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Phenylcyanoguanidines as Inhibitors of Glucose-Induced Insulin Secretion from Beta Cells

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Abstract—3,5-Disubstituted-phenylcyanoguanidines have been identified as activators of SUR1/Kir6.2 potassium channels and as potent inhibitors of insulin release from pancreatic beta cells in vitro. © 2001 Elsevier Science Ltd. All rights reserved.

The ATP regulated potassium (K_{ATP}) channels, which are present in different tissues, for example smooth muscle, the heart and in pancreatic beta cells are important in coupling the effects of hormones and transmitters and cell metabolism to cellular membrane potential.[1] K_{ATP} channel blockers have been developed for treatment of diabetes whereas K_{ATP} channel activators are being investigated as potential drugs for the treatment of, for example, hypertension urinary incontinence, asthma and cardiac or neuronal ischemic damage.[2,3] Certain cyanoguanidines act as potent potassium channel openers (PCOs) on vascular smooth muscle to reduce blood pressure. These include *N*-pyridyl derivatives (e.g., pinacidil and P1075; Fig. 1)[4,5] and chloro, cyano and nitro substituted phenyl cyanoguanidines.[6] While pinacidil and P1075 have only minimal effects on the K_{ATP} channels of pancreatic beta cells, reducing the conformational flexibility of the aryl-cyanoguanidine to form, for example, BPDZ 44, an increase in potency with respect to inhibition of insulin release from rat islets could be obtained.[7] BPDZ 44, and the even more potent analogue BPDZ 73,[8] are related to the 1,2,4-benzothiadiazine 1,1-dioxide, diazoxide, which nonselectively opens K_{ATP} channels of smooth muscle and beta cells to induce vasodilatation and inhibition of insulin release. Diazoxide has been found to protect beta cells in newly diagnosed patients suffering from Type 1 diabetes,[9] to potentiate weight loss in obese individuals[10] and to be active in animal models of Type 2 diabetes,[11,12] but are associated with several side effects.

To investigate the structural requirements of phenyl cyanoguanidines as openers of beta cell K_{ATP} channels, a series of compounds having a phenyl group substituted with electron withdrawing lipophilic groups and different *N*-alkyl groups were prepared and studied for effects on glucose stimulated beta cell membrane potential and insulin release versus relaxation of vascular smooth muscle in vitro.

The method described by Yoshizumi et al. was applied for the synthesis of 3,5-substituted phenyl-cyanoguanidines[13]

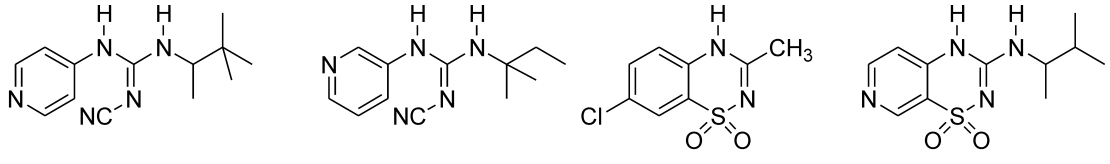

Figure 1. Activators of ATP regulated potassium channels.

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(Fig. 2). By this method, which involves reacting diphenylcyanocarbonimidate with anilines and subsequently treating the obtained cyano-*O*-phenylisourea with alkylamines, the 3,5-disubstituted phenyl-cyanoguanidines derivatives are produced in moderate to good yield.[14]

The synthesized compounds (Table 1) were evaluated for their ability to relax phenylepherine contracted rat aorta rings[15] and by their effects on glucose stimulated insulin release from murine βTC6 cells[16] and on murine β-cell (βTC3) membrane potential as measured by changes in $DIBAC_4(3)$ fluorescence.[17] To further substantiate the effects of the compounds on insulin release, selected compounds (**6** and **7**) were examined on freshly isolated rat islets[18] and on recombined human K_{ATP} channels (Fig. 3).[19]

3,5-Bistrifluoromethylphenyl cyanoguanidines and 3,5-dichlorophenyl cyanoguanidines, having the pinacidil and P1075 alkyl side chains were found to inhibit insulin secretion and to relax vascular smooth muscle. The effects of several derivatives on rat aorta rings were as potent as that of pinacidil ($IC_{50} = 1.5$ μM) and P1075 ($IC_{50} = 0.25$ μM). In contrast, the prepared monotrifluoromethylphenyl cyanoguanidine (**5**) was nearly inactive both on aorta and beta cells. The 3-methoxy-5-trifluoromethylphenyl cyanoguanidine (**6**) was considerably less potent on vascular smooth muscle than on murine beta cells and rat islets. Among the 3,5-disubstituted phenyl cyanoguanidines the branching in the α position was of major importance for the vascular activity. Removing the methyl groups α to the nitrogen one by one decreases the activity on aorta (**2**→**1**→**3**).

In the present series, all compounds, with one exception (**7**) potently inhibited glucose stimulated insulin release.

The inhibitory effect on insulin release was in general accompanied by an effect on membrane potential of βTC3 cells with the exception that compound **9** was found to be a potent inhibitor of insulin release ($IC_{50} = 3.7$ μM) with minimal effects on beta cell membrane potential. This lack of correlation could be due to non-specific interactions caused by the lipophilic compound or interaction with other parts of the insulin secretory process. In addition to being potent inhibitors of insulin release, **3** and **10** also were tissue-selective. **6** and **7** were tested on freshly prepared rat islets and found to be potent inhibitors of insulin release ($IC_{50} = 4.8$ and 2.2 μM, respectively) both being approximately as potent as diazoxide ($IC_{50} = 6.1$ μM) (data not shown). To further evaluate the present compounds, **6** (3–30 μM) was shown in a whole cell patch clamp configuration to activate human SUR1/Kir6.2 K_{ATP} channels co-expressed in HEK 293 cells[18] (Fig. 3) whereas the compound (1–100 μM) did not activate human SUR2A/Kir6.2 channels (data not shown). The K_{ATP} channel blocker glibenclamide (1 μM) was able to counteract the effects of **6** on the ion current through the SUR1/Kir6.2 channels substantiating that **6** acts through the K_{ATP} channels.

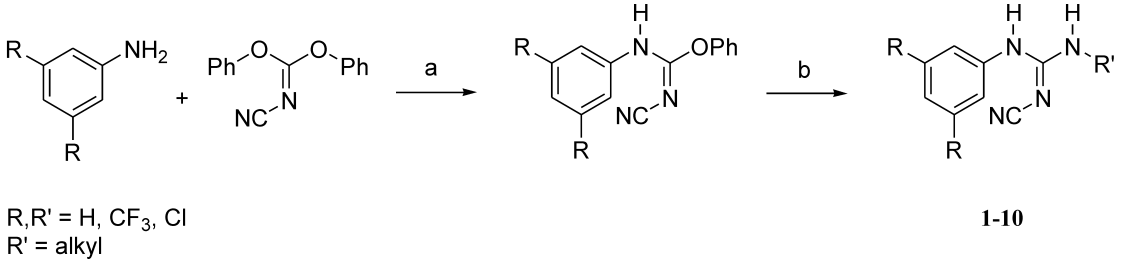

Figure 2. Synthesis of phenyl-cyanoguanidines: (a) rt, 14 h; (b) R′-NH_2, 75 °C, 8 h.

Table 1. Effects of compounds **1**–**10** and reference K_{ATP} channel activators on vascular tissue and beta cells in vitro

Compounds	R, R	R′	Relaxation of rat aorta rings IC_{50} (μM)[a]	Inhibition of insulin release βTC6 cells		Membrane potential βTC3 cells IC_{50} (μM)[b]
				IC_{50} (μM)[b]	Efficacy[b] (%)	
1	CF_3,CF_3	$CH(CH_3)C(CH_3)_3$	8.7	4.6 (1.2)	78 (7)	NA
2	CF_3,CF_3	$C(CH_3)_2CH_2CH_3$	0.9	NT		NA
3	CF_3,CF_3	$CH_2CH_2CH_3$	12.4	5 (5.7)	58 (13)	0.9 (0.8)
4	CF_3,CF_3	$CH_2CH_2CH(CH_3)_2$	5.6	2 (0.2)	77 (7)	0.5 (0.4)
5	H, CF_3	$CH(CH_3)C(CH_3)_3$	88	>100	29 (8)	>100
6	OCH_3,CF_3	$CH_2CH_2CH(CH_3)_2$	79	3.9 (3)	79 (9)	13 (6)
7	Cl,Cl	$CH(CH_3)C(CH_3)_3$	0.5	26.7 (2.9)	86 (4)	19 (14)
8	Cl,Cl	$C(CH_3)_2CH_2CH_3$	0.3	2.3 (3.3)	57 (2)	NA
9	Cl,Cl	$CH_2CH_2CH_3$	31	3.7 (4.7)	50 (31)	36 (450)
10	Cl,Cl	$CH_2CH_2CH(CH_3)_2$	18.7	0.7 (0)	72 (8)	4 (6)
Diazoxide			11.5	22.4 (15)	23 (22)	77.4 (4.3)

NA = not active; NT = not tested.
[a]Values are an average of two experiments.
[b]Values are means of at least two experiments, standard deviation is given in parentheses.

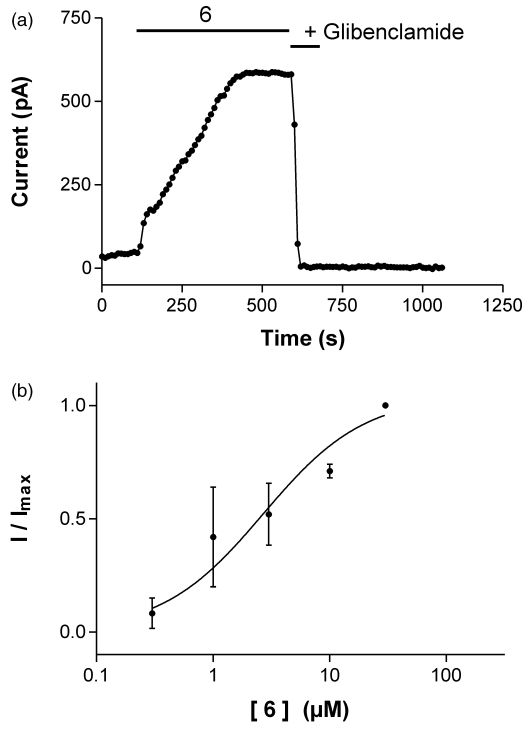

Figure 3. Effect of **6** on currents through human SUR1/KIR6.2 channels. (a) Representative experiment from HEK 293 cells stably expressing human SUR1/Kir6.2 showing typical currents changes upon application of **6** (30 μM) and glibenclamide (1 μM) plus **6** (30 μM). Currents were evoked by a 10 mV depolarizing pulse for 200 ms every 10 s from a holding potential of −80 mV. (b) Dose–response relationship for the current activated by increasing concentrations of **6**. The current amplitude induced by each concentration was normalized to the current induced by 30 μM **6** in each cell. Symbols and bars indicate the mean and SE values, respectively. The number of the observations at each point was three or four.

The present data suggest that by changing the structure of pinacidil, which is nearly inactive on beta cells, to 3,5-disubstituted phenyl cyanoguanidines, it is possible to identify compounds that are able to activate K_{ATP} channels of pancreatic beta cells and to inhibit insulin release. The *N*-alkyl side chain is important for potency and selectivity as previously described by Yoshiizumi et al.[6] who found that 3,5-disubstituted phenyl cyanoguandines having bulky alkyl substituents are potent dilators of vascular smooth muscle, and by Pirotte et al., showing that in a series of 4*H*-pyrido[4,3-*e*]-1,2,4-thiadiazine 1,1-dioxides the branching in the α position was important for the inhibitory effects on insulin.[7]

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14. **Experimental and physical data for test compounds: *N*-(3,5-(bistrifluoromethyl)phenyl)-*N*′-cyano-*O*-phenylisourea**. A solution of diphenylcyanocarbonimidate (2 mmol, 476 mg), 3,5-bis(trifluoromethyl)aniline (2 mmol, 458 mg) and triethylamine (2 mmol, 202 mg) in dichloromethane (15 mL) was stirred under nitrogen for 12 h. After concentration, the residue was stirred with toluene (5 mL) for 2 h and the solid collected by filtration giving 550 mg of *N*-(3,5-(bistrifluoromethyl)phenyl)-*N*′-cyano-*O*-phenylisourea (73.6%); mp 190.5–191.5 °C; ^{1}H NMR (DMSO-d_6): 7.25 (m, 5H), 7.95 (s, 1H), 8.15 (s, 2H), 11.2 (s, 1H).

***N*-Cyano-*N*′-(3,5-bis-(trifluoromethyl)phenyl)-*N*″-(1,2,2-trimethylpropyl)guanidine (1).** A solution of *N*-(3,5-(bistrifluoromethyl)phenyl)-*N*′-cyano-*O*-phenylisourea (0.8 mmol, 300 mg), 2-amino-3,3-dimethylbutane (0.88 mmol, 0.09 g) and triethylamine (0.88 mmol, 0.123 mL) in acetonitrile (2 mL) was stirred for 8 h at 75 °C. After concentration the residue was purified by column chromatography (heptane/ethyl acetate 2:1) to give the title compound (140 mg, 46%) as white crystals. Mp 165.5–166.5 °C; EI SP/MS: 380 (M^+); ^{1}H NMR ($CDCl_3$): 0.92 (s, 9H), 1.13 (d, 3H), 3.8 (m, 1H), 4.8 (br d, 1H), 7.74 (br s, 3H), 8.5 (br, 1H); MA calcd for $C_{16}H_{18}F_6N_4$: C 50.53%, H 4.77%, N 14.73%. Found: C 50.48%, H 4.74%, N 14.45%.

2: Yield: 28%, mp 149–150 °C; **3**: yield: 28%, mp: 142.5–143.5 °C; **4**: yield: 27%, mp 130–132 °C; **5**: yield: 63%, mp: 114.5–117 °C; **6**: yield: 81%, mp 105.5–108.5 °C; **7**: yield: 68%, mp 158.5–160.5 °C; **8**: yield: 65%, mp 158.5–160 °C; **9**: yield: 23%, mp 141–143 °C; **10**: yield: 54%, mp 146.5–151.5 °C.
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16. Inhibition of glucose induced insulin release in βTC6: βTC6 was cultured at 5×10^4 cells/well in microtiter plates in DMEM + 10%FCS, 1 g/L glucose, 1% Glutamax and 20 mM Hepes for 3 days. Cells were washed twice with NN buffer (NaCl, 114 mM; KCl, 4.7 mM; KH_2PO_4, 1.21 mM; $MgSO_4$, 1.16 mM; $NaHCO_3$, 25.5 mM; $CaCl{\cdot}2H_2O$, 2.5 mM; HEPES, 10 mM) supplemented with 0.1% BSA and incubated for 60 min in this buffer. All wells were aspirated and the cells incubated for 3 h with NN buffer, 22 mM glucose and a serial dilution of the compounds. 0.1 mM IBMX was added in order to potentiate the glucose stimulated insulin release. A reference compound and a series of different glucose concentrations without compound were used as references. The supernatant from each well was harvested and insulin content was measured by a competition Elisa. In brief, Elisa microtiter plates were coated with anti-guinea pig IgG and incubated overnight at 4 °C in PBS. 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 (NN)] was added, followed by an incubation for 2 h at rt. Plates were

washed five times in washing buffer and test samples were added together with Peroxidase-labelled insulin and incubated for another 2 h at rt in Assay buffer (Washing buffer + 0.5% BSA). A series of standards of rat insulin were made to cover a range of 1000 to 1 μg/mL insulin and the standards were incubated with PO-insulin as well. Eventually, TMB substrate was added to all wells and the enzyme reaction stopped after 5 min by adding H_3PO_4. Absorption was measured in an Elisa reader and converted into ng/mL insulin. The results were analyzed in Prism and expressed as EC_{50} (potency) and percentage inhibition (efficacy) of insulin release.

17. Effects on membrane potential of βTC3 cells were measured according to ref 8.

18. Inhibition of glucose induced insulin release in rat islets. Islets were isolated by collagenase and gradient centrifugation in Ficoll (40–13%). Isolated islets were incubated in bulk overnight in RPMI, 10% FCS, 11 mM glucose. The islets were handpicked and placed at 10 islets/microtiter well and cultured overnight in DMEM, 10% FCS, and 3 mM glucose.

Essentially, the islets were tested as described for the βTC6 but with no addition of IBMX. The insulin content was measured in the same Elisa as used for the βTC6.

19. Electrophysiological recordings. The whole cell patch-clamp technique was employed to measure the currents in a HEK 293 cell line stably expressing the human $SUR1/K_{IR}6.2$ receptor channel. The microelectrodes were pulled from borosilicate glass and had resistances between 3 and 5 MΩ and were filled with internal solution containing (in mM): 120 KCl, 1 $MgCl_2$, 5 EGTA, 2 $CaCl_2$, 20 Hepes, 0.3 NaADP, 5 K_2ATP (pH 7.3). The external recording solution was (in mM): 140 NaCl, 3 KCl, 1 $CaCl_2$, 1 $MgCl_2$, 20 mannitol, 10 Hepes (pH 7.2). Currents were recorded on an EPC9 amplifier (HEKA Electronic GmbH, Lambrecht, Germany) and filtered at 2 kHz and sampled at 10 kHz. Data analysis was performed using Pulse + PulseFit software (HEKA Electronic GmbH, Lambrecht, Germany). Measuring the maximal current induced by increasing concentrations of agonist produced agonist dose–response curves. Data were fitted to the logistic equation: $I = I_{max}/\{1 + (EC_{50}/[\mathbf{6}]^n\}$, where I is the current induced by **6**. The parameters I_{max} (maximal current at infinite **6** concentration), n (the Hill coefficient, and EC_{50} (concentration of **6** producing a current of 50% of I_{max}) were determined by an iterative least-squares fitting routine (Origen, MicroCal Software, MA, USA).