excitation and emission wavelengths of 488 and 520 nm, respectively (multiwells reader, EnSpire, Perkin-Elmer). In particular, an increase of fluorescence, corresponding to an inward flow of the dye, reflects a membrane depolarization, while a decrease in fluorescence, due to an outward flow of the dye, is linked to membrane hyperpolarizing effects. After the assessment of baseline fluorescence, the tested compounds or the reference drug LCRK was added to evaluate the influence on membrane potential. The involvement of specific ion channels is detected using a selective channel blocker, glibenclamide, which was added 10 min before the tested compounds and LCRK, when required.

Changes in fluorescence were calculated as relative fluorescence decrease $((F_t - F_0)/F_0)$ where F_0 is the basal fluorescence (before the addition of the tested compounds or LCRK) and F_t is the fluorescence at time t (after the addition of the tested compounds or LCRK). Then changes in fluorescence were expressed as % of the maximal effect produced by LCRK 100 μM. The potency parameter pEC_{50} is the negative logarithm of the molar concentration of the test compounds, evoking a half-maximal decrease of relative fluorescence. The experiments were performed in six replicates.

Isolation and Functional Stimulation of Rat Pancreatic Islets. Pancreatic islets were isolated from male Wistar rats of 200−250 g body weight (Harlan, Italy) by the collagenase method using the procedure of pancreatic duct cannulation and density gradient purification as described elsewhere.[36] Freshly isolated islets were resuspended in RPMI culture medium (containing 5.6 mM glucose, antibiotics, and 10% adult bovine serum), cultured at 37 °C in 5% CO_2 for 24 h, and used the next day for functional studies.

Functional Studies. Cultured islets were picked up under a dissecting microscope and preincubated for 30 min in a 24-well plate (10 islets/well) in Krebs−Ringer−HEPES (KRH) buffer, pH 7.4, containing 2.8 mM glucose and 1% bovine serum albumin. After preincubation, the buffer was removed and islets were incubated for 1 h in the same buffer containing 2.8 or 16.7 mM glucose in the presence or absence of various concentrations of either compound **8** (1, 10, and 100 nM) or compound **9** (10, 100, and 1000 nM), dissolved in DMSO and properly diluted to obtain work concentrations. The K_{ATP} blocker glibenclamide (1 μM) and the K_{ATP} activator diazoxide (80 μM) were also used to modulate glucose-stimulated insulin release and serve as suitable controls in this experimental setting. At the end of the incubation period, the buffer was taken for measurement of released insulin and 1 mL of cold acidified EtOH (EtOH/H_2O/concentrated HCl, 150:47:3, v/v) was added into the wells for extraction and subsequent measurement of islet insulin content. Determination of total insulin content was made to verify homogeneity of islet batches in incubation experiments. Insulin was measured by radioimmunoassay (rat insulin RIA kit, Millipore, St. Charles, MO, U.S.).

■ ASSOCIATED CONTENT

Ⓢ Supporting Information

Crystal structure analysis of compound **25**, additional references, and combustion analyss results for compounds **2**−**11**. This material is available free of charge via the Internet at http://pubs.acs.org.

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Author Contributions

All authors have given approval to the final version of the manuscript.

Notes

The authors declare no competing financial interest.

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■ ABBREVIATIONS USED

DiBAC4(3), bis(1,3-dibutylbarbituric acid)trimethine oxonol; DIPEA, *N*,*N*-diisopropylethylamine; HASMC, human aortic smooth muscle cell; KRH, Krebs−Ringer−HEPES; KATP, ATP-sensitive potassium; Kir, inwardly rectifying potassium channel; KCO, potassium channel opener; SAR, structure−activity relationship; LCRK, levcromakalim; RPMI, Roswell Park Memorial Institute; SUR, sulfonylurea receptor; TBAB, tetra-*n*-butylammonium bromide

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