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Triphenylphosphonium salts of 1,2,4-benzothiadiazine 1,1-dioxides related to diazoxide targeting mitochondrial ATP-sensitive potassium channels

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ABSTRACT

The present work aims at identifying new ion channel modulators able to target mitochondrial ATP-sensitive potassium channels (mitoK$_{ATP}$ channels). An innovative approach should consist in fixing a cationic and hydrophobic triphenylphosphonium fragment on the structure of known K$_{ATP}$ channel openers. Such phosphonium salts are expected to cross the biological membranes and to accumulate into mitochondria.

Previous works revealed that the presence of an (*R*)-1-hydroxy-2-propylamino chain at the 3-position of 4*H*-1,2,4-benzothiadiazine 1,1-dioxides K$_{ATP}$ channel openers increased, in most cases, the selectivity towards the pancreatic-type (SUR1/Kir6.2) K$_{ATP}$ channel. In order to target cardiac mitoK$_{ATP}$ channels, we decided to introduce a triphenylphosphonium group through an ester link on the SUR1-selective (*R*)-7-chloro-3-(1-hydroxy-2-propyl)amino-4*H*-1,2,4-benzothiadiazine 1,1-dioxide. The new compounds were found to preserve an inhibitory activity on insulin secretion (SUR1-type K$_{ATP}$ channel openers) while no clear demonstration of an impact on mitochondria from cardiomyocytes (measurement of oxygen consumption, respiratory parameters and ATP production on H9C2 cells) was observed. However, the most active (inhibition of insulin release) compound **17** was found to penetrate the cardiac cells and to reach mitochondria.

Mitochondria are membrane-enclosed organelles found in most eukaryotic cells. Their crucial role consists in generating most of the cell's supply of adenosine triphosphate (ATP) used as a source of chemical energy. In addition to supplying cellular energy, mitochondria are involved in a range of other processes such as cellular differentiation, cell death as well as the control of the cell cycle and cell growth. Mitochondria have been involved in several human diseases, including cardiac dysfunction and pathologies linked to mitochondrial disorders, and may play a role in the aging process.[1]

Potassium channels regulated by variations of intracellular ATP concentrations (K$_{ATP}$ channels) have been identified in numerous tissues, not only at the level of the cell membrane but also on the internal membrane of mitochondria.[2] Several data indicated that the mitochondrial K$_{ATP}$ channels (mitoK$_{ATP}$ channels) could play a role in the protection of tissues in case of ischemia-reperfusion.[3] It is well documented that the cardioprotective effect of some activators of K$_{ATP}$ channels, such as diazoxide and cromakalim, could be related to their effects on mitoK$_{ATP}$ channels.[4] Through activation of such channels, mitochondria are able to maintain an efficient energy exchange with the cytosol during inotropic stress.[5]

Potassium channel openers are able to mimic the cardioprotective effect of ischemic preconditioning, an effect abolished by K$_{ATP}$ channel blockers.[6] Other therapeutic benefits are expected with mitoK$_{ATP}$ channel openers. According to cell protection against oxidative stress induced by such compounds, potassium channel

Abbreviations: ATP, adenosine triphosphate; SUR, sulfonylurea receptor; Kir, inwardly rectifying potassium channel; mitoK$_{ATP}$ channel, mitochondrial ATP-sensitive potassium channel; DCC, *N*,*N*′-dicyclohexylcarbodiimide; NHS, *N*-hydroxysuccinimide; HOBt, hydroxybenzotriazole; TMS, tetramethylsilane; DMSO, dimethylsulfoxide; DCVC, dry column vacuum chromatography.

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openers like diazoxide, a reference mitoK$_{ATP}$ channel opener, have been proposed to improve organ and cells survival before transplantation.[7]

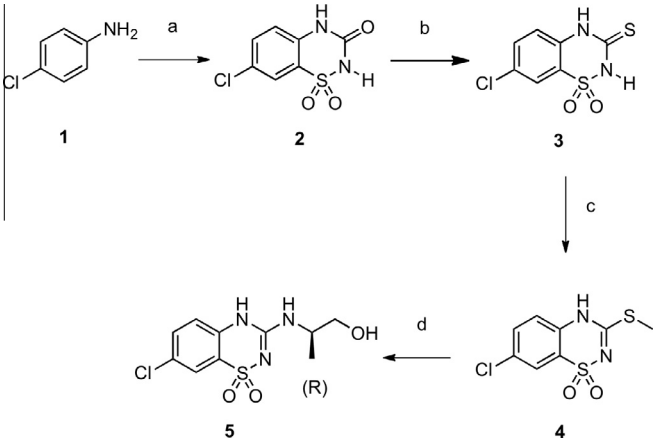
In spite of numerous studies performed on the mitoK$_{ATP}$ channel, the molecular structure of the latter channel remains the object of scientific controversy. State of the art indicates that, among potassium channel openers already studied on cardiomyocytes, diazoxide (interacting with SUR1/Kir6.2 and SUR2B/Kir6.1/Kir6.2-type channels) was more efficient at opening mitoK$_{ATP}$ channels than sarcolemmal K$_{ATP}$ channels (cell membrane SUR2A/Kir6.2-type channels), positioning diazoxide as a pharmacological tool able to discriminate between sarcolemmal and mitochondrial K$_{ATP}$ channels in cardiomyocytes.[6a] Moreover, a recent study demonstrated the existence of a mitoK$_{ATP}$ channel composed of Kir6.1 and SUR1 in rat hepatocytes,[7c] supporting the view that K$_{ATP}$ channel openers interacting with the SUR1-type channel could become interesting tools to target mitochondria.

Our laboratories have been involved for a long time in the development of K$_{ATP}$ channel openers structurally related to diazoxide (a benzothiadiazine dioxide) and cromakalim (a dihydrobenzopyran).[8] Several original benzothiadiazines were found to be highly active and selective for the SUR1-type K$_{ATP}$ channel (expressed i.e. on insulin-secreting cells and neurons), while others strongly and specifically activated the SUR2B-type K$_{ATP}$ channel (expressed i.e. on smooth muscle cells).[8,9]

Recently, we found that the presence of an (*R*)-1-hydroxy-2-propylamino chain at the 3-position of 4*H*-1,2,4-benzothiadiazine 1,1-dioxides K$_{ATP}$ channel openers considerably increased the selectivity for the pancreatic-type (SUR1/Kir6.2) versus the vascular smooth muscle-type (SUR2B/Kir6.1) channels.[8l] Thus, the SUR1-selective diazoxide analogue (*R*)-7-chloro-3-(1-hydroxy-2-propyl)amino-4*H*-1,2,4-benzothiadiazine 1,1-dioxide[8l] was chosen as the starting point for the design of new compounds targeting mitochondria and mitoK$_{ATP}$ channels of cardiomyocytes, supposing that the SUR1 subunit would be present on the cardiac mitoK$_{ATP}$ channels. We decided to introduce a triphenylphosphonium group through an ester link on (*R*)-7-chloro-3-(1-hydroxy-2-propyl)amino-4*H*-1,2,4-benzothiadiazine 1,1-dioxide so as to obtain a new generation of mitoK$_{ATP}$ channel activators or to design prodrugs of the SUR1-selective opener (*R*)-7-chloro-3-(1-hydroxy-2-propyl)amino-4*H*-1,2,4-benzothiadiazine 1,1-dioxide which, after accumulation into the mitochondria, could be converted, by hydrolysis of the ester link, to the active compound.

Lipophilic triphenylphosphonium cations are known to pass directly through phospholipid bilayers due to their large hydrophobic surface area lowering the activation energy for uptake.[10] The inherent positive charge causes these cations to selectively accumulate several hundred folds within mitochondria, driven by the plasma and mitochondrial membrane potentials.[10a,b]

The synthetic pathway giving access to (*R*)-7-chloro-3-(1-hydroxy-2-propyl)amino-4*H*-1,2,4-benzothiadiazine 1,1-dioxide (**5**) is described in Scheme 1. The key intermediate for the synthesis of this compound was the previously reported 7-chloro-3-methylsulfanyl-4*H*-1,2,4-benzothiadiazine 1,1-dioxide **4**.[8c] This intermediate was obtained from the corresponding aniline **1** in three steps (Scheme 1). The first step allowed ring closure of a chlorosulfonylurea intermediate through Friedel–Crafts conditions. The ring-closed sulfonylurea **2** obtained was then converted into its sulfonylthiourea analogue **3** by the action of phosphorus pentasulfide in pyridine. In the next step, 7-chloro-3-thioxo-4*H*-1,2,4-benzothiadiazine 1,1-dioxide **3** was alkylated with methyl iodide to give the corresponding 3-methylsulfanyl-substituted key intermediate **4**. The product **4** was then heated during five hours with optically pure (*R*)-1-hydroxy-2-propylamine at 150 °C, leading to the expected *R* stereoisomer **5** with a global yield of 25% on five steps.



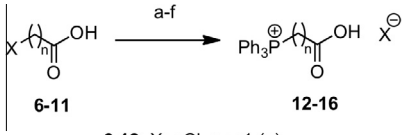
Scheme 1. Reagents and conditions: (a) $ClSO_2NCO$, $AlCl_3$, CH_3NO_2 (52%); (b) P_2S_5, pyridine (85%); (c) CH_3I, $NaHCO_3$, CH_3OH/H_2O (80%); (d) (*R*)-1-hydroxy-2-propylamine, 150 °C, 5 h (71%).

The introduction of a phosphonium group on compound **5** was realized by esterification between the hydroxy function of **5** and the acid function of triphenylphosphonium fragments. The different phosphonium salts were easily synthesized via alkylation (quaternization) of triphenylphosphine with the corresponding commercially available haloalkylcarboxylic acids in toluene, dichloromethane or without solvent, except for compound **9** (Scheme 2).[11] Conditions were dependent on starting material.

The esterification reaction between the precursor **5** and triphenylphosphonium fragments was not very easy (Scheme 3). In classical acid catalysis conditions, the expected products were formed in very few yields. Then, various coupling agents such as DCC/NHS or DCC/HOBt were tested but without better results.

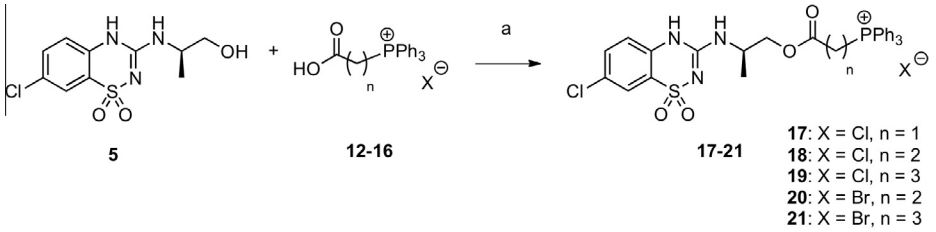
Esterification was finally realized with the coupling agent *N,N′*-dicyclohexylcarbodiimide (DCC) alone to activate the carboxylic acid group of compounds **12**–**16**. Five different compounds from the five phosphonium salts, with variable carbon chains ($n = 1, 2, 3$), were obtained (compounds **17**–**21**). Three of them (the chloride salts **17**, **18** and **19**) were selected for biological evaluation. The final compounds were characterized by RMN ^1H, ^{13}C and elemental analysis.

The first in vitro model used to characterize the phosphonium salts determined the ability of the compounds at inhibiting the glucose-induced insulin secretion from rat pancreatic islets, a SUR1-expressing tissue. Data collected with this model were expressed as the percentage of residual insulin release recorded at a 10 μM drug concentration. Results obtained with the new compounds were compared to previously reported data obtained with (*R*)-7-chloro-3-(1-hydroxy-2-propyl)amino-4*H*-1,2,4-benzothiadiazine 1,1-dioxide **5** and diazoxide (Table 1).



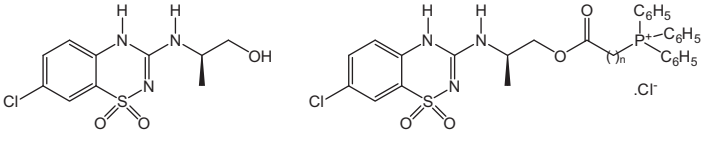
6,**12**: X = Cl, n = 1 (a)
7,**13**: X = Cl, n = 2 (b)
8,**14**: X = Cl, n = 3 (c)
9: X = Br, n = 1 (d)
10,**15**: X = Br, n = 2 (e)
11,**16**: X = Br, n = 3 (f)

Scheme 2. Reagents and conditions: (a) PPh_3, CH_2Cl_2, rt, 18 h (97%); (b) PPh_3, toluene, 110 °C, 18 h (93%); (c) PPh_3, toluene, 180 °C, 3 h (78%); (d) various conditions tested but without success; (e) PPh_3, toluene, 120 °C, 4 h (93%); (f) PPh_3, toluene, 110 °C, 48 h (64%).



Scheme 3. Reagents and conditions: (a) DCC, CH_3CN, reflux, 18 h (16–63%).

Table 1
Effects of the triphenylphosphonium salts of 3-alkylamino-4*H*-1,2,4-benzothiadiazine 1,1-dioxides on insulin secretion from rat pancreatic islets



compound	*n*	RIS[a] (10 μM)
17	1	27.0 ± 1.6 (15)
18	2	56.0 ± 3.0 (15)
19	3	63.7 ± 3.8 (15)
5[b]		8.5 ± 0.6 (13)
Diazoxide[b]		73.9 ± 4.4 (16)

[a] RIS: percentage of residual insulin release from rat pancreatic islets incubated in the presence of 16.7 mM glucose (mean ± SEM (*n*)).
[b] Published results (Refs. 8c,l).

It was found that the three phosphonium chlorides esters **17**, **18** and **19** (10 μM) were more potent than diazoxide (10 μM) at inhibiting insulin release. However, none of them reached the activity of the 3-(1-hydroxy-2-propyl)amino-substituted analogue **5** (10 μM). Interestingly, the activity on insulin-secreting cells decreased with the increase in the number of carbon atoms linking the phosphonium group to the ester function. It was expected that these phosphonium salts kept the pharmacological profile of K_{ATP} channel openers.

A second set of biological investigations consisted in the measurement of the oxygen consumption and respiratory parameters using High Resolution Oxygraphy (HRO) on cultured cardiomyocytes (H9C2 cell line).[12] Results obtained with the three compounds **17**, **18** and **19** were compared to those obtained with diazoxide (see Fig. 1).

Briefly, the cells were trypsinized and introduced in the oxygraph. After stabilization, the compound to be tested was injected in the recording chamber. In such conditions, the routine respiration was measured. After a second step of stabilization, the ATP synthase inhibitor oligomycin was injected. In these conditions, the leak (non-phosphorylating) respiration was measured. After further stabilization, carbonyl cyanide 4-(trifluoromethoxy)phenylhydrazone (FCCP), a mitochondrial oxidative phosphorylation inhibitor, was injected allowing the measure of the maximal respiratory capacity and the effect on the electron transfer system (ETS max).

Overall, the three phosphonium salts did not show any effect on routine and ETS assays, even though compound **17** seemed to be weakly active on electron transfer system but there was no significant effect. Amongst those compounds, only **17** was able to slightly modify the leak following the use of oligomycin, meaning that this compound might act at the ATP synthase level. To confirm this effect on the leak, a correlation with the ATP production was suggested.

Thus, in a third set of experiments, the effects of the phosphonium salts on the metabolic activity of cardiomyocytes (H9C2 cell line) in presence or absence of the electron acceptor paraquat and on the ATP production were evaluated. None of the tested compounds, up to a 10 μM concentration, were able to affect the metabolic activity (results not shown) even in the presence of paraquat (200 μM). Moreover, at the same drug concentrations (up to 10 μM), none of these phosponium salts were able to influence the ATP production (results not shown).

Lastly, the most active phosphonium salt on insulin secretion (compound **17**) was selected for investigating cell membrane

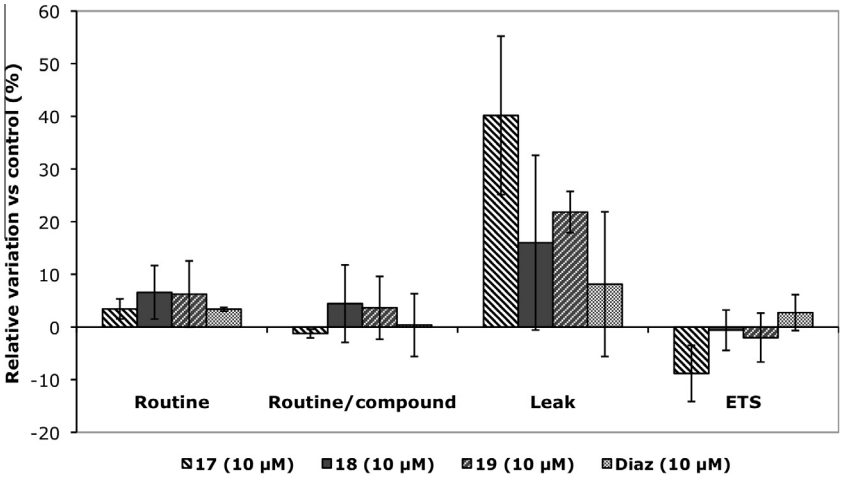


Figure 1. Modifications of the oxygen fluxes. Effect of compounds **17**, **18**, **19** and diazoxide (Diaz) on the cellular routine respiration (routine/compound), the oligomycin-inhibited leak respiration (Leak) and the maximal respiratory capacity (ETS max) of cultured cardiomyocytes (H9C2 cell line). Control without compound (Routine). Each assay was done in triplicate (mean ± SD).

permeation and accumulation in the mitochondria of H9C2 cells. After preincubation of a concentrated solution of compound **17** with a population of H9C2 cardiomyocytes grown to confluence, and then dissociated by trypsinization, the cell suspension was subjected to gentle disruption by means of a potter homogenizer. The mitochondrial fraction was isolated from the cell homogenate by differential centrifugation and further purified after an appropriate washing treatment of the mitochondrial pellet. The presence of the phosphonium salt **17** in the cytosol as well as in the mitochondrial fraction was demonstrated by HPLC analysis and formally identified by means of a QTOF MS detection performed on the peak found at the retention time of compound **17**. Although such an analysis may not be considered as quantitative, it was however observed that the signal corresponding to the phosphonium salt (using a specific detection at the molecular mass of **17**) was clearly more important in the mitochondrial fraction than in the cytosol (see LC–MS chromatograms and MS spectra in the Supplementary data section).

These data suggest that compound **17** crosses the cell membrane, disperses in the cytosol and then reaches the mitochondria where it appears to accumulate.

The present work described the synthesis of phosphonium salts derived from the SUR1-selective K_{ATP} channel opener (*R*)-7-chloro-3-(1-hydroxy-2-propyl)amino-4*H*-1,2,4-benzothiadiazine 1,1-dioxide and their evaluation as new ion channel modulators able to target mitoK_{ATP} channels. The new compounds were found to preserve an inhibitory activity on insulin secretion, supporting the view that such compounds interacted with the SUR1-type K_{ATP} channels expressed on the pancreatic endocrine tissue. However, no clear demonstration of an impact on mitochondria activity from H9C2 cells was observed, even if the most potent compound **17** (inhibition of insulin secretion) was found to reach the mitochondria.

In the future, it would be interesting to develop the same strategy with other prototypical K_{ATP} channel openers and to introduce the triphenylphosphonium moiety on a SUR2-selective opener (i.e. cromakalim).

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Supplementary data

Supplementary data (all details about the synthesis of the triphenylphosphonium salts and the biological investigations) associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.bmcl.2013.08.091.

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