Available online at www.sciencedirect.com
SCIENCE DIRECT®
Bioorganic & Medicinal Chemistry 13 (2005) 141–155
Bioorganic & Medicinal Chemistry

Synthesis and pharmacological evaluation of 4*H*-1,4-benzothiazine-2-carbonitrile 1,1-dioxide and *N*-(2-cyanomethylsulfonylphenyl)acylamide derivatives as potential activators of ATP sensitive potassium channels

Søren C. Schou,[a] Holger C. Hansen,[a] Tina M. Tagmose,[a] Harrie C. M. Boonen,[a] Anne Worsaae,[a] Michael Drabowski,[a] Philip Wahl,[a] Per O. G. Arkhammar,[a] Thora Bodvarsdottir,[a] Marie-Hélène Antoine,[b] Philippe Lebrun[b] and John Bondo Hansen[a,*]

[a]*Discovery, Novo Nordisk A/S, Novo Nordisk Park, DK-2760 Måløv, Denmark*
[b]*Laboratory of Pharmacology, Faculty of Medicine, Université Libre de Bruxelles, Route de Lennik 808-CP 617, Brussels B-1070, Belgium*

Received 2 June 2004; accepted 28 September 2004

Abstract—1,2,4-Thiadiazine derivatives, like 3-methyl-7-chlorobenzo-4*H*-1,2,4-thiadiazine 1,1-dioxide, diazoxide and 7-chloro-3-isopropylamino-4*H*-benzo-1,2,4-thiadiazine 1,1-dioxide, BPDZ 73, are potent openers of Kir6.2/SUR1 K_{ATP} channels. To explore the structure–activity relationship of this series of K_{ATP} openers, 4*H*-1,4-benzothiazine-2-carbonitrile 1,1-dioxide and *N*-(2-cyanomethylsulfonylphenyl)acylamide derivatives were synthesized from 2-acetylamino-5-chloro-benzenesulfonic acid pyridinium salt or 2-aminobenzenethiols. The 4*H*-1,4-benzothiazine-2-carbonitrile 1,1-dioxide derivatives (e.g., 7-chloro-3-isopropylamino-4*H*-1,4-benzothiazine-2-carbonitrile 1,1-dioxide, **3f**) were found to activate K_{ATP} channels as indicated by their ability to hyperpolarize beta cell membrane potential, to inhibit glucose-stimulated insulin release in vitro and to increase ion currents through Kir6.2/SUR1 channel as measured by patch clamp. The potency and efficacy of, for example, **3f** is however significantly reduced compared to the corresponding 4*H*-1,2,4-benzothiadiazine 1,1-dioxide derivatives. Opening of the 4*H*-1,2,4-thiadiazine ring to get (e.g., 2-cyanomethylsulfonyl-4-fluorophenyl) carbamic acid isopropyl ester (**4c**) gives rise to compounds, which are able to open K_{ATP} channels but with considerable reduced potency compared to, for example, diazoxide. Compound **3a**, 7-chloro-3-methyl-4*H*-1,4-benzothiazine-2-carbonitrile 1,1-dioxide, which inhibits insulin release in vitro from beta cells and rat islets, reduces plasma insulin levels and blood pressure in anaesthetized rats upon intravenous administration.

1. Introduction

ATP sensitive potassium (K_{ATP}) channels are found in different tissues, such as the heart, vascular smooth muscle, central neurons and pancreatic β-cells.[1–4] By regulating the open state probability of the channel, it is possible to affect cellular membrane potential and thereby the influx of Ca^{2+} through the voltage-gated calcium channels. Blockers of beta cell K_{ATP} channels (e.g., repaglinide, tolbutamide and glibenclamide) stimulate insulin release and are used in treatment of type 2 diabetes mellitus. Openers of K_{ATP} channels of smooth muscle (e.g., diazoxide and pinacidil) have been explored as drugs for treatment of cardiovascular diseases. Recently it has been suggested that openers of beta cell K_{ATP} channels, which reduce insulin release to induce beta cell rest, can be used in treatment of metabolic diseases.[5–7]

The K_{ATP} channels are constructed as 4+4 hetero-octamers made from the regulatory sulfonylurea receptor SUR and the inward rectifier Kir6.2 or Kir6.1. Different forms of SUR have been cloned and characterized.[4,8] SUR1 have been found to combine with Kir6.2 to form the K_{ATP} channels of beta cells

* Corresponding author. Tel.: +45 4443 4857; fax: +45 4443 4547; e-mail: jbha@novonordisk.com

0968-0896/$ - see front matter © 2004 Elsevier Ltd. All rights reserved.
doi:10.1016/j.bmc.2004.09.051

and neurones whereas SUR2A and SUR2B in combination with Kir6.1 and Kir6.2 form the channels of the heart and vascular smooth muscle, respectively.

Pinacidil opens SUR2B K_{ATP} channels to induce vasorelaxation but has nearly no effects on the SUR1 channels of beta cells.[9] In contrast, diazoxide is a moderately potent and non-selective activator of SUR1 and SUR2B channels with only minimal effects on SUR2A. Optimizations of diazoxide have given compounds, for example, BPDZ 62, BPDZ 73 and NN414 (Fig. 1), which potently and selectively open the SUR1 K_{ATP} channels of beta cells.[10–13] In analogy, it has recently been shown that certain pinacidil derivatives, for example, **1**[14] and **2**[15] are able to inhibit insulin release with only minimal effects on smooth muscle.

Structural requirements for K_{ATP} channel openers acting on Kir6.2/SUR1 have been explored. These include an aromatic group, possibly substituted with one or more halogen atoms, a space filling alkyl group, an acidic (hydrogen bond donating) NH group (e.g., N4 of the 1,2,4-thiadiazine derivatives), and an electronegative (hydrogen bond accepting) group (SO_2 or NCN) (Fig. 2).[10,16,17] By combining structural component from diazoxide and pinacidil to substitute the 3-methyl group of diazoxide with 3-alkylamino groups, it has been possible to improve potency and change efficacy highlighting the importance of the exocyclic amino-group.[10,12,13,16,17]

In the present study the structure–activity relationship for the K_{ATP} channel activity of these diazoxide derivatives have been investigated by a series of 4*H*-1,4-benzothiazine-2-carbonitrile 1,1-dioxide derivatives (**3**) and the ring-opened *N*-(2-cyanomethylsulfonylphenyl)acylamide derivatives (**4**) (Fig. 2). One of the objectives of this study was to examine the consequences of introducing a substituent in the 2-position of the 4*H*-1,4-benzothiazine entity. Using the cyano group, we expected to achieve this while retaining the acidic proton in position 4 necessary for the activity of the compounds as K_{ATP} channel openers. Another objective was to study the effects of opening the 4*H*-1,4-benzothiazine ring to maintain certain aspects of the parent compound by keeping the relative positions of the SO_2 group versus the NH (position '4') and the hydrophobic side chain.

2. Chemistry

3-Methylbenzothiazines of the general formula **3** were synthesized by base catalyzed ring closure of cyanomethylsulfones **4**, which in turn were prepared by different routes (Scheme 1 path A, B and C).

Path A: the unstable 2-acetylamino-5-chlorobenzenesulfonyl chloride was prepared from the pyridinium sulfonate **5a**[18] and reduced without purification to the sulfinic acid **6a** following the general procedure reported by Lee and Field.[19] Alkylation of **6a** with iodoacetonitrile provided the sulfone **4a**.

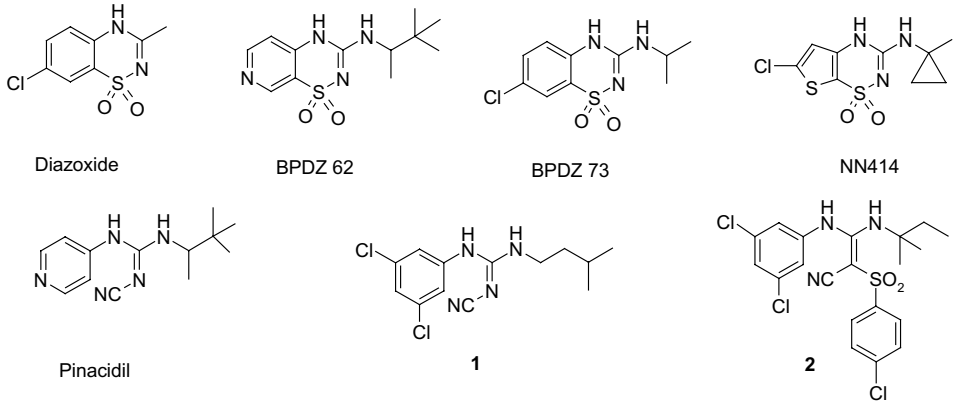


Figure 1. Openers of K_{ATP} channels.

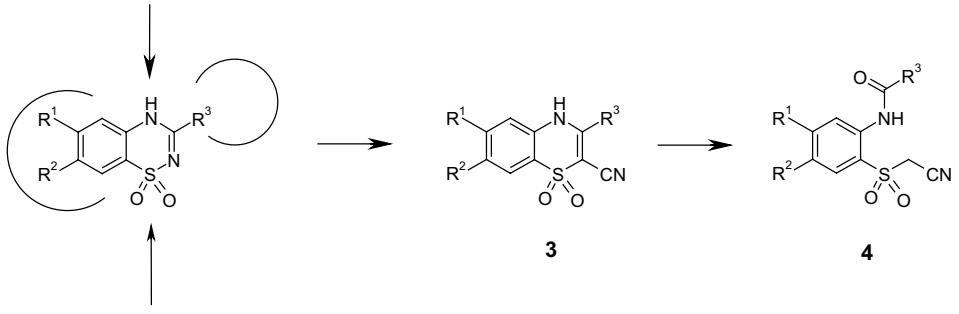
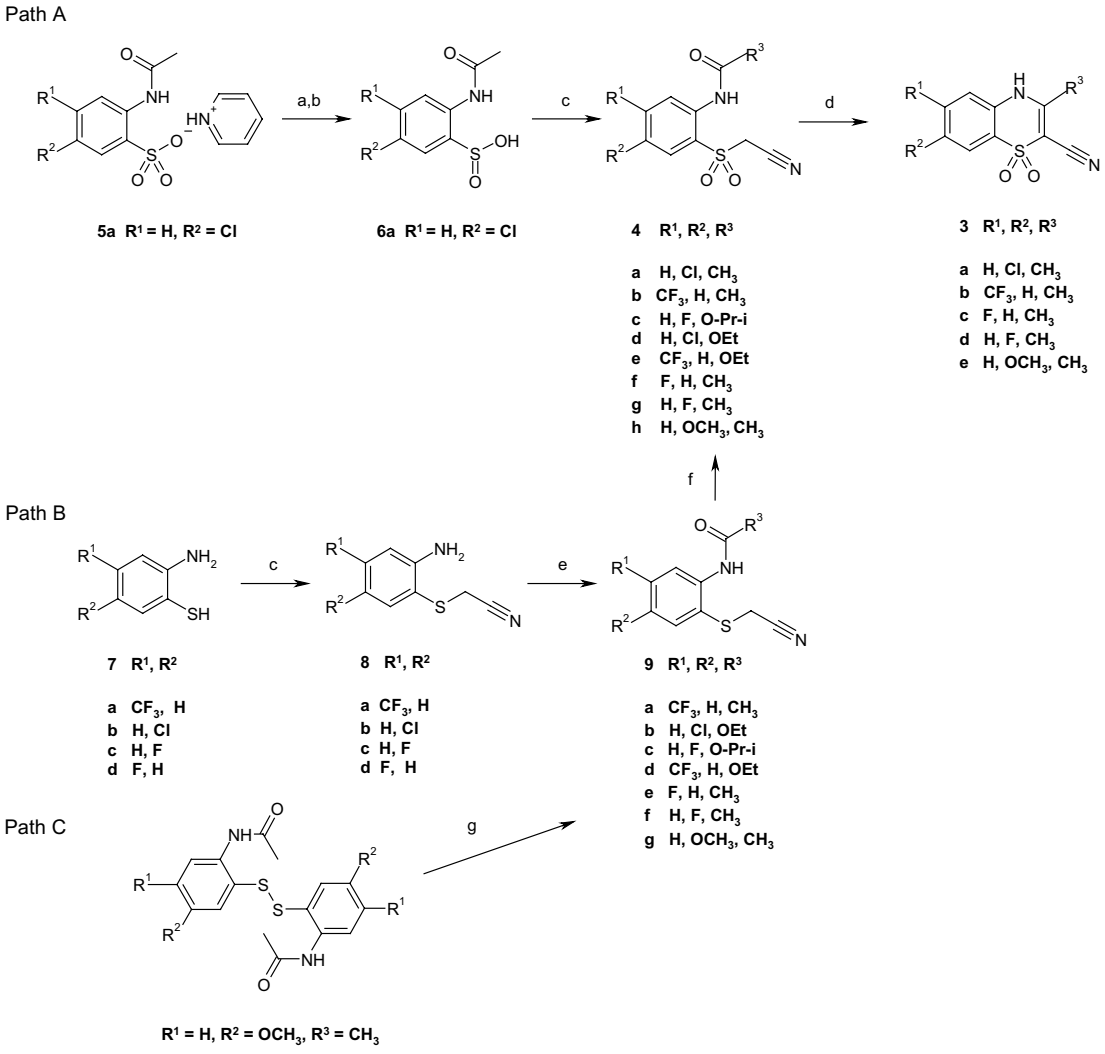


Figure 2.

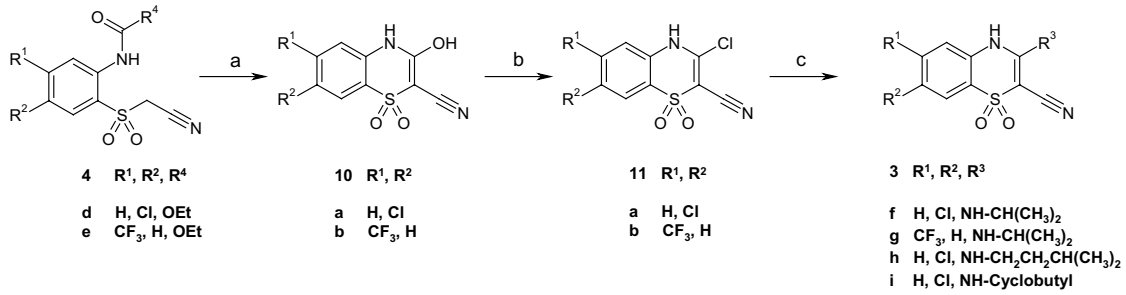


Scheme 1. Synthesis of *N*-(2-cyanomethylsulfonylphenyl)acetamides (**4a**–**h**) and 3-methylbenzothiazines (**3a**–**e**). Reagents and conditions: (a) PCl_5, 60 °C, 2 h; (b) *p*-TolSH/Et_3N, CH_2Cl_2; (c) NaH, I–CH_2–CN/DMF; (d) (1) 0.5 M NaOH, rt 1 h, (2) 1 M HCl; (e) acetic anhydride or appropriate chloroformates; (f) *m*-CPBA/CH_2Cl_2 or H_2O_2/HOAc; (g) (1) $NaBH_4$/EtOH, 70 °C, 30 min, (2) I–CH_2–CN, rt, 15 min.

Path B: 2-aminobenzenethiols **7**, which were either commercially available (e.g., **7a**) or prepared by alkaline hydrolysis of the appropriately substituted 2-aminobenzothiazoles[20] were cyanomethylated to get the cyanomethylsulfanyl derivatives by a modification of the procedure previously described for **8b**.[21] In our hands, however, a much better yield and a more pure product was obtained if the aminobenzenethiol was not isolated but instead generated in situ from the 2-aminobenzothiazole as the thiolate salt and immediately alkylated with iodoacetonitrile under nitrogen atmosphere to avoid air oxidation of the thiolate. Acylation of **8** with acetic anhydride or chloroformates gave acetamides **9a**,**e**–**g**, and carbamates **9b**–**d**, respectively, which were oxidized with either *m*-CPBA in dichloromethane or, preferentially, with hydrogen peroxide in acetic acid to give the sulfones. By treatment with diluted aqueous base at room temperature, the *N*-(2-cyanomethylsulfonylphenyl)acetamides **4a**,**b**,**f**–**h** underwent a ring closure to give the 4*H*-1,4-benzothiazines **3a**–**e**.

Path C: in an attempted synthesis of 2-aminobenzenethiol **7** (R^1 = H, R^2 = OCH_3) from 2-amino-6-methoxy-benzothiazole we only obtained the corresponding disulfide.[22] Acetylation gave bis(2-acetylamino-5-methoxyphenyl)-disulfane,[23] which was reduced with sodium borohydride, and the resulting thiolate alkylated in situ to give **9g**.

Spectroscopic data supports the assigned 4*H* structures of **3** rather than the 2*H* tautomer form; for example, in the ^{1}H NMR spectrum of **3a** the acidic proton was observed at δ = 12.11, similar to what has been found for HN(4) in 1,2,4-benzothiadiazine 1,1-dioxides,[10,24] and thus at a much lower field than would be expected for a 2*H* tautomer. Furthermore, the ^{13}C NMR spectrum showed C2 and C3 signals at δ 85.51 and 155.72, respectively. The predicted values for the 4*H* tautomer are δ 83.57 and 157.78, whereas for the 2*H* tautomer values of δ 52.83 and 172.61 are predicted.[25] The C–H correlation spectrum showed no interaction of the acidic proton with any carbon atom, again suggesting a N–H rather than a C–H proton.



Scheme 2. Synthesis of 3-alkylamino-4*H*-1,4-benzothiazine-2-carbonitriles (**3f–i**). Reagents and conditions: (a) (1) 0.2 M NaOH, rt 1 h, (2) 1 M HCl; (b) $POCl_3$, pyridine hydrochloride/H_3PO_4; (c) amine (R^3–H) in CH_3CN or neat.

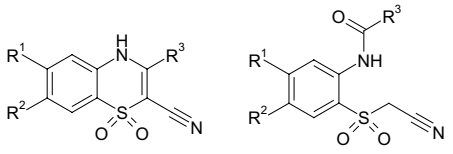
In IR, the signal at ν 2215 cm^{-1} corresponds to a cyano group bound to an unsaturated carbon.

3-Alkylamino-4*H*-1,4-benzothiazine-2-carbonitriles were prepared starting from the carbamates **4d**,**e** (Scheme 2). Base catalyzed ring-closure of **4d**,**e** gave **10a**,**b**, which were treated with phosphoroxychloride using the procedure developed by Andersen and Begtrup[26] to get **11a**,**b**. Prolonged heating of **11** in a closed vial[27] with an excess of the appropriate amine was required in order to obtain the desired 3-amino substituted derivatives **3f–i**. Low to moderate yields were obtained, the sterically hindered amines giving the lowest yields.

3. Results and discussion

The synthesized compounds (Table 1) were initially evaluated for their ability to repolarize the membrane potential of βTC3 beta cells depolarized by 10 mM glucose; to inhibit 22 mM glucose-stimulated insulin release from βTC6 beta cells and to relax phenylephrine contracted

Table 1. Structures and biology screening data for compounds **3a–i** and **4a–e**



3 **4**

Compound	R^1, R^2	R^3	Membrane potential[a] βTC3 cells IC_{50} (μM)	Inhibition of insulin release[b] βTC6 cells		Relaxation of rat aorta rings[c] EC_{50} (μM)	Membrane potential[d] HEK 293 cells IC_{50} (μM)
				IC_{50} (μM)	Efficacy (%)		
3a	H, Cl	CH_3	NA	6.78 4.25	18 ± 2.39	30.5 ± 5.4	>30
3b	CF_3, H	CH_3	47.7 ± 23	>100	8 ± 4.71	44.3 ± 14.1	14 ± 6
3c	F, H	CH_3	NA	NA	NA	35 ± 9	NT
3d	H, F	CH_3	NA	3.95 ± 3.25[e]	25 ± 1.75[e]	24 ± 3	NT
3e	H, OCH_3	CH_3	NA	NA	NA	18 ± 1	NT
3f	H, Cl	$NHCH(CH_3)_2$	17.6 ± 6	42.3 ± 22.85	14 ± 6.22	4.3 ± 0.8	13 ± 4
3g	CF_3, H	$NHCH(CH_3)_2$	NA	NA	NA	4.2 ± 5	>30
3h	H, Cl	$NH(CH_2)_2CH(CH_3)_2$	10.7 ± 6	>100	16 ± 4.77	18.6 ± 5.4	NT
3i	H, Cl	NHCyclobutyl	NA	NA	NA	9 ± 6	NT
4a	H, Cl	CH_3	NA	>100	12 ± 5.92	11.4 ± 5.2	NT
4b	CF_3, H	CH_3	NA	>100	14 ± 6.50	84.1 ± 7.3	NT
4c	H, F	$OCH(CH_3)_2$	NA	NA	NA	109 ± 8.6	6 ± 2
4d	H, Cl	OCH_2CH_3	NA	>100	6 ± 2.65	4.7 ± 2.6	NT
4e	H, CF_3	OCH_2CH_3	NA	0.36 ± 0.20	9 ± 6.75	183.0 ± 80.7	NT
Diazoxide			13.7 ± 0.25	22.98 ± 4.1	12.8 ± 2.5	25 ± 3.8	33 ± 11
BPDZ 73			0.25 ± 0.02	0.46 ± 0.31	73 ± 7.5	36.3 ± 2.2[f,g]	0.8 ± 0.01
Pinacidil			NA	NA	NA	0.8 ± 0.2[h]	NT

[a] Effects on membrane potential in βTC3 cells. Values are means of at least three measurements ± SD.
[b] Inhibition of glucose-stimulated insulin release from βTC6 cells. Values are means ± SEM of at least four experiments.
[c] Relaxation of phenylephrine or KCl induced contraction of rat aorta rings. Values are means ± SEM of at least three experiments.
[d] Effects on membrane potential of HEK 293 cells expressing Kir6.2/SUR 1 channels. Values are means ± SEM of at least three experiments.
[e] $n = 2$.
[f] Values from Lebrun et al.[11]
[g] Relaxation of KCl induced contraction.
[h] Value from Nielsen et al.[12] NA = not active. NT = not tested.

rat aortic rings. These assays have previously been used to characterize activators of K_{ATP} channels.[11,12,14,15]

Among the 4*H*-1,4-benzothiazine-2-carbonitrile 1,1-dioxide derivatives, compounds **3b**, **3f** and **3h** were able to hyperpolarize βTC3 cell membranes, whereas compounds **3a**,**c**,**d**,**g**,**i** and the ring-opened *N*-(2-cyano-methylsulfonylphenyl)acylamide derivatives (**4a**–**e**) where inactive in this test. Compounds **3b**, **3f** and **3h** were found to be approximately equipotent with diazoxide but considerable less potent than BPDZ 73; efficacies were moderate (30–60%) and lower than determined for BPDZ 73 or diazoxide (100%) (data not shown).

The compounds of the present series were in general not potent inhibitors of glucose-stimulated insulin release from βTC6 beta cells. Compounds **3a**, **3b**, **3d**, **3f**, **3h**, **4a**, **4b**, **4d** and **4e** significantly inhibit insulin release although with low efficacy. In comparison are **3a**, **3d**, **3f** and **4e** at least as potent as diazoxide and with comparable efficacy. Whereas compounds **3b**, **3h**, **4a**, **4b** and **4d** weakly inhibit insulin release with IC_{50}'s above 100 μM, compounds **3g**, **3i** and **4c** did not affect insulin release at concentration up to 50 μM.

All compounds induced vasodilatation of isolated aorta, yet with different order of potency. The most potent of these (i.e., **3f**, **3g**, **3i** and **4d**) being more potent than the non-selective potassium channel opener diazoxide but equal or less potent than pinacidil.

Overall the screening data suggests that 3-methyl-4*H*-1,4-benzothiazine-2-carbonitrile 1,1-dioxide derivatives with a chlorine or fluorine in position 7 have a profile similar to that of diazoxide whereas a methoxy substituent in position 7 seems to reduce potency slightly in analogy with previous findings.[11] Compounds with substituents in position 6 appear to be less potent. Substituting the 3-methylgroup with a 3-alkylaminogroup, which in the 4*H*-1,4-benzothiadiazine 1,1-dioxide series strongly increases beta cell activity,[10,16] has little beneficial effect on potency.

In order to evaluate their mechanism of action, selected compounds were examined for effects on HEK 293 cells expressing Kir6.2/SUR1 (Table 1) or Kir6.2/SUR2B channels (Fig. 3).

Compound **3b** and **3f**, which weakly reduced insulin release from βTC6 cells and which hyperpolarized βTC3 cell membranes in presence of glucose, did repolarize the HEK 293 cell membranes depolarized by the K_{ATP} channel blocker tolbutamide (Table 1), suggesting that these compounds reduce insulin release through an activation of Kir6.2/SUR1 potassium channels. In conflict with its inability to reduce glucose-stimulated insulin release, compound **4c** was found to be able to hyperpolarize membrane of cells expressing Kir6.2/SUR1 K_{ATP} channels (IC_{50} = 6 ± 2 μM).

The effect of selected compounds on the membrane potential of HEK 293 cells expressing Kir6.2/SUR2B potassium channels were examined (Fig. 3). The reference compound diazoxide and the pinacidil derivative P1075 potently hyperpolarized the cell membrane with IC_{50}'s of 50 ± 19 μM and 0.18 ± 0.01 μM, respectively, which are comparable to their ability to relax pre-contracted rat aorta rings.[12,28] Compounds **3a** (IC_{50} = 16 ± 3.6 μM) and **3b** (IC_{50} = 8.5 ± 4.9 μM) significantly hyperpolarized the membranes, which indicate that these compounds were able to activate the Kir6.2/SUR2B channel. At high concentrations compound **3f** and **3g** also appeared to hyperpolarize the membranes. Compounds **4c** and **4d** were found to be inactive in this assay. While the ability of compounds **3a** and **3b** to relax vascular smooth muscle therefore could be mediated through a direct activation of the Kir6.2/SUR2B K_{ATP} channels, it is however likely that some compounds of these series (exemplified by compounds **4c** and **4d**) affect smooth muscle through a mechanism not involving Kir6.2/SUR2B K_{ATP} channels.

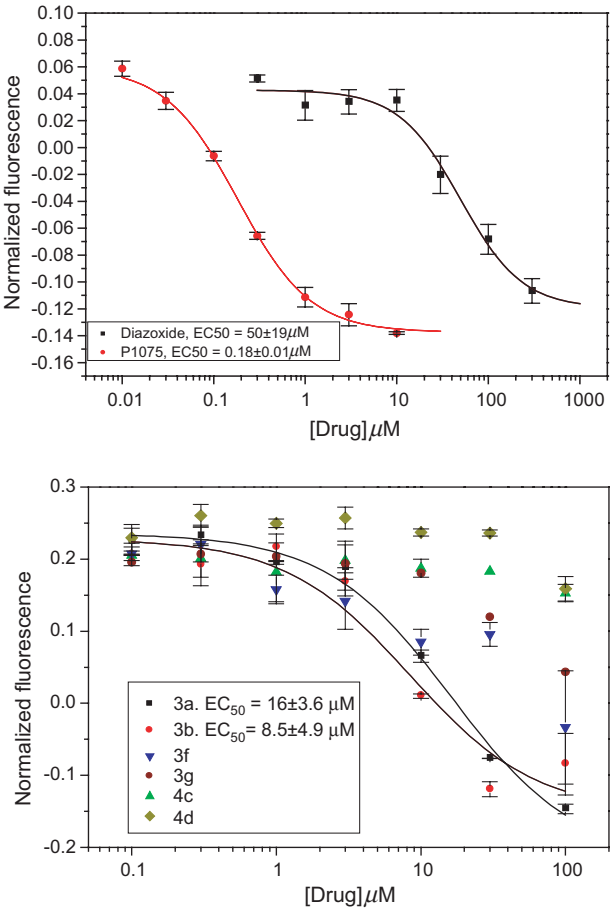


Figure 3. Concentration-response curves for P1075 and diazoxide (upper panel) and compounds **3a**–**d**, **4c**, **4d** (lower panel)-induced membrane potential responses in HEK293 cells expressing Kir6.2/SUR2B. Data points are mean ± SEM from 2 to 3 experiments performed in triplicate.

The apparent conflict between the ability of compound **4c** to hyperpolarize membranes of HEK 293 cells expressing Kir6.2/SUR1 and its lack of effects on glucose-stimulated insulin release from βTC6 cells was also investigated using the more sensitive patch-clamp technique. Compound **4c** potently activates Kir6.2/SUR1

channels expressed in HEK293 cells when measured by patch clamp in the whole cell configuration or Kir6.2/SUR1 expressed in inside-out macropatches excised from *Xenopus* oocytes (Fig. 4). The efficacy of the activation was, however considerable lower than that of diazoxide. Apparently, the partial agonist effect of **4c** as measured by patch clamp is not sufficient to cause a hyperpolarization of the cell membrane and subsequent inhibition of insulin release from the beta cells.

Compound **3a**, which inhibits glucose-stimulated insulin release from βTC6 cells with a potency similar to diazoxide was further examined using freshly isolated rat islets.

Incubation of pancreatic islets in the presence of **3a** revealed an inhibitory effect of the drug on 16.7 mM glucose-induced insulin release. Residual insulin release after the addition of 10 μM and 50 μM **3a** represented 88.6 ± 3.8% ($n = 16$ samples) and 49.6 ± 1.7% ($n = 16$ samples) of the control value, respectively. Under the same experimental conditions and after the addition of 10 μM and 50 μM diazoxide, the residual insulin release averaged 70.0 ± 3.6% ($n = 22$ samples) and 28.8 ± 2.5% ($n = 21$ samples) of the control value, respectively. The data clearly revealed that micromolar concentrations of **3a** inhibited the glucose-induced insulin release. However, the capacity of **3a** to inhibit the secretory process was not highly pronounced and the compound appeared to be less potent than diazoxide.

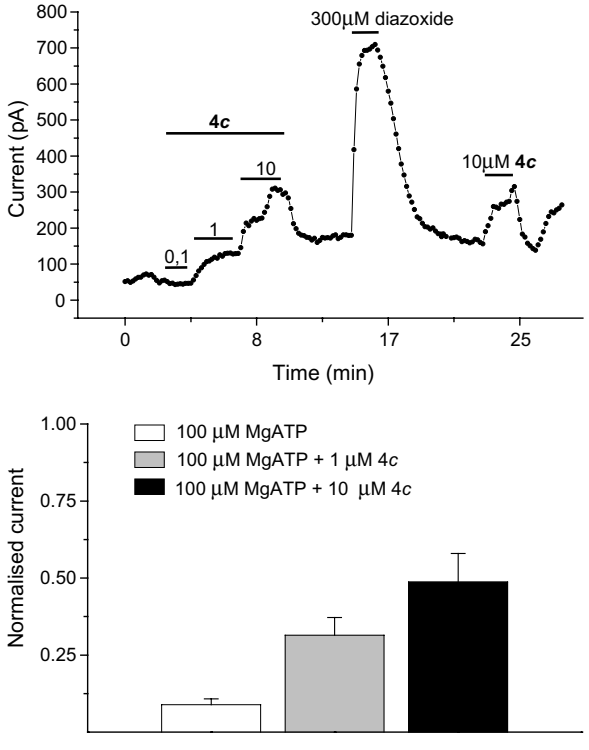


Figure 4. Effects of **4c** on Kir6.2/SUR1 channels. Upper panel: whole cell recording from an HEK293 cell expressing Kir6.2/SUR1. The points indicate the current obtained in response to a 10 mV depolarizing pulse applied every 10 s from a holding potential of −80 mV. Diazoxide (300 μM) and **4c** (0.1, 1 and 10 μM) were applied as indicated by the horizontal bars. Lower panel: effect of **4c** on macroscopic currents through Kir6.2/SUR1 channels in inside-out patches. Mean macroscopic conductances (G) are expressed as a fraction of the mean slope conductance in nucleotide and drug free solution ($G_{control}$). Data were obtained in the presence of 100 μM MgATP (white column), 100 μM MgATP plus 1 μM **4c** (grey column) or 100 μM MgATP plus 10 μM **4c** (black column).

In the presence of 16.7 mM glucose and extracellular Ca^{2+} in the perifusing medium, the addition of 10 μM **3a** provoked a minor reduction in both ^{45}Ca outflow and insulin output (Fig. 5, left panels). On removal of **3a** from the perifusate, a modest increase in ^{45}Ca (fractional outflow rate) FOR and insulin release was noticed. The latter increases could reflect relief from inhibitory effects of the drug.

Under identical experimental conditions, the addition of a higher concentration (50 μM) of **3a** provoked more pronounced inhibitory effects on $^{45}Ca^{2+}$ FOR and insulin release from pre-labelled and perifused rat pancreatic islets (Fig. 5, right panels).

To study the effects of **3a** on $^{45}Ca^{2+}$ movements in detail, similar experiments were conducted in the presence of 16.7 mM glucose but absence of extracellular Ca^{2+}. In islets exposed to Ca^{2+}-depleted media, the basal rate of $^{45}Ca^{2+}$ outflow (min 40–44) was significant lower than in presence of Ca^{2+} ($P < 0.05$) and the addition of either 10 μM or 50 μM **3a** did not affect $^{45}Ca^{2+}$ FOR.

These dynamic experiments indicated that the addition of **3a** reduced the $^{45}Ca^{2+}$ FOR and insulin release from pre-labelled islets perifused in the presence of 16.7 mM glucose and extracellular Ca^{2+}. Under the latter experimental conditions, that is, in islets exposed throughout to Ca^{2+} and insulinotropic concentrations of glucose, the $^{45}Ca^{2+}$ fractional outflow rate is known to reflect a sustained stimulation of isotopic exchange between influent $^{40}Ca^{2+}$ and effluent $^{45}Ca^{2+}$.[11,29,30] Thus, the modest inhibitory effect of **3a** on $^{45}Ca^{2+}$ outflow can be interpreted as the result of K_{ATP} channel opening followed by a subsequent reduction in $^{40}Ca^{2+}$ entry into the islet cells. In agreement with such a view, the **3a**-induced decrease in $^{45}Ca^{2+}$ outflow did not occur when the islets were perifused in the absence of extracellular Ca^{2+}. The decrease in Ca^{2+} entry mediated by **3a** may lead, ultimately, to a reduction in insulin output.

In order to examine if the effect of **3a** in vitro are reflected in vivo, 3 mg/kg of the compound was administered intravenously to anaesthetized rats.[12] Compound **3a** reduced plasma insulin levels by 48 ± 18% from basal levels (104 ± 1 pM), which is comparable to diazoxide.[12] The effect, however, was of shorter duration (less than 20 min). Blood pressure was not affected by this dose (from 78 ± 5 to 76 ± 6 mmHg) within the observation period (0.5 h). **3a** was also given by gavage (30 mg/kg) to hyperinsulinemic Zucker obese rats[12] but no significant effect on plasma insulin levels could be observed (data not shown). Together, these studies support that **3a** is able to inhibit insulin release in vivo but that the duration of action is short and the oral bioavailability is probably to low to see effects after peroral administration.

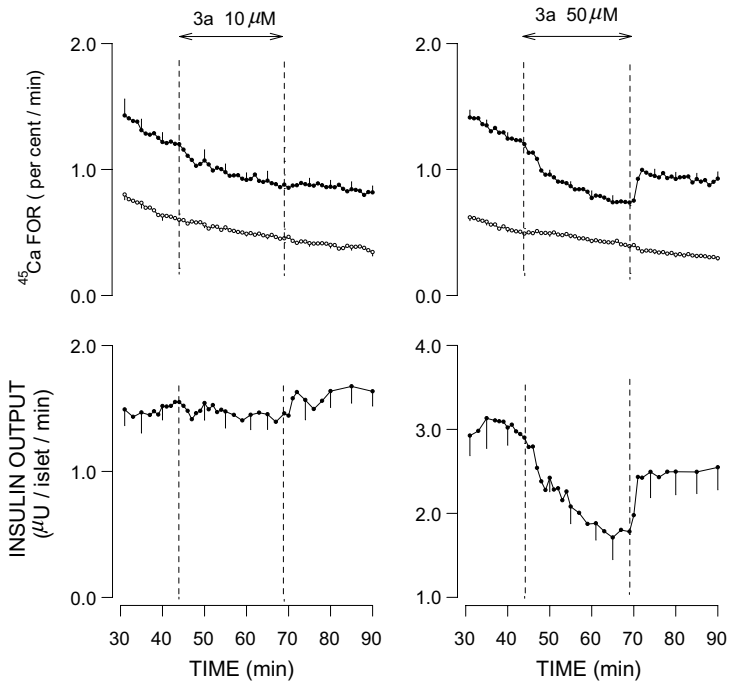


Figure 5. Upper panels: effect of 10 μM (left panel) and 50 μM (right panel) **3a** on $^{45}Ca^{2+}$ outflow from rat pancreatic islets perifused throughout in the presence of 16.7 mM glucose. Basal media contained extracellular Ca^{2+} (●, 2.56 mM) or were deprived of Ca^{2+} and enriched with EGTA (○, 0.5 mM). Lower panels: effect of 10 μM (left panel) and 50 μM (right panel) **3a** on insulin release from rat pancreatic islets perifused throughout in the presence of 16.7 mM glucose. Basal media contained extracellular Ca^{2+} (●, 2.56 mM). Mean values ± SEM refer to 4–6 individual experiments.

4. Conclusion

7-Chloro-3-alkylamino-4*H*-1,2,4-benzothiadiazine 1,1-dioxide derivatives are potent activators of ATP sensitive potassium (K_{ATP}) channels. The 3-alkylamino group, the acidic proton of position 4 as well as the electronegative, H-bond accepting SO_2-group, are important for the activity.[10] In the present study, it has been found that substituents in the 2-position, as found in the 4*H*-1,4-benzothiazine-2-carbonitrile 1,1-dioxide derivatives, retain the ability of the compounds to activate K_{ATP} channels. The potency is however significantly reduced compared to the corresponding 3-alkylamino-4*H*-1,2,4-benzothiadiazine 1,1-dioxide derivatives. We furthermore have found that opening of 4*H*-1,2,4-thiadiazine ring to get *N*-(2-cyanomethylsulfonylphenyl)acylamide derivatives gives rise to compounds, which are able to open K_{ATP} channels but with considerable reduced potency compared to, for example, diazoxide. The present study also suggests that low efficiency opening of ATP sensitive K^+-currents might not be enough to inhibit insulin release induced by high concentrations of glucose.

5. Experimental section

5.1. Chemistry

5.1.1. General. Reagents, starting materials and solvents were purchased from common commercial suppliers and were used as received. All dry solvents were dried overnight over molecular sieves (0.3 or 0.4 nm). Evaporation was carried out on a rotary evaporator at bath temperatures <40 °C and under appropriate vacuum. Flash chromatography was carried out on a Biotage flash 40 using Biotage flash columns (KP-SIL 60 Å, particle size 32–63 μm). Melting points were determined with a Büchi B545 apparatus and are uncorrected. Proton NMR spectra were recorded at ambient temperature using a Brucker Avance DPX 200 (200 MHz) and Brucker Avance DPX 300 (300 MHz) with tetramethylsilane as an internal standard for proton spectra, and DMSO-d_6 at 39.50 ppm or $CDCl_3$ at 77.00 ppm as internal standards for carbon spectra. Chemical shifts are given in parts per million (δ) and splitting patterns are designated as follows: s, singlet; d, doublet; dd, double doublet; t, triplet; q, quartet; sept, septet; m, multiplet and br = broad. The 70 eV E.I. solid mass spectra were recorded on a Finnigan MAT-TSQ 70 mass spectrometer. Reactions were followed by thin layer chromatography performed on silica gel 60 F254 (Merck) or ALUGRAM®SIL G/UV_{254} (MACHEREY-NAGEL) TLC aluminium sheets. Elemental analyses (C, H, N, Cl) were performed by Novo Nordisk, Microanalytical Laboratory, Denmark.

5.1.2. 7-Chloro-3-methyl-4*H*-1,4-benzothiazine-2-carbonitrile 1,1-dioxide (3a). Compound **4a** (0.73 g, 2.7 mmol) was added at room temperature to 0.5 M aqueous NaOH (10 mL, 5 mmol) to form a yellow solution. After 45 min charcoal (0.1 g) was added and the mixture was filtered through celite. The filtrate was cooled to 0 °C and 1 M HCl (7.5 mL) was added. After 30 min the precipitate was collected by filtration and dried to

give 0.66 g of the title compound. Recrystallization from methanol gave pale crystals (0.45 g, 66%); mp 297–299 °C; ^{1}H NMR (CD_3OD-d_4): δ 7.91 (d, J = 2.8 Hz, 1H); 7.68 (dd, 1H); 7.37 (d, J = 9 Hz, 1H); 4.88 (br, H_2O+NH); 2.51 (s, 3H); ^{1}H NMR (DMSO-d_6): δ 12.11 (br, 1H, NH), 8.02 (d, J = 2.3 Hz, 1H, H8), 7.84–7.75 (dd, 1H, H6), 7.48 (d, J = 8.8 Hz, 1H, H5), 2.47 (s, CH_3; partly overlapping with the DMSO-signal); ^{13}C NMR (DMSO-d_6): δ 155.72 (C3), 133.81 (C4a), 133.72 (C6), 129.38, 124.78, 121.23 and 121.18 (C5 and C8), 112.48, 85.51 (C2), 20.16 (CH_3) (assignments are based on C–H correlation and HMBC-spectra); IR (KBr) ν 2214 (CN) cm^{-1}. Anal. Calcd for $C_{10}H_7ClN_2O_2S$: C 47.16%, H 2.77%, N 11.00%, Cl 13.92%. Found: C 47.08%, H 2.71%, N 10.79%, Cl 14.02%. An additional crop of the title compound (0.09 g, 13%) was obtained from the mother liquid.

5.1.3. 3-Methyl-6-trifluoromethyl-4*H*-1,4-benzothiazine-2-carbonitrile 1,1-dioxide (3b). Following the procedure described for **3a** the title compound was prepared from **4b**, white flakes (84%), mp 325–330 °C (from MeOH); ^{1}H NMR (CD_3OD): δ 8.20–8.10 (br d, 1H), 7.78–7.62 (m, 2H), 4.88 (br, H_2O+NH), 2.52 (s, 3H); ^{13}C NMR (DMSO-d_6): δ 156.26, 135.45, 132.97, 126.46, 124.07, 122.91, 121.81, 116.07, 112.27, 86.21, 20.32; IR (KBr) ν 3301 (NH), 2214 (CN) cm^{-1}; MS *m*/*z* (rel abundance): 288 (74) M$^+$, 224 (74), 223 (100), 203 (8), 155 (9), 69 (16). Anal. Calcd for $C_{11}H_7F_3N_2O_2S$: C 45.84%, H 2.45%, N 9.72%, S 11.12%. Found: C 45.83%, H 2.46%, N 9.66%, S 11.49%.

5.1.4. 6-Fluoro-3-methyl-4*H*-1,4-benzothiazine-2-carbonitrile 1,1-dioxide (3c). The compound was prepared from **4f** by a procedure similar to the procedure described for **3a**. White crystals, mp 261–263 °C (sinters at 240–251 °C); ^{1}H NMR (DMSO-d_6): δ 12.03 (br s, 1H, NH), 8.06 (dd, 9.0 Hz/5.5 Hz, 1H, H8), 7.35 (m, 1H, H7), 7.20 (dd, 10.0/2.5 Hz, 1H, H5), 2.46 (s, 3H, CH_3); ^{13}C NMR (DMSO-d_6): δ 163.89 (d, J = 251 Hz), 155.86, 136.98 (d, J = 12 Hz), 125.49 (d, J = 11 Hz), 120.58 (d, J = 2.7 Hz), 114.03 (d, J = 24 Hz), 112.46, 104.99 (d, J = 26 Hz), 86.31, 20.16; IR (KBr) ν 3273 (NH), 2215 (CN) cm^{-1}. Anal. Calcd for $C_{10}H_7FN_2O_2S$: C 50.42%, H 2.96%, N 11.76%. Found: C 50.10%, H 2.85%, N 11.45%.

5.1.5. 7-Fluoro-3-methyl-4*H*-1,4-benzothiazine-2-carbonitrile 1,1-dioxide (3d). The compound was prepared from **4g** in 85% yield by a procedure similar to the procedure described for **3a**. White solid, mp 326–327 °C (decomp.); ^{1}H NMR (DMSO-d_6): δ 12.07 (s, 1H, NH), 7.95–7.81 (m, 1H), 7.75–7.45 (m, 2H), 2.47 (s, CH_3+DMSO); ^{13}C NMR (DMSO-d_6): δ 158.50 (d, J = 247 Hz), 155.47, 131.73 (d, J = 2.3 Hz), 124.53 (d, J = 7.3 Hz), 121.89 (d, J = 22 Hz), 121.73 (d, J = 9.7 Hz), 112.65, 108.02 (d, J = 26 Hz), 84.29, 20.08; MS *m*/*z* (rel abundance) 238 (M+, 42), 174 (M−64, 49), 173 (M−65, 100); IR (KBr) ν 3291 (NH), 2214 (CN) cm^{-1}. Anal. Calcd for $C_{10}H_7FN_2O_2S$: C 50.42%; H 2.96%, N 11.76%. Found: C 50.38%, H 2.93%, N 11.60%.

5.1.6. 7-Methoxy-3-methyl-4*H*-1,4-benzothiazine-2-carbonitrile 1,1-dioxide (3e). The compound was prepared from **4h** in 23% yield by a procedure similar to the procedure described for **3a**. White crystals; mp 311–313 °C (from methanol); ^{1}H NMR (DMSO-d_6): δ 11.89 (br s, 1H, NH), 7.51–7.27 (m, 3H), 3.86 (s, 3H, CH_3O), 2.44 (s, 3H, CH_3); IR (KBr) ν 3281 (NH), 2207 (CN) cm^{-1}. Anal. Calcd for $C_{11}H_{10}N_2O_3S$: C 52.79%, H 4.03%, N 11.19%. Found: C 52.57%, H 3.89%, N 10.95%.

5.1.7. 7-Chloro-3-isopropylamino-4*H*-1,4-benzothiazine-2-carbonitrile 1,1-dioxide (3f). Compound **11a** (275.1 mg, 1 mmol) and isopropylamine (95 μL, 1.1 mmol) was dissolved in 3 mL of CH_3CN. To the stirred solution was added dry Et_3N (153 μL, 1.1 mmol) and the mixture was stirred in a screw-cap sealed reaction vessel at 80 °C for 52 h. The mixture was concentrated in vacuo and purified by column chromatography (EtOAc–heptane 2:1, V/V) to give the title compound (40 mg, 13%) as white crystals, mp 291–292 °C; ^{1}H NMR (DMSO-d_6): δ 10.68 (s, 1H), 7.89 (d, J = 8.67 Hz, 1H), 7.77 (d, J = 2.26 Hz, 1H), 7.71 (m, 1H), 7.58 (d, J = 8.67 Hz, 1H), 4.10 (m, 1H), 1.24 (d, J = 6.41 Hz, 6H). ^{13}C NMR (DMSO-d_6): δ 153.88, 133.41, 132.40, 128.35, 128.29, 121.54, 119.38, 114.04, 65.02, 44.98, 22.66. Anal. Calcd for $C_{12}H_{12}ClN_3O_2S$: C 48.41%, H 4.06%, N 14.11%, Cl 11.91%l. Found: C 48.03%, H 4.05%, N 13.83%, Cl 11.70%.

5.1.8. 3-Isopropylamino-6-trifluoromethyl-4*H*-1,4-benzothiazine-2-carbonitrile 1,1-dioxide (3g). A mixture of **11b** (0.135 g, 0.44 mmol) and isopropylamine (1 mL) was heated for 42 h at 60–65 °C in a screw-cap sealed reaction vessel, cooled to room temperature and concentrated on a rotary evaporator. The residual yellow oil was stirred for 1 h with H_2O (8 mL), pH was adjusted to 1 by 4 M HCl (~10 drops) and the precipitate was filtered off, rinsed with H_2O and dried to give a beige powder (0.106 g). Purification of the crude product by flash column chromatography (R_f = 0.8; silica, EtOAc) gave white voluminous crystals (0.021 g, 14%); mp 311–311.5 °C; ^{1}H NMR (DMSO-d_6): δ 10.75 (s, 1H), 8.12–7.94 (m, 3H), 7.78–7.68 (m, 1H), 4.24–4.01 (m, 1H), 1.26 (d, 6H); MS *m*/*z* (rel abundance): 331 (26) M$^+$, 289 (17), 267 (12), 266 8 (12), 252 (83), 225 (100), 199 (22), 176 (15), 160 (34), 156 (23), 107 (14), 67 (16), 58 (22), 43 (43), 41 (39); IR (KBr) ν 2196 (CN) cm^{-1}. Anal. Calcd for $C_{13}H_{12}F_3N_3O_2S$: C 47.13%, H 3.65%, N 12.68%. Found: C 47.30%, H 3.81%, N 12.28%.

5.1.9. 7-Chloro-3-isopentylamino-4*H*-1,4-benzothiazine-2-carbonitrile 1,1-dioxide (3h). A mixture of **11a** (0.50 g, 1.8 mmol) and isopentylamine (2 mL) was heated for 24 h at 60–65 °C in a screw-cap sealed reaction vessel, cooled to room temperature and condensed on a rotary evaporator. The residual yellow oil was stirred for 1 h with a mixture of H_2O (20 mL) and 4 M HCl (1 mL) and the precipitate was filtered off, rinsed with H_2O and dried to give a beige powder (0.52 g). Recrystallization from glacial acetic acid gave the title compound (0.37 g, 63%), mp 252–254 °C. R_f = 0.7 (silica, EtOAc); ^{1}H NMR (DMSO-d_6): δ 10.79 (s, 1H, NH), 7.99 (br t, 1H, NH), 7.81–7.67 (m, 3H, 6H+8H), 7.56 (d,

$J = 9.2$ Hz, 1H, 5H), 3.42 (q, ~4H, CH_2+H_2O), 1.60 (octet, 1H, methin), 1.48 (q, 2H, CH_2), 0.90 (d, 6H, CH_3). ^{13}C NMR (DMSO-d_6): δ 154.77, 133.30, 132.44, 128.31, 121.45, 119.44, 113.94, 65.00, 41.06, 37.49, 25.04, 22.23; MS *m*/*z* (rel abundance) 325 (30) M^+, 321 (18), 269 (20), 255 (57), 205 (46), 204 (99), 191 (36), 190 (23), 176 (44), 170 (24), 165 (25),156 (47), 148 (21), 126 (22), 70 (25), 43 (100) *i*-Pr^+; IR (KBr) *v* 3276 (NH), 2199 (CN) cm^{-1}. Anal. Calcd for $C_{14}H_{16}ClN_3O_2S$: C 51.61%, H 4.95%, N 12.90%. Found: C 51.57%, H 4.90%, N 12.68%.

5.1.10. 7-Chloro-3-cyclobutylamino-4*H*-1,4-benzothiazine-2-carbonitrile 1,1-dioxide (3i). This compound was prepared from **11a** and cyclobutylamine according to the procedure described for **3h**. White crystals (55 mg, 10%); mp 316–318 °C (decomp.); ^{1}H NMR (DMSO-d_6): δ 10.70 (br, 1H), 8.32 (br d, 1H), 7.82–7.65 (d+dd, 2H), 7.61–7.48 (d, 1H), 4.30 (m, 1H), 2.48–1.54 (m, 6H); ^{13}C NMR (DMSO-d_6): δ 153.50, 133.37 132.44, 128.28, 121.55, 119.41, 113.87, 65.19, 47.68, 30.38, 14.29; IR (KBr) *v* 2198 (CN) cm^{-1}; MS *m*/*z* (rel abundance) 309 (30; M+), 281 (11), 217 (68), 216 (86), 190 (29), 189 (32), 182 (100; M−SO_2−Cl−C_2H_4), 155 (38), 55 (24). Anal. Calcd for $C_{13}H_{12}ClN_3O_2S$: C 50.41%, H 3.90%, N 13.56%. Found: C 50.27%, H 3.66%, N 13.30%.

5.1.11. *N*-(4-Chloro-2-cyanomethylsulfonylphenyl)acetamide (4a). NaH (0.10 g, 60% dispersion in mineral oil, 2.5 mmol) was added at ambient temperature under nitrogen in small portions to a stirred solution of **6a** (0.55 g, 2.4 mmol) in dry dimethylformamide (7.5 mL). After stirring for 35 min iodoacetonitrile (0.18 mL) was added and the temperature was raised to 70 °C for 45 min. Then the solvent was removed in vacuo. The residue was triturated with H_2O (30 mL) and filtered. The filter cake was dried, stirred with a mixture of ether (5 mL) and petroleum ether (10 mL) for 1 h, filtered off and dried to give the title compound as white needles (0.52 g, 81%); mp 173.5–174 °C: ^{1}H NMR (DMSO-d_6): δ 9.69 (s, 1H); 8.06–7.87 (m, 3H); 5.37 (s, 2H), 2.16 (s, 3H); ^{13}C NMR (DMSO-d_6): δ 169.25, 136.22, 136.02, 130.10, 129.41, 129.13, 128.16, 111.66, 44.57, 23.97 ppm; IR (KBr) *v* 3343(NH), 2258 (CN), 1682 (C=O), 1330 (SO_2), 1156 and 1140 (SO_2) cm^{-1}. Anal. Calcd for $C_{10}H_9ClN_2O_3S$: C 44.04% H 3.33% N 10.11% Cl 13.00%. Found: C 43.86% H 3.30% N 10.11% Cl 13.09%.

5.1.12. *N*-(2-Cyanomethylsulfonyl-5-trifluoromethylphenyl)acetamide (4b). A solution of 3-chloroperbenzoic acid (0.78 g, purity ~70%, 3.2 mmol) in CH_2Cl_2 was added to a stirred solution of **9a** (0.31 g, 1.1 mmol) in CH_2Cl_2 (10 mL) at 0 °C. After $6\frac{1}{2}$ h an additional amount of 3-chloroperbenzoic acid (0.2 g) was added and the solution was left at room temperature overnight. Excess of peracid was destroyed by washing the reaction mixture with a solution of sodium disulfite (0.5 g) in H_2O (10 mL). The solution was dried over Na_2SO_4 and evaporated. The residue was recrystallized from 96% ethanol to give the title compound as white voluminous crystals (0.22 g, 62%); mp 146–147 °C; ^{1}H NMR ($CDCl_3$): δ 9.54 (br, 1H, NH), 9.00 (br, 1H), 8.10 (d, $J = 8.3$ Hz, 1H), 7.55 (dd, $J1 = 8.3$ Hz, $J2 = 1.4$ Hz, 1H), 4.18 (s, 2H), 2.30 (s, 3H); ^{13}C NMR ($CDCl_3$): δ 168.80, 139.07, 138.55 ($J = 34$ Hz), 131.59, 125.72, 122.6 ($J = 274$ Hz), 120.61 ($J = 3.7$ Hz), 120.21 ($J = 4.0$ Hz), 109.26, 45.92, 25.24; MS *m*/*z* (rel abundance): 306 (8) M^+, 264 (55), 224 (35), 202 (7.6), 183 (5), 176 (12), 160 (28), 140 (6), 43 (100). Anal. Calcd for $C_{11}H_9F_3N_2O_3S$: C 43.14%, H 2.96%, N 9.15%. Found: C 43.33% H 2.94% N 8.96%. An additional crop of the title compound (0.05 g, 14%) was obtained from the mother liquor.

5.1.13. (2-Cyanomethylsulfonyl-4-fluorophenyl)carbamic acid isopropyl ester (4c). To a solution of **9c** (0.23 g, 0.89 mmol) in glacial acetic (1.5 mL) acid was added 35% hydrogen peroxide (0.5 mL, 5 mmol). The solution was heated at 100 °C for 1 h 45 min, cooled to 0 °C and diluted with H_2O (10 mL). After stirring at 0 °C for 20 min the precipitate was collected by filtration, rinsed with H_2O (5 mL) and dried to yield the title compound as white needles (0.21 g, 81%); mp 123–123.5 °C; ^{1}H NMR (DMSO-d_6): δ 9.00 (s, 1H, NH), 8.10–7.95 (m, 1H), 7.85–7.67 (m, 2H), 5.37 (s, 2H), 4.90 (septet, 1H), 1.28 (d, 6H); ^{13}C NMR (DMSO-d_6): δ 157.40 ($J = 246$ Hz), 153.08, 134.29 ($J = 2.9$ Hz), 127.96 ($J = 6.6$ Hz), 127.14 ($J = 7.6$ Hz), 124.24 ($J = 22$ Hz), 117.36 ($J = 26$ Hz), 111.57, 68.94, 44.89, 21.74. EI/MS *m*/*z* (rel abundance): 300 (14) M^+, 241 (34) M^+−PrO, 214 (19), 174 (20), 126 (11), 110 (16), 108 (15), 43 (100); IR (KBr) *v* 3343 (NH), 2260 (CN), 1712 (C=O) cm^{-1}. Anal. Calcd for $C_{12}H_{13}FN_2O_4S$: C 47.99%, H 4.36%, N 9.33%. Found: C 48.06%, H 4.42%, N 9.13%.

5.1.14. (4-Chloro-2-cyanomethylsulfonylphenyl)carbamic acid ethyl ester (4d). Hydrogen peroxide (3 mL, 35%) was added drop wise to a stirred solution of **9b** (3.22 g, 12 mmol) in glacial acetic acid (30 mL) at room temperature. After 1 h an additional amount of hydrogen peroxide (2 mL) was added. The mixture was heated at 100 °C for 2 h. After standing overnight at room temperature voluminous crystals had precipitated. Water (100 mL) was added and the crystals were collected by filtration. The filter cake was washed on the filter with portions of H_2O (50 mL in total) and dried to give the title compound as pale needles (3.20 g, 89%). Recrystallization from 96% ethanol gave white, voluminous needles (76% recovered), mp 131–133 °C; ^{1}H NMR (DMSO-d_6): δ 9.09 (s, 1H, NH), 8.18–8.07 (m, 1H), 7.98–7.87 (m, 2H), 5.42 (s, 2H), 4.18 (q, 2H), 1.25 (t, 3H); ^{13}C NMR (DMSO-d_6): δ 152.95 (C=O), 136.82, 136.38, 130.11, 127.82, 126.71, 125.14, 111.56 (CN), 61.49 (O–CH_2), 45.12 (SO_2–CH_2), 14.26 (CH_3); MS *m*/*z* (rel abundance): 302 (34) M^+, 230 (17), 190 (38), 170 (26), 142 (47), 126 (100); IR (KBr) *v* 3358 (NH), 2259 (CN), 1722 (C=O) cm^{-1}. Anal. Calcd for $C_{11}H_{11}N_2O_4ClS$; C 43.64%, H 3.66%, N 9.25%. Found: C 43.74%, H 3.73%, N 9.12%.

5.1.15. (2-Cyanomethylsulfonyl-5-trifluoromethylphenyl)carbamic acid ethyl ester (4e). Oxidation of **9d** (3.55 g) with 3-chloroperbenzoic acid in CH_2Cl_2 as described for **4b** gave the title compound as white crystals

(2.61 g, 90%; practically pure for further synthesis). An analytically pure sample was obtained by further recrystallization, mp 109–110 °C (from EtOH); ^{1}H NMR ($CDCl_3$): δ 8.93 (br, 1H, NH), 8.82 (br, 1H), 8.10 (d, $J1 = 8.3$ Hz, 1H), 7.50 (dd, $J2 = 1.4$ Hz, 1H), 4.30 (q, 2H), 4.17 (s, 3H), 1.38 (t, 3H); ^{13}C NMR ($CDCl_3$): δ 152.68, 139.50, 138.58 (q, $J = 33$ Hz), 131.94, 124.75, 122.59 (CF_3, $J = 274$ Hz), 119.64 ($J = 3.6$ Hz), 118.51 ($J = 4.0$ Hz), 109.13, 62.59, 45.43, 14.31; IR (KBr) ν 2258 (CN), 1716 (C=O), cm^{-1}. Anal. Calcd for $C_{12}H_{11}F_3N_2O_4S$: C 42.86%, H 3.30%, N 8.05%. Found: C 42.99%, H 3.26%, N 8.15%.

5.1.16. *N*-(2-Cyanomethylsulfonyl-5-fluorophenyl)acetamide (4f). The title compound was prepared by oxidation of **9e** with H_2O_2 in glacial acetic acid according to the procedure described for **4c**. White crystals, mp 131–132 °C; ^{1}H NMR (DMSO-d_6): δ 9.65 (s, 1H, NH), 8.07–7.93 (m, 2H), 7.41–7.28 (m, 1H), 5.32 (s, 2H, CH_2), 2.16 (s, 3H, CH_3); ^{13}C NMR (DMSO-d_6): δ 169.24, 165.91 (d, $J = 254$ Hz), 139.92 (d, $J = 13$ Hz), 134.12 (d, $J = 11.5$ Hz), 123.19 (d, $J = 2.9$ Hz), 112.28 (d, $J = 23$ Hz), 11.90, 111.60 (d, $J = 24$ Hz), 44.89, 24.37; IR ν 3343 (NH), 2253 (CN), 1693 (C=O) cm^{-1}.

5.1.17. *N*-(2-Cyanomethylsulfonyl-4-fluorophenyl)acetamide (4g). The title compound was prepared in 58% yield by oxidation of **9f** with H_2O_2 in glacial acetic acid according to the procedure described for **4c**. White needles, mp 154–157 °C (from 96% EtOH); ^{1}H NMR (DMSO-d_6): δ 9.64 (s, 1H, NH), 7.99–7.64 (m, 3H), 5.30 (s, 2H, CH_2), 2.08 (s, 3H, CH_3); ^{13}C NMR (DMSO-d_6): δ 169.43, 158.24 (d, $J = 247$ Hz), 133.66 (d, $J = 3$ Hz), 130.75 (d, $J = 6.5$ Hz), 130.04 (d, $J = 7.3$ Hz), 123.34 (d, $J = 22$ Hz), 117.39 (d, $J = 26$ Hz), 111.66, 44.35, 23.63; MS *m*/*z* (rel abundance) 256 (M+, 21), 214 (M−42, 70), 174 (31), 126 (16), 110 (31); IR ν 2261 (CN), 1667, 1638 (C=O) cm^{-1}.

5.1.18. *N*-(2-Cyanomethylsulfonyl-4-methoxyphenyl)acetamide (4h). The title compound was prepared in 58% yield by oxidation of **9g** with H_2O_2 in glacial acetic acid according to the procedure described for **4c**. Pale yellow crystals, mp 189–190 °C (from 96% EtOH); ^{1}H NMR (DMSO-d_6): δ 9.54 (br s, 1H, NH), 7.70–7.62 (m, 1H), 7.46–7.35 (m, 2H), 5.20 (s, 2H, CH_2), 3.84 (s, 3H, CH_3O), 2.07 (s, 3H, CH_3); ^{13}C NMR (DMSO): δ 169.46, 156.58, 131.09, ca. 129.62, 129.62, 121.72, 115.07, 111.86, 55.95, 44.21, 23.44.

5.1.19. 2-Acetylamino-5-chloro-benzenesulfinic acid (6a). A mixture of ground phosphorus pentachloride (2.28 g, 11 mmol) and 2-acetylamino-5-chloro-benzenesulfonic acid pyridinium salt (**5a**) (3.00 g, 9 mmol) was stirred at 60 °C. After 2 h the resulting yellow fluid was poured onto crushed ice/water (100 mL) and stirred vigorously for 10 min. The aqueous phase was decanted and the sticky precipitate was dissolved in CH_2Cl_2 (75 mL) and dried over Na_2SO_4 and filtered. The filtrate was cooled to −75 °C and a solution of *p*-toluenethiol (2.28 g) and triethylamine (2.55 mL) in CH_2Cl_2 (25 mL) was added drop wise with stirring. After 15 min an additional amount of triethylamine (1 mL) was added. The mixture was stirred at −75 °C for 1 h, heated to room temperature and extracted with H_2O (5 × 20 mL). The combined aqueous phases were extracted with ether (20 mL) and cooled to 0 °C. The solution was acidified with concentrated HCl (5 mL), NaCl (15 g) was added and the solution was extracted with ether (5 × 20 mL). The combined extracts were dried over Na_2SO_4, filtered and evaporated to give **6a** as a white solid (0.58 g, 27%). The crude product was practically pure and was used without purification; mp 116–119 °C; ^{1}H NMR (DMSO-d_6): δ 10.07 (br, 1H), 7.7–7.48 (m, 3H), 5.7 (br, –SO_2H+H_2O), 2.08 (s, 3H).

5.1.20. (2-Amino-4-trifluoromethylphenylsulfanyl)acetonitrile (8a). NaH (2.93 g, 60% dispersion in mineral oil 73 mmol) was added at 5–10 °C under nitrogen in small portions to a stirred and cooled solution of 2-amino-4-trifluoromethylbenzenethiol hydrochloride (8.00 g, 35 mmol) dissolved in dry dimethylformamide (125 mL). After stirring for 45 min iodoacetonitrile (2.65 mL) was added. Stirring was continued at ~0 °C for 15 min and then at room temperature for 1 h. The mixture was concentrated in vacuo at 55 °C and the oily residue was extracted with petroleum ether (3 × 50 mL) in order to remove the mineral oil. The residue was partitioned between H_2O (50 mL) and EtOAc (100 mL). The organic phase was washed with H_2O (2 × 50 mL) and then with brine (30 mL), dried over Na_2SO_4, filtered and concentrated in vacuo to give a yellow oil. The crude product was purified by flash chromatography on silica gel eluted with EtOAc–heptane 3:7 (V/V) giving the title compound as a yellow oil (6.1 g, 75%); ^{1}H NMR (DMSO-d_6): δ 7.54 (d, $J = 8.3$ Hz, 1H), 7.09 (d, $J = 1.8$ Hz, 1H), 6.86 (dd, 1H), 5.97 (br, 2H), 4.02 (s, 2H); ^{13}C NMR (DMSO-d_6): δ 149,.88, 135.66, 130.74 ($J = 31$ Hz), 124.12 ($J = 272$ Hz), 117.58, 116.96 ($J = 1.3$ Hz), 112.04 ($J = 3.8$ Hz), 110.53 ($J = 3.9$ Hz), 17.95.

5.1.21. (2-Amino-5-chloro-phenylsulfanyl)acetonitrile (8b). A mixture of 2-amino-6-chloro-benzothiazole (4.90 g, 26.5 mmol) and a solution of potassium hydroxide (8.9 g, 159 mmol) in H_2O (18 mL) was stirred at 160 °C under nitrogen for 5.5 h. The mixture was cooled to 0–5 °C and acetic acid (6.25 mL) in H_2O (6 mL) was added in order to neutralize the excess of NaOH but not the thiolate. To the resulting slurry was added ethanol (30 mL) and then iodoacetonitrile (1.9 mL, 26.5 mmol). After 0.5 h acetic acid (1.55 mL) was added and the mixture was concentrated in vacuo to approx. 20 mL and diluted with H_2O (100 mL). The precipitated pale yellow crystals were filtered off, rinsed with H_2O and dried to give the title compound (4.98 g, 94%) practically pure, mp 71–73 °C. A sample was recrystallization from ethanol to give pure crystals, mp 72–73 °C; $R_f = 0.39$ (EtOAc–heptane 1:1); ^{1}H NMR ($CDCl_3$): δ 7.49 (d, $J = 2.3$ Hz, 1H), 7.18 (dd, 1H), 6.70 (d, $J = 9.2$ Hz, 1H), 4.42 (br, 2H), 3.47 (s, 2H); ^{13}C NMR ($CDCl_3$): δ 147.49, 136.07, 131.93, 122.87, 116.46, 116.31, 114.75, 19.98; IR (KBr) ν 3362 (NH_2), 2243 (CN) cm^{-1}.

5.1.22. (2-Amino-5-fluorophenylsulfanyl)acetonitrile (8c). A mixture of 2-amino-6-fluorobenzothiazole (5.13 g, 30.5 mmol) and a solution of potassium hydroxide (10.3 g, 183 mmol) in H_2O (20 mL) was stirred at 165–170 °C under nitrogen in a flask with a condenser for $6\frac{1}{2}$ h. The mixture was cooled to 10 °C and acetic acid (7.2 mL) in H_2O (7 mL) was added. Then pH was adjusted to 8 by the addition of 1 M NaOH and ethanol (30 mL) and iodoacetonitrile (2.20 mL) was added. After 15 min the reaction was quenched by the addition of acetic acid (1.8 mL) and the mixture was concentrated in vacuo to approx. 20 mL and diluted with H_2O (150 mL). A yellow oil separated. The mixture was extracted with EtOAc (50 mL). A white precipitate in the EtOAc phase was removed by filtration and was identified as thiocarbamic acid S-(2-amino-5-fluorophenyl) ester, yield 0.35 g. The filtrate was dried over Na_2SO_4, filtered and the solvent was removed in vacuo to give the title compound (4.88 g, 88%) as a yellow oil containing a small amount of crystalline precipitate. The product was used without further purification. ^{1}H NMR (DMSO-d_6): δ 7.26–7.14 (m, 1H), 7.09–6.95 (m, 1H), 6.85–6.72 (m, 1H), 5.36 (br, 2H, NH_2), 3.99 (s, 2H). ^{13}C NMR (DMSO-d_6): δ 153.53 (d, J = 234 Hz), 146.20 (d, J = 1.4 Hz), 120.30 (d, J = 22.3 Hz), 117.68, 117.54 (d, J = 22.0 Hz), 115.69 (d, J = 7.6 Hz), 113.29 (d, J = 7.9 Hz), 18.19.

5.1.23. (2-Amino-4-fluorophenylsulfanyl)acetonitrile (8d). The title compound was prepared in 96% yield from 2-amino-5-fluorobenzothiazole according to the procedure described for **8c**. Yellow oil; ^{1}H NMR (DMSO-d_6): δ 7.89 (dd, 1H, H6), 6.56 (dd, 1H, H3), 6.37 (ddd, 1H, H5), 5.88 (br, 2H, NH_2), 3.83 (s, 2H, CH_2); ^{13}C NMR ($CDCl_3$): δ 164.41 (d, J = 244 Hz), 152.24 (d, J = 12.8 Hz), 138.67 (d, J = 11.0 Hz), 117.75, 107.76 (d, J = 2.1 Hz), 103.27 (d, J = 22.4 Hz), 100.40 (d, J = 25.1 Hz), 18.93.

5.1.24. *N*-(2-Cyanomethylsulfanyl-5-trifluoromethylphenyl)acetamide (9a). Compound **8a** (0.66 g, 2.8 mmol) was stirred on an ice bath at 0 °C. Acetic acid anhydride (2.5 mL) was added, and the resulting solution was allowed to reach room temperature. After 1 h the mixture was concentrated in vacuo and the solid residue was triturated with petroleum ether (2 × 2 mL), filtered off and dried to give the title compound as a white solid (0.75 g, 96%), mp 103–103.3 °C; ^{1}H NMR ($CDCl_3$): δ 8.79 (br, 1H), 8.36 (br, 1H), 7.78 (d, 1H), 7.38 (dd, 1H, J: 8 Hz/2 Hz), 3.50 (s, 2H), 2.29 (s, 3H); ^{13}C NMR (DMSO-d_6): δ 169.06, 137.02, 134.25, 129.49, 127.56 (J = 33 Hz), 123.81 (J = 272 Hz), 122.61 (two very close signals), 117.49, 23.06, 17.46; IR (KBr) ν 2244 (C≡N) cm^{-1}.

5.1.25. (4-Chloro-2-cyanomethylsulfanyl-phenyl)carbamic acid ethyl ester (9b). Compound **8b** (3.1 g, 15.5 mmol) was dissolved dry THF (in 25 mL) and cooled in an ice bath at 0 °C. To the stirred solution was added first a solution of ethyl chloroformate (1.50 mL) in THF (5 mL) and then a solution of pyridine (1.25 mL) in THF (5 mL). The ice bath was removed after completion of the addition and the mixture was stirred for 2 h. The solvent was removed in vacuo on a rotary evaporator, ether (100 mL) was added and the mixture was washed with H_2O (2 × 25 mL) and finally with brine (10 mL). The organic phase was dried over Na_2SO_4, filtered and evaporated to give the title compound as a practically pure off-white solid (3.4 g, 75%); mp 93–95 °C; ^{1}H NMR (DMSO-d_6): δ 9.11 (s, 1H, NH), 7.71–7.56 (m, 1H), 7.48–7.32 (m, 2H), 4.25 (s, 2H), 4.10 (q, 2H), 1.22 (t, 3H); ^{13}C NMR (DMSO-d_6): δ 154.21 (C=O), 136.10, 131.86, 129.98, 129.03, 127.75, 127.14, 117.54 (CN), 60.68 (O–CH_2), 18.09 (S-CH_2), 14.46 (CH_3); IR (KBr) ν 3362 (NH), 2243 (CN), 1719 (C=O) cm^{-1}.

5.1.26. (2-Cyanomethylsulfanyl-4-fluorophenyl)carbamic acid isopropyl ester (9c). To a stirred solution of **8c** (0.20 g, 1.1 mmol) in dry THF (5 mL) at 0 °C was added drop wise a 1 M solution of isopropyl chloroformate in toluene (1.15 mL) and then pyridine (93 μL, 1.16 mmol). After $\frac{1}{2}$ h the solvent was stripped off and the oily residue was triturated with H_2O (10 mL) at 0 °C. A solid precipitate was collected by filtration, rinsed with H_2O (5 mL) and dried to give the title compound as a beige powder (0.24 g, 81%). The crude product was used without further purification. Mp 68–69 °C; ^{1}H NMR (DMSO-d_6): δ 9.00 (s, 1H, NH), 7.50–7.28 (m, 2H), 7.22–7.07 (m, 1H), 4.82 (septet, 1H), 4.24 (s, 2H), 1.22 (d, 6H); ^{13}C NMR (DMSO-d_6): δ 159.84 (J = 237 Hz), 154.15, 132.90 (J = 2.9 Hz), 128.34 (J = 9.2 Hz), 117.59, 115.22 (J = 25 Hz), 114.10 (J = 22 Hz), 67.90, 21.92, 17.55; MS m/z (rel abundance): 268 (30) M$^+$, 226 (6), 209 (9), 184 (7), 182 (20), 168 (9), 154 (22), 142 (92), 114 (15, 98 (10), 45 (16), 43 (100), 42 (43); IR (KBr) ν 3304 (NH), 2249 (CN), 1693 and 1534 (C=O) cm^{-1}.

5.1.27. (2-Cyanomethylsulfanyl-5-trifluoromethylphenyl)carbamic acid ethyl ester (9d). Ethyl chloroformate (1.67 mL, 17.4 mmol) and subsequently triethylamine (2.41 mL, 17.4 mmol) were added drop wise to a stirred solution of **8a** (3.68 g, 17.4 mmol) in dry THF (25 mL) at 0 °C. The ice bath was removed and stirring was continued at ambient temperature for 30 min and then $1\frac{1}{2}$ h at 60 °C. The solvent was removed on a rotary evaporator and the residue was partitioned between ether (75 mL) and H_2O (25 mL). The organic layer was washed with H_2O (2 × 20 mL) and finally with brine (20 mL), dried over Na_2SO_4, filtered and evaporated to give the title compound as yellow crystals (3.06 g, 63%). Recrystallization from 96% ethanol gave pale prisms (2.42 g, 50%), pure enough for further synthesis. A pure sample was obtained from ether–petroleum ether 1:1; mp 87–88 °C, ^{1}H NMR (DMSO-d_6): δ 9.30 (s, 1H), 7.85–7.59 (m, 3H), 4.29 (s, 2H), 4.14 (q, 2H), 1.25 (t, 3H); ^{13}C NMR (DMSO-d_6): δ 154.22 (C=O), 137.29, 134.31, 129.84, 127.82 (q, J = 32 Hz, CF_3), 123.78 (q, J = 272 Hz, CF3), 122.38 (J = 3.8 Hz), 121.90 (J = 3.6 Hz), 117.49 (CN), 60.88, 17.70, 14.42; EI-MS: m/z (rel abundance): 304 (100) M$^+$, 285 (18), 258 (14), 232 (50), 204 (53), 192 (99), 45 (16), 40 (22); IR (KBr) ν 3361 (NH), 2247 (CN), 1725 (C=O) cm^{-1}.

5.1.28. *N*-(2-Cyanomethylsulfanyl-5-fluorophenyl)acetamide (9e). Acetyl chloride (1.5 mL) in THF (10 mL) and subsequently pyridine (1.7 mL) in THF (10 mL) was added to a stirred solution of **8d** (3.5 g) in THF

(30 mL) at 0 °C. The mixture was stirred at 0 °C for 50 min. Then the solvent was evaporated and the residue was triturated with water (80 mL). The precipitate was filtered off and dried yielding 3.7 g (86%) of the title compound. Yellow crystals; mp 113–115 °C; ^{1}H NMR (DMSO-d_6): δ 9.59 (s, 1H, NH), 7.73–7.57 (m, 2H), 7.19–7.05 (m, 1H), 4.05 (s, 2H, CH_2), 2.12 (s, 3H, CH_3); ^{13}C NMR (DMSO-d_6): δ 169.03, 162.16 (d, J = 245 Hz), 140.67 (d, J = 12 Hz), 135.36 (d, J = 9.7 Hz), 120.34 (br), 117.64, 112.35 (d, J = 22 Hz), 111.05 (d, J = 26 Hz), 23.60, 19.79; IR (KBr) ν 3314 (NH), 2250 (CN), 1686 (C=O) cm^{-1}.

5.1.29. *N*-(2-Cyanomethylsulfanyl-4-fluorophenyl)acetamide (9f). This compound was prepared in 82% crude yield by acetylation of **8c** according to the procedure for **9e**. Recrystallization from 96% ethanol gave white crystals, mp 100.5–101.5 °C; ^{1}H NMR (DMSO-d_6): δ 9.68 (s, 1H, NH), 7.50–7.30 (m, 2H), 7.23–7.07 (m, 1H), 4.26 (s, 2H, CH_2), 2.05 (s, 3H, CH_3); ^{13}C NMR (DMSO-d_6) 168.80 (C=O), 159.90 (d, J = 244 Hz), 132.82 (d, J = 2.8 Hz), 132.11 (d, J = 8.6 Hz), 128.42 (d, J = 8.9 Hz), 117.60, 115.18 (d, J = 25.3 Hz), 114.05 (d, J = 22.3 Hz), 22.84, 17.50; IR (KBr) ν 3239 (NH), 2244 (CN), 1641 and 1579 (amide) cm^{-1}.

5.1.30. *N*-(2-Cyanomethylsulfanyl-4-methoxyphenyl)acetamide (9g). Sodium borohydride (0.19 g) was added in small portions over 30 min to a stirred solution of bis(2-acetylamino-5-methoxyphenyl)disulfane (1.0 g) in dry ethanol (100 mL) at 70 °C under nitrogen. The mixture was cooled to room temperature and iodoacetonitrile (355 μL) was added. After stirring for 15 min the solvent was evaporated. The residue was partitioned between ethyl acetate (50 mL) and water (50 mL); the organic phase was washed with water (2 × 50 mL) and dried over sodium sulfate. Removal of the solvent gave 1.06 g (91%) of the pure title compound. White crystals, mp 130.5–131.5 °C; ^{1}H NMR (DMSO-d_6): δ 9.48 (s, 1H, NH), 7.23 (d, J = 9 Hz, 1H), 7.08 (d, J = 3 Hz, 1H), 6.88 (dd, 1H), 4.18 (s, 2H, CH_2), 3.78 (s, 3H, CH_3O), 2.01 (s, 3H, CH_3); ^{13}C NMR (DMSO-d_6): δ 168.73, 157.44, 130.86, 129.64, 128.00, 117.85, 114.41, 112.84, 55.49, 22.83, 17.68; IR (KBr) ν 3286 (NH), 2250 (CN), 1658 (C=O) cm^{-1}.

5.1.31. 7-Chloro-3-hydroxy-4*H*-1,4-benzothiazine-2-carbonitrile 1,1-dioxide (10a). Compound **4d** (5.47 g, 18 mmol) was stirred at room temperature with 0.25 M NaOH (180 mL). After 2 h the resulting yellow solution was extracted with EtOAc (3 × 50 mL). The extracts were discarded and the aqueous phase was acidified with 4 M HCl (11.5 mL). The solvent was removed on a rotary evaporator and the yellow solid residue was extracted with petroleum ether–ether (3:1 (V/V), 25 + 40 mL)). The undissolved material was extracted with EtOAc (50 + 20 + 20 mL). The combined EtOAc extracts were evaporated to give the title compound as a beige solid (3.70 g, 80%); mp 204–206 °C (ebullition); ^{1}H NMR (DMSO-d_6): δ 10.09 (br, 1H, NH), 7.54 (d, J = 2.3 Hz, 1H), 7.47 (dd, 1H, C(6)–H), 7.12 (d, J = 8.7 Hz, 1H, C(5)–H), 5.31 (br, H_2O and possibly OH); ^{13}C NMR (DMSO-d_6): δ 162.51, 134.50, 131.74, 126.27, 124.65, 119.82, 118.75, 117.24, 66.44 ppm; MS m/z (rel abundance) 256 (100) M$^+$, 189 (31), 137 (25), 129 (19), 126 (22), 125 (78), 90 (20), 67 (31), 63 (53); IR (KBr) ν 3253 (NH), 2222 (CN) cm^{-1}.

5.1.32. 3-Hydroxy-6-trifluoromethyl-4*H*-1,4-benzothiazine-2-carbonitrile 1,1-dioxide (10b). Compound **4e** (2.61 g, 7.8 mmol) was stirred at room temperature in 0.2 M NaOH (50 mL). During the next 6$\frac{1}{2}$ h additional amounts of aqueous base were added: After 1.5 h 0.2 M NaOH (10 mL), and after 3 h 1 M NaOH (2 mL). The turbid mixture was filtered through celite and acidified to pH = 1 by the addition of 4 M HCl (3 mL) giving a white precipitate. The water was removed by evaporation and the white residue was extracted with two portions (30 + 10 mL) of EtOAc. The combined extracts were dried over Na_2SO_4 and the solvent was evaporated to give an amorphous residue. Trituration with a mixture of petroleum ether (20 mL) and ether (5 mL) gave white crystals, which were filtered off and dried to give the title compound, practically pure (2.08 g, 95%). The compound may be purified further by recrystallization from glacial acetic acid; mp 222–224 °C (ebullition; discolourization started at ~200 °C); ^{1}H NMR (DMSO-d_6): δ 10.20 (br, 1H, NH), 7.89–7.76 (~d at 7.82, J = 8.3 Hz,1H), 7.51–7.30 (m, 2H, d at 7.82 and dd at 7.38), 6.6–6.2 (br, two peaks: 6.44 and 6.39; 1H+H_2O); ^{13}C NMR ($CDCl_3$): δ 162.46 (C3), 136.32, 131.48 (J = 32 Hz, C6), 128.16, 123.52 (J = 273 Hz, CF_3), 122.10, 117.51 (CN), 116.86 (J = 3.7 Hz), 113.19 (J = 4.1 Hz), 66.06 (C2); EI-MS: m/z (rel abundance): 290 (100) M$^+$, 271 (18) M$^+$−F, 247 (4) M$^+$−CN−OH, 226 (28) M$^+$−SO_2, 223 (73) M$^+$−67, 206 (38), 171 (60), 159 (78), 132 (67), 113 (31), 109 (29),88 (22), 75 (34) CF_2C_2H, 69 (32) CF_3, 67 (49) NCCCOH, 63 (85); IR (KBr) ν 3278 (NH), 2233 and 2215 (CN) cm^{-1}.

5.1.33. 3,7-Dichloro-4*H*-1,4-benzothiazine-2-carbonitrile 1,1-dioxide (11a). Compound **10a** (1.75 g, 6.8 mmol) was added to a stirred solution of phosphoric acid (0.2 mL) in phosphorus oxychloride (6 mL). Then pyridine hydrochloride (1.98 g) was added. The mixture was heated at 100 °C overnight. The resulting dark solution was cooled on an ice bath and crushed ice (50 mL) was added with vigorous stirring. The mixture was filtered after 10 min, the beige filter cake washed on the filter with H_2O and dried to give the title compound (1.78 g, 95%). The product was almost pure and was used without further purification; mp 269–271 °C (decomp.); ^{1}H NMR (DMSO-d_6): δ 8.01 (d, J = 2.8 Hz, 1H), 7.79 (dd, 1H), 7.50 (d, J = 9.2 Hz, 1H), 6.33 (br, NH+H_2O); ^{13}C NMR (DMSO-d_6): δ 146.59, 134.87, 133.61, 129.75, 125.53, 123.23, 120.94, 112.02 (apparently two aromatic signals are coinciding); IR (KBr) ν 2217 (CN) cm^{-1}.

5.1.34. 3-Chloro-6-trifluoromethyl-4*H*-1,4-benzothiazine-2-carbonitrile 1,1-dioxide (11b). Compound **10b** (0.52 g, 1.8 mmol) was added to a stirred solution of phosphoric acid (0.05 mL) in phosphorus oxychloride (2.5 mL). Then pyridine hydrochloride (0.5 g) was added. The mixture was heated at 100 °C overnight. The resulting yellow solution was concentrated in a rotary evaporator in vacuo at 50–60 °C for 0.5 h. The oily residue was

cooled on an ice bath at 0 °C and stirred with ice/water (10 mL) for 0.5 h and the beige precipitate was filtered off, washed on the filter with water and dried to give the title compound (0.51 g, 93%). The product was almost pure and was used without further purification; mp 275–285 °C (decomp.); ^{1}H NMR (DMSO-d_6): δ 8.20–8.10 (m, 1H), 7.83–7.69 (m, 2H), 5.75 (br, NH+H_2O); ^{13}C NMR (DMSO-d_6): δ 147.87, 138.05, 132.67 (J = 33 Hz), 127.52, 127.31, 123.08 (J = 273 Hz), 121.58 (J = 3.4 Hz), 119.53, 112.62; MS m/z (rel abundance): 310 (11) M^++2, 308 (31) M^+, 289 (<1) M^+−F, 244 (100) M^+-SO_2, 225 (25), 209 (11), 194 (25); IR (KBr) ν 2218 (CN) cm^{-1}.

5.2. Biology

5.2.1. Cell culture and transfection. HEK 293 cells were cultured at 37 °C in a humidified atmosphere of 95% air and 5% CO_2 in Dulbecco's modified Eagles medium with 4.5 g/L glucose supplemented with 10% FCS, penicillin (100 units/mL) and streptomycin (0.1 mg/mL). Transient transfections were performed using Fugene 6 Transfection reagent (Roche), according to the manufacturer's instruction. Cells were seeded at 50% confluency and transfected with murine SUR2B (GenBank D86038) and human Kir6.2 (GenBank D50582). Cells were co-transfected with green fluorescent protein (GFP) to enable visual identification of transfected cells. Experiments were performed 2 days after transfection. HEK293 cells stably expressing human Kir6.2 and human SUR1 (Genbank L78207) were used for some experiments.[31]

5.2.2. Measurements of effects on membrane potential. Two days before experiments 50,000 cells/well, in 100 μL culture medium, were seeded out in black walled 96-well plates (Greiner). At the day of experiment, Loading Buffer from the Membrane potential kit (Molecular Devices) made in accordance with the kit prescription (100 μL/well) was added to all wells.

Plates were then incubated at 37 °C, 5.0% CO_2 for 30 min. Polypropylene plates (Greiner) were used to plate out 5× concentrated dilution series (starting at a final assay concentration of 30 μM at a 1:3 dilution). Compounds were tested as triple dilution series, each with eight points in 1:3 dilution steps. Both cell plate and compound plate were put in a NovoStar™ fluorescence plate reader for about 30 min at 34 °C and the measurements were subsequently begun. An initial reading was taken from the plate. Compounds were added manually from the compound plate with an eight-channel multipipette (50 μL/well) and the assay was then incubated for 10 min. A second reading was taken using the same measurement protocol without any adjustment of the signal gain. Measurement data were ported over to MS Excel where a before–after subtraction and normalization to the measurement level preceding any addition was made. Concentrations of test and control (BPDZ 73)[11] compounds were compared and E_{max} values were determined using BPDZ 73[11] as the reference at 30 μM. Data from three parallel runs on the same plate were copied to Graph Pad Prism where a four-parameter logistic curve fit was made (Hill slope locked at −1.5) for each run of eight points separately. The IC_{50} values were extracted and put into MS Excel to calculate a mean estimate of IC_{50} with SD. If the curves showed no slope the abbreviation is NA. If the IC_{50} value was above 100 the notation is >100 and no SD is given. Three or more observations from one or two assay runs were used for the compilation of data given in Table 1.

5.2.3. Membrane potential studies in HEK 293 cells expressing Kir6.2/SUR1 or Kir6.2/SUR2B. K_{ATP} channel activity was monitored by evaluating changes in membrane potential, using a membrane potential kit (Molecular Devices, USA). Assays were carried out in black clear-bottomed 96-well plates at 34 °C on a NovoStar™ machine (BMG, Germany) and K_{ATP} channels activated by the addition of the K_{ATP} channel opener in a mixed assay medium containing 10 μM tolbutamide. Changes in fluorescence were measured using an excitation wavelength of 490 nm and an emission wavelength of 520 nm, before and 5–10 min after addition of test compounds.

5.2.4. Inhibition of glucose-stimulated insulin release from βTC6 cells. βTC6 cells[32] were cultured at 5×10^4 cells/microtitre well in DMEM+10% FCS, 11 mM glucose, 1% Glutamax and 20 mM Hepes for 3 days (95% humidity, 37 °C, 5% CO_2). Cells were washed twice with NN buffer (all in mM: NaCl 114; KCl 47; KH_2PO_4, 1.21; $MgSO_4$, 1.16; $NaHCO_3$, 25.5; CaCl, $2H_2O$, 2.5; HEPES, 10) supplemented with 0.1% BSA and incubated 60 min in this buffer. All wells were aspirated and the cells incubated for 3 h with NN buffer, 22 mM glucose, 0.1 mM IBMX and serial dilutions of the compounds. A reference compound (BPDZ 73)[11] served as positive control. A test for responsiveness towards a series of glucose concentrations was included in every assay to ensure functionality. The supernatant from each well was harvested and insulin content was measured by an in-house ELISA using guinea pig anti-insulin antibodies and a rat insulin as standard. ELISA microtitre plates were coated with anti-guinea pig IgG (Dako Z 108, 1:1000) and incubated overnight at 4 °C in PBS pH 7.2. All plates were washed five times with washing buffer (PBS diluted 1:4 in H_2O+0.05% Tween20) and incubated 30 min in this buffer at rt. Wells were aspirated and anti-insulin antibodies (polyclonal GP4 (Novo Nordisk 1:75.000) was added, followed by an incubation for 2 h at room temperature (rt). Plates were washed five times in washing buffer and 10 μL test samples were added together with 60 μL Peroxidase-labelled (PO)-insulin (Sigma I 2133 1:2400) and incubated for another 2 h at rt in Assay buffer (washing buffer+0.5% BSA). A series of standards of rat insulin (NN) were made to cover a range from 1000 down to 1 ng/mL insulin and the standards were incubated with PO-insulin as well. Eventually, 120 μL TMB-substrate (3.3′, 5,5′-tetramethylbenzidine hydrogen peroxide) was added to all wells and the enzyme reaction stopped after 5 min by adding 120 μL 4 M H_3PO_4. Absorption was measured in an Elisa reader (450 nm with 620 nm as reference) and converted into nanogram per millilitre insulin. The results were

analyzed in Prism (Graph Pad software) and expressed as IC_{50} and E_{max}. The IC_{50} value was calculated as the concentration of test compound needed to inhibit the insulin release half of the maximal inhibition for that particular compound. The E_{max} was defined as the percentage inhibition of a compound tested at 10 μM relative to the maximum release when no compound was added. SEM was calculated for all compounds.

5.2.5. Relaxation of rat aorta rings. Female Wistar rats weighing approximately 150–200 g were killed by cervical dislocation, and the thoracic aorta was removed. Aorta was cut into rings of approximately 5 mm wide. Ring preparations were mounted in 5 mL organ baths (Danish Myo Technology, Aarhus, Denmark) with a resting tension of 2 g, and bathed in Krebs Ringer solution with the following composition (in mM: 118.5 NaCl; 25 $NaHCO_3$; 4.7 KCl; 6.8 $CaCl_2$; 2.4 $MgCl_2$; and 11.1 glucose in double distilled water). The Ringer solution was continuously aerated with 95% O_2/5% CO_2 at 37 °C. Cumulative concentration response curves (0.1–300 μM) were constructed for potassium channel openers on top of a precontraction induced by 0.3 μM phenylephrine. All test compounds were freshly dissolved in dimethylsulfoxide. Minimum values of each concentration step of potassium channel openers were measured. Sensitivity of vasodilating effect is expressed as the concentration inducing half of the maximal effect (EC_{50}), estimated using a four parameter logistic regression (Graphpad Prism, San Diego, CA, USA). Phenylephrine was obtained from Sigma Chemicals, St. Louis, MA, USA.

5.2.6. Measurements of insulin release from incubated rat pancreatic islets. Experiments were performed with pancreatic islets isolated by the collagenase method from fed female Wistar rats (Iffa Credo, Belgium).

Groups of 10 islets were preincubated for 30 min in a bicarbonate-buffered solution (in mM: 115 NaCl, 5 KCl, 2.56 $CaCl_2$, 1 $MgCl_2$, 24 $NaHCO_3$) supplemented with 2.8 mM glucose and 0.5% (w/v) dialyzed albumin (fraction V, Sigma Chemical Co.). The islets were then incubated for a further 90 min in 1 mL of the same bicarbonate-buffered medium containing 16.7 mM glucose and, in addition, the required compound. Individual experiments were repeated on different islets populations with the values corresponding to the number of samples pooled. Due to the islet-related variations, insulin release was expressed as a percentage of the value recorded in control experiments (100%), that is, in the absence of drug and presence of 16.7 mM glucose. The release of insulin was measured radioimmunologically as reported previously.[33] Compound **3a** and diazoxide were dissolved in dimethylsulfoxide, which was added to both control and test media. At the final concentrations used, dimethylsulfoxide fails to affect islet function.[29,34]

5.2.7. Measurements of ^{45}Ca outflow and insulin release from perifused rat pancreatic islets. The methods used to measure ^{45}Ca outflow and insulin release from perifused islets have been described previously.[11,29,30] Briefly, groups of 100 islets were incubated for 60 min in a bicarbonate buffered medium (in mM: 115 NaCl, 5 KCl, 2.56 $CaCl_2$, 1 $MgCl_2$, 24 $NaHCO_3$) containing 16.7 mM glucose and ^{45}Ca ion (0.02–0.04 mM; 100 μCi/mL). After incubation, the islets were washed four times with a non-radioactive medium and then placed in a perifusion chamber. The perifusate was delivered at a constant rate (1.0 mL/min). From the 31st to the 90th min of perifusion, the effluent was continuously collected over successive periods of 1 min each. An aliquot of the effluent (0.5 mL) was used for scintillation counting while the remainder was stored at −20 °C for further radioimmunological measurement of insulin release.[33] At the end of the experiment, the radioactive content of the islets was also determined. The outflow of $^{45}Ca^{2+}$ ion (cpm/min) was expressed as a fractional outflow rate (% of instantaneous islet content/min, FOR). Insulin release was expressed as μU/islet/min. Results are expressed as the mean (±SEM). The statistical significance of the differences between mean data was assessed by use of Student's *t*-test.

5.2.8. Patch-clamp measurements

5.2.8.1. RNA preparation. Mouse Kir6.2 (Genbank D50581) and rat SUR1 (Genbank L40624) cDNAs were cloned into the pBF expression vector. Capped mRNA was prepared using the mMESSAGE mMACHINE in vitro transcription kit (Ambion, Austin, USA).

5.2.8.2. Patch-clamp recordings. Whole cell currents were recorded from HEK 293 cells stably expressing human Kir6.2/SUR1 channels, using an EPC9 patch-clamp amplifier (HEKA Electronic GmbH, Lambrecht, Germany). Cells were clamped at −70 mV and currents evoked by repetitive 250 ms, 10 mV depolarizing voltage steps. Currents were filtered at 2 kHz and sampled at 10 kHz. The internal solution contained (in mM): 120 KCl, 1 $MgCl_2$, 5 EGTA, 2 $CaCl_2$, 20 Hepes, 0.3 MgADP, 5 MgATP (pH 7.3). The external solution was (in mM): 140 NaCl, 3 KCl, 1 $CaCl_2$, 1 $MgCl_2$, 20 mannitol, 10 HEPES (pH 7.2 with NaOH).

5.2.8.3. Xenopus oocyte macropatches. Currents were recorded from giant inside-out patches excised from oocytes expressing various types of K_{ATP} channel using an EPC7 patch-clamp amplifier. Currents were evoked by repetitive 3 s voltage ramps from −110 to +100 mV, filtered at 0.2 kHz and digitized at 0.4 kHz. The external (pipette) solution contained (in mM): 140 KCl, 1.2 $MgCl_2$, 2.6 $CaCl_2$, 10 HEPES (pH 7.4 with KOH). The intracellular (bath) solution contained (in mM): 110 KCl, 2 $MgCl_2$, 1 $CaCl_2$ 10 EGTA, 10 HEPES (pH 7.2 with KOH); final $[K^+]$: 140 mM). The slope conductance (G) was measured by fitting a straight line to the current–voltage relation between −20 mV and −100 mV. Drug effects were calculated as the conductance in the presence of drug (G) relative to the conductance in drug and nucleotide-free solution (G_c).

5.2.9. Beta cell selectivity studies after intravenous administration to rats. Post-prandial, adult male Sprague Dawley rats, weighing 280–320 g (M&B Breeding Cen-

ter, Ll. Skensved, Denmark), were anaesthetized subcutaneously with 2 mL/kg of a 1:1:1 mixture of Hypnorm (fentanyl citrate 0.315 mg/mL and fluanisone 10 mg/mL, Janssen Pharmaceutica, Belgium), Dormicum (midazolam, 5 mg/mL, Hoffmann-La Roche AG, Switzerland) and 0.9% NaCl aqueous solution, receiving supplemental anesthetic (1 mL/kg) every 20 min.[35] Body temperature was maintained at 37 °C by a homeothermic blanket system (Harvard Homeothermic Blanket System, Harvard Apparatus Ltd, UK). A polythene cannula (PE50) coupled to a pressure transducer (Statham P23XC) and a pressure coupler (HSE 561, Hugo Sachs Elektronik Kg, Germany) was introduced into the carotid artery to measure mean arterial blood pressure, which was recorded continuously and fed to a plotter (Linearcorder Mark VII, WR 3310, Hugo Sachs Elektronik Kg, Germany) and a computer and analyzed offline. A polythene cannula (PE50) was inserted into the jugular vein for drug infusion and blood sampling. Cannulas were filled with 25 u/mL heparin (LEO Pharma Nordic, Sweden) dissolved in 0.9% NaCl aqueous solution. Due to the short time frame of the experiment (<1 h), the trachea was not cannulated. After a stabilization period, the test substances were injected as a bolus lasting for 20 s. Blood samples were drawn at 5–10 min intervals, from 15 min pre-injection until 30 min post-injection. Compounds were dissolved in 5% NaOH (0.15% in 0.9% NaCl aqueous solution) and 95% in 0.9% NaCl aqueous solution, and were administered in a fixed dose of 3 mg/kg rat. Plasma insulin levels were determined using a mono/polyclonal ELISA assay with monoclonal antibodies as catching antibodies. Standards were rat insulin (Novo Nordisk, Bagsvaerd, Denmark). Catching antibody was HUI-018 (Novo Nordisk, Bagsvaerd, Denmark) raised against the A-chain of the insulin. Detecting antibody, a pool of polyclonal guinea pig antibodies from GP4042D (Novo Nordisk, Bagsvaerd, Denmark), used as non-purified serum.

References and notes

1. Aguilar-Bryan, L.; Bryan, J. *Endocr. Rev.* **1999**, *20*, 101.
2. Ashcroft, F. M.; Gribble, F. M. *Trends Neurosci.* **1998**, *21*, 288.
3. Coghlan, M. J.; Carroll, W. A.; Gopalakrishnan, M. *J. Med. Chem.* **2001**, *44*, 1627.
4. Seino, S.; Inagaki, N.; Namba, N.; Wang, C. H.; Kotake, K.; Nagashima, K.; Miki, T.; Aguilar-Bryan, L.; Bryan, J.; Gonoi, T. *Jpn. J. Physiol.* **1997**, *47*, S3.
5. Aizawa, T.; Taguchi, N.; Sato, Y.; Nakabayashi, T.; Kobuchi, H.; Hidaka, H.; Nagasawa, T.; Ishihara, F.; Itoh, N.; Hashizume, K. *J. Pharmacol. Exp. Ther.* **1995**, *275*, 194.
6. Carr, R. D.; Brand, C. L.; Bödvarsdottir, T. B.; Hansen, J. B.; Sturis, J. *Diabetes* **2003**, *52*, 2513.
7. Alemzadeh, R.; Slonim, A. E.; Zdanowicz, M. M.; Maturo, J. *Endocrinology* **1993**, *133*, 705.
8. Seino, S.; Miki, T. *Prog. Biophys. Mol. Biol.* **2003**, *81*, 133.
9. Gribble, F. M.; Reimann, F.; Ashfield, R.; Ashcroft, F. M. *Mol. Pharmacol.* **2000**, *57*, 1256.
10. de Tullio, P.; Pirotte, B.; Lebrun, P.; Fontaine, J.; Dupont, L.; Antoine, M. H.; Ouedraogo, R.; Khelili, S.; Maggetto, C.; Masereel, B.; Diouf, O.; Podona, T.; Delarge, J. *J. Med. Chem.* **1996**, *39*, 937.
11. Lebrun, P.; Arkhammar, P.; Antoine, M. H.; Nguyen, Q. A.; Hansen, J. B.; Pirotte, B. *Diabetologia* **2000**, *43*, 723.
12. Nielsen, F. E.; Bodvarsdottir, T. B.; Worsaae, A.; MacKay, P.; Stidsen, C. E.; Boonen, H. C. M.; Pridal, L.; Arkhammar, P. O. G.; Wahl, P.; Ynddal, L.; Junager, F.; Dragsted, N.; Tagmose, T. M.; Mogensen, J. P.; Koch, A.; Treppendahl, S. P.; Hansen, J. B. *J. Med. Chem.* **2002**, *45*, 4171.
13. Pirotte, B.; de Tullio, P.; Lebrun, P.; Antoine, M. H.; Fontaine, J.; Masereel, B.; Schynts, M.; Dupont, L.; Herchuelz, A.; Delarge, J. *J. Med. Chem.* **1993**, *36*, 3211.
14. Tagmose, T. M.; Mogensen, J. P.; Agerholm, P. C.; Arkhammar, P. O. G.; Wahl, P.; Worsaae, A.; Hansen, J. B. *Bioorg. Med. Chem. Lett.* **2001**, *11*, 1749.
15. Tagmose, T. M.; Zaragoza, F.; Boonen, H. C.; Worsaae, A.; Mogensen, J. P.; Nielsen, F. E.; Jensen, A. F.; Hansen, J. B. *Bioorg. Med. Chem.* **2003**, *11*, 931.
16. de Tullio, P.; Becker, B.; Boverie, S.; Dabrowski, M.; Wahl, P.; Antoine, M. H.; Somers, F.; Sebille, S.; Ouedraogo, R.; Hansen, J. B.; Lebrun, P.; Pirotte, B. *J. Med. Chem.* **2003**, *46*, 3342.
17. Cosgrove, K. E.; Antoine, M. H.; Lee, A. T.; Barnes, P. D.; de Tullio, P.; Clayton, P.; McCloy, R.; De Lonlay, P.; Nihoul, F.; Robert, J. J.; Saudubray, J. M.; Rahier, J.; Lindley, K. J.; Hussain, K.; Aynsley-Green, A.; Pirotte, B.; Lebrun, P.; Dunne, M. J. *J. Clin. Endocrinol. Metab.* **2002**, *87*, 4860.
18. Barco, A.; Benetti, S.; Pollini, G. P.; Taddia, R. *Synthesis* **1974**, 877.
19. Lee, C.; Field, L. *Synthesis* **1990**, 391.
20. Mital, R. L.; Jain, S. K. *J. Chem. Soc.* (*C*) **1969**, 2148.
21. Shridhar, D. R.; Sastry, C. V. R.; Bansal, O. P.; Rao, P. P. *Indian J. Chem. Sect. B* **1986**, *25*, 874.
22. White, E. H.; Bursey, M. M.; Roswell, D. F.; Hill, J. H. *J. Org. Chem.* **1967**, *32*, 1198.
23. Hodgson, H. H.; Handley, F. W. *J. Chem. Soc.* **1928**, 625.
24. Jacobsen, P.; Treppendahl, S. P. *Tetrahedron* **1979**, *35*, 2151.
25. ^{13}C NMR spectra were predicted using the ACD SpecManager CNMR Predictor, ACD/Labs Version 7.08 23Apr 2003, Advanced Chemistry Development Inc., Toronto, Canada, 2004.
26. Andersen, K.; Begtrup, M. *Acta Chem. Scand.* **1992**, *46*, 1130.
27. Begtrup, M. *J. Chem. Educ.* **1987**, *64*, 974.
28. Manley, P. W.; Quast, U. *J. Med. Chem.* **1992**, *35*, 2327.
29. Lebrun, P.; Malaisse, W. J.; Herchuelz, A. *Am. J. Physiol. Endocrinol. Metab.* **1982**, *5*, E59.
30. Lebrun, P.; Antoine, M. H.; Ouedraogo, R.; Kane, C.; Dunne, M.; Hermann, M.; Herchuelz, A.; Masereel, B.; Delarge, J.; de Tullio, P.; Pirotte, B. *J. Pharmacol. Exp. Ther.* **1996**, *277*, 156.
31. Dabrowski, M.; Wahl, P.; Holmes, W. E.; Ashcroft, F. M. *Diabetologia* **2001**, *44*, 747.
32. Efrat, S.; Linde, S.; Kofod, H.; Spector, D.; Delannoy, M.; Grant, S.; Hanahan, D.; Baekkeskov, S. *Proc. Natl. Acad. Sci. U.S.A.* **1988**, *85*, 9037.
33. Lebrun, P.; Antoine, M. H.; Devreux, V.; Hermann, M.; Herchuelz, A. *J. Pharmacol. Exp. Ther.* **1990**, *255*, 948.
34. Lebrun, P.; Atwater, I. *Biophys. J.* **1985**, *48*, 919.
35. Brand, C. L.; Rolin, B.; Jorgensen, P. N.; Svendsen, I.; Kristensen, J. S.; Holst, J. J. *Diabetologia* **1994**, *37*, 985.