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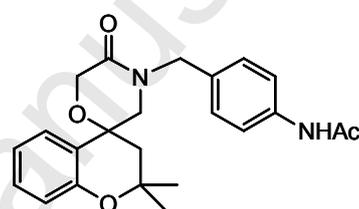
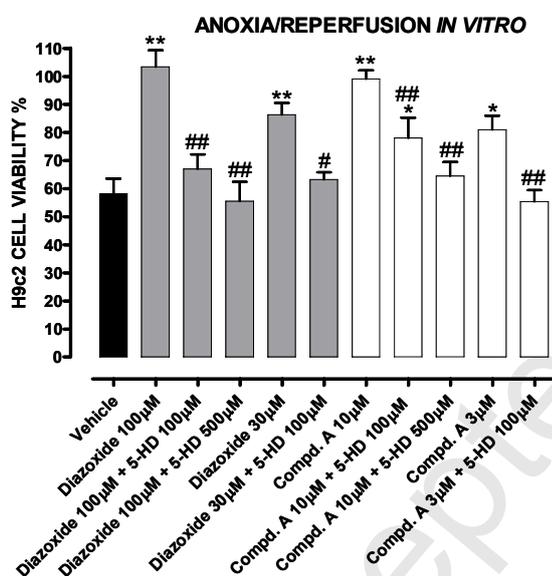
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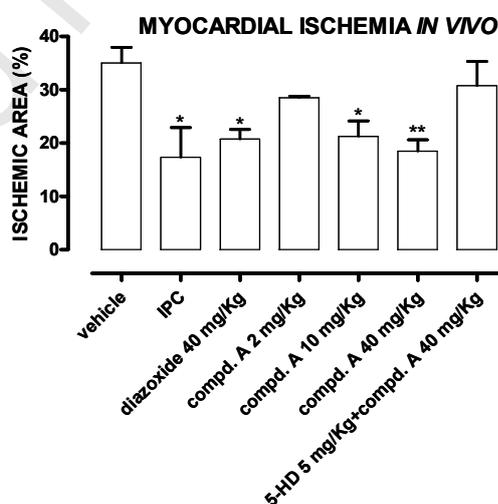
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Anti-ischemic properties of a new spiro-cyclic benzopyran activator of the cardiac mitoK_{ATP} channelVincenzo Calderone*¹, Lara Testai¹, Alma Martelli¹, Simona Rapposelli²,Maria Digiaco², Aldo Balsamo², Maria C. Breschi¹¹Dipartimento di Psichiatria, Neurobiologia, Farmacologia, Biotecnologie, Università di Pisa²Dipartimento di Scienze Farmaceutiche, Università di Pisa

Graphical abstract



Compound A



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3 **Anti-ischemic properties of a new spiro-cyclic benzopyran activator of**
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6 **the cardiac mitoK_{ATP} channel**
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53 **Abbreviations:**

54 CsA, cyclosporine A; 5-HD, 5-hydroxydecanoic acid; IPC, ischemic pre-conditioning; I/R,
55 ischemia/reperfusion; LAD, left anterior descending coronary artery; K_{ATP}, ATP-sensitive
56 potassium channel; MPTP, mitochondrial permeability transition pore.
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Abstract

1
2 Many activators of K_{ATP} channels exhibit cardioprotective effects, mainly mediated by channels
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4 expressed on mitochondria (mito- K_{ATP}). Previous results showed anti-ischemic effects of the spiro-
5
6 cyclic derivative **A**, on isolated rat hearts. In this work this molecule was more extensively studied
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8 and diazoxide was used as reference mito- K_{ATP} opener. The studies were performed on an *in vivo*
9
10 rat model of myocardial infarct and on heart-derived H9c2 cells exposed to an anoxic environment.
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12 The mechanism of action was further investigated on isolated rat heart mitochondria. In the model
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14 of myocardial infarct compound **A** and diazoxide produced significant cardioprotective effects,
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16 antagonised by the selective mito- K_{ATP} blocker 5-hydroxydecanoic acid (5-HD). Compound **A**, like
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18 diazoxide, produced modest and non-significant hypotensive responses, while the hyperglycaemic
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20 effects of diazoxide were not observed for the new compound. Protective effects of compound **A**
21
22 and diazoxide were also recorded in H9c2 cells and again were inhibited by 5-HD. Compound **A**
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24 and diazoxide caused swelling of cardiac mitochondria, in agreement with the profile of mito- K_{ATP}
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26 openers. Both compounds evoked concentration-dependent Ca^{++} - release from Ca^{++} pre-loaded
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28 mitochondria, prevented mitochondrial Ca^{++} - uptake and caused mitochondrial membrane
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30 depolarisation. These effects were antagonised by ATP, the endogenous K_{ATP} inhibitor. In
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32 conclusion, compound **A** exhibits a promising profile of an anti-ischemic agent, with a mechanism
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34 likely to be linked to the activation of mito- K_{ATP} channels, and, because of its chemical
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36 characteristics such as structural rigidity and chirality due to the spiro-cyclic moiety, represents an
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38 interesting template for development of analogues further improved in activity and selectivity.
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48 **Key-words:** myocardial ischemia/reperfusion; anti-ischemic drugs; mitochondrial K_{ATP} channel;
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50 benzopyran K_{ATP} activators; spirocyclic derivatives.
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1. Introduction

In 1986, Murry and colleagues described an endogenous mechanism for the survival of cardiac tissue, defined as ischemic preconditioning (IPC), in which brief periods of ischemia protect myocardium from following and more sustained ones [1]. IPC is a multi-factorial phenomenon which requires the interaction of several receptor-dependent and receptor-independent processes. Presently, it is widely accepted that ATP-sensitive potassium channels (K_{ATP}), and in particular those expressed in the mitochondrial inner membrane (mito- K_{ATP}) rather than the sarcoplasmic ones (sarc- K_{ATP}), play a key role in IPC.

Really, it should be reported that some authoritative authors attribute the mitochondrial activity of well-known K_{ATP} -activators to other mechanisms [2,3]. Nevertheless, many K_{ATP} -openers, belonging to heterogeneous chemical classes (benzopyrans such as cromakalim; cyanoguanidines such as pinacidil; benzothiadiazines, such as diazoxide; etc), have cardioprotective effects [4], thus the sharing of other common ancillary mechanisms is quite improbable. Moreover, the presence of K_{ATP} channels (identical to the mammalian ones) and their involvement in IPC phenomena have been recognised in mitochondria of invertebrate animals, suggesting that the mito- K_{ATP} channel is actually an evolutionarily well conserved milestone of self-defence pathways [5].

The activation of mito- K_{ATP} allows a net influx of K^+ ions into the matrix, depolarisation of the inner membrane and reduction of the driving force for Ca^{++} uptake [6,7].

During reperfusion, but not during ischemia [8], high mitochondrial Ca^{++} levels promote the irreversible formation of the membrane permeability transition pore (MPTP), which represents the cause of irreversible injury to the cardiac tissue, through the release of pro-apoptotic proteins, such as cytochrome c [9]. Therefore, the activation of mito- K_{ATP} in the ischemic phase might prevent Ca^{++} accumulation in the matrix, blunting the following irreversible opening of MPTP in the reperfusion phase [10]. The reduced Ca^{++} - accumulation in the matrix can account for cardioprotection [11], but this is not an exhaustive explanation for the effects of mito- K_{ATP} openers:

1 a reduced production of reactive oxygen species by these drugs has been observed in isolated
2 mitochondria, and attributed to K^+ influx-induced matrix alkalisation [12], mild uncoupling and/or
3 increased oxidation of fatty acids [13]. The K^+ influx in the mitochondrial matrix is also
4 accompanied by diffusion of water and uptake of anions, resulting in matrix swelling, which is
5 responsible for the preservation of the low permeability of the outer membrane for nucleotides and
6 for the creation of a favourable gradient for ATP synthesis [6,7].
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14 Generally, K_{ATP} -openers are non-selective activators that target also the sarc- K_{ATP} channels
15 expressed in myocardiocytes, in vascular smooth muscle cells and in pancreatic β -cells, producing
16 many undesired effects, such as vasodilatation and hyperglycaemia [14,15]. Therefore, the
17 development of selective mito- K_{ATP} openers is viewed as a challenging issue to obtain anti-
18 ischemic drugs devoid of side effects related to affinity versus the other subtypes of the K_{ATP}
19 channel. For these purposes, the benzopyran scaffold has been subjected to many structural
20 modifications in order to increase the selectivity for the mito- K_{ATP} channels. In particular, BMS
21 180448 and BMS 191095 have been early reported to possess cardioprotective profiles comparable
22 with cromakalim but devoid of vasodilator activity [16,17].
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More recently, our group developed new benzopyran derivatives bearing a spiro-cyclic substituent [18,19]. Some molecules, such as 4'-(N-(4-acetamidobenzyl))- 2,2-dimethyl- 2,3-dihydro- 5'H-spiro[chromene- 4,2'- [1,4] oxazinan]- 5'- one (compound **A**, Fig.1), showed cardioprotective activity on Langendorff-perfused rat hearts submitted to ischemia-reperfusion (I/R), with modest effects on vascular smooth muscle. The cardioprotection was antagonised by 5-hydroxydecanoic acid (5-HD), which is widely used as a selective mito- K_{ATP} inhibitor, but unfortunately can have further effects at the mitochondrial level [20,21]. Furthermore, compound **A** showed an enantioselectivity of action: its **S**-(-)-enantiomer was responsible for the cardioprotective effect, whereas the **R**-(+)-enantiomer was almost ineffective [22].

In this paper, compound **A** has been submitted to a more detailed pharmacological study. Although diazoxide is likely to be endowed with further mechanisms, other than its activity on channels

[21,23,24], this benzothiadiazine is the most commonly used mito- K_{ATP} activator, exhibiting a satisfactory level of selectivity. Therefore, diazoxide was selected as reference drug.

2. Methods

Male Wistar rats (250-350 g) were housed and cared for in conformity with the Guidelines of the European Community Council Directive 86/609, adopted by Italian law D.L. 116/92, and with the Guide for the Care and Use of Laboratory Animals (NIH n° 85-23, revised 1996). The protocols were approved by the ethical committee of University of Pisa. Compound **A** was synthesised and chemically characterised as previously published [18].

2.1 *In vivo* model of coronary occlusion-reperfusion

The experimental protocol for coronary occlusion-reperfusion has been performed as described in [25], with minor modifications. Two hours before the experimental procedures, rats received an i.p. injection of the reference drug diazoxide (40 mg/Kg, Sigma-Aldrich, St. Louis, MO, USA), compound **A** (2, 10 or 40 mg/Kg), or vehicle (dimethylsulphoxide, DMSO, Sigma-Aldrich, St. Louis, MO, USA). When required, the mito- K_{ATP} blocker 5-HD (5 mg/Kg, Sigma-Aldrich, St. Louis, MO, USA) was injected 20 min before the treatment with the mito- K_{ATP} opener. The doses of the tested compounds were selected on the basis of the previous work [18].

Then, rats were anaesthetised with sodium pentobarbital (70 mg/kg, i.p.). The trachea was intubated and connected to a rodent ventilator (mod. 7025 Ugo Basile, Comerio, Italy) for artificial ventilation with room air (stroke volume, 1ml/100g body weight; 70 strokes/min). Electrocardiogram (ECG) was continuously measured by lead II (Mindray, PM5000). The chest was opened by a left thoracotomy. A 6-0 surgical needle was passed around the left anterior descending coronary artery (LAD), located between the base of the pulmonary artery and left atrium. The ends of the suture were passed through a polypropylene tube (PE50) to form a snare. To induce infarction, the LAD was occluded by pulling the snare and then fixing it in place by

1 clamping the tube with a haemostat, when the snare was released the reperfusion was initiated. The
2 acute infarct protocol consisted of 30' occlusion/120' reperfusion; successful occlusion was
3 confirmed visually by regional cyanosis downstream of the ligature and by ST elevation on the
4 ECG.
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9 A group of vehicle-pretreated animals was submitted to an IPC procedure, achieved by 2 cycles of
10 5' occlusion/10' reperfusion, followed by 30' coronary occlusion and 120' reperfusion. Each
11 experimental group was composed of at least 6 animals.
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16 At the end of reperfusion, rats were euthanised by an overdose of pentobarbital sodium, then hearts
17 were quickly excised, mounted on a Langendorff apparatus and perfused for 10' with Krebs
18 solution at 37°C to wash out the coronary blood vessels. Then, hearts were deprived of the atria and
19 right ventricle. The left ventricular tissue was dried, frozen, and cut into 4-5 transverse slices from
20 apex to base of equal thickness (about 2 mm). The slices were then incubated in a 1% 2,3,5-
21 triphenyltetrazolium chloride (TTC, Sigma-Aldrich, St. Louis, MO, USA) solution in a phosphate
22 buffer (pH 7.4) at 37 °C for 20 min.
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33 TTC reacts with NADH in the presence of dehydrogenase enzymes, to form a formazan derivative
34 causing the staining of viable cells with intense red colour. Then, the slices were fixed overnight in
35 10% formaldehyde and finally they were photographed. Red-stained viable tissue was easily
36 distinguished from the infarcted white-unstained necrotic tissue. The infarct area (A_i) was
37 planimetrically evaluated using an image analyser program (The GIMP 2). The infarct size was
38 calculated as a percentage of the whole area of the left ventricle ($A_i/A_{LV}\%$).
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51 **2.2 *In vivo* assessment of systolic blood pressure**

52 The experimental protocol was performed as already described in [18]. In order to reproduce the
53 same conditions used for the evaluation of the cardioprotective effects, the animals were
54 anaesthetised with sodium pentobarbital (60 mg/Kg). After the administration of the anaesthetic
55 drug, the animal tails were exposed for 15-20 minutes to irradiation with an I.R. lamp to cause
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1 vasodilation of the tail-vessels. Then, three basal systolic blood pressure values were
2 sphygmomanometrically recorded with the “tail-cuff” method by a BP recorder (Ugo Basile 58500,
3 Italy) at 5-min intervals. Then, the animals were treated with an i.p. administration of vehicle,
4 diazoxide 40 mg/Kg, compound A 40 mg/Kg or the potent K_{ATP} -activator cromakalim 1 mg/Kg, i.e.
5 the dose which exhibited significant anti-ischemic effects in the previous work [18]. The systolic
6 blood pressure values were recorded for 1 hour at intervals of 5 min (during this period, a
7 maintenance dose of 10 mg/Kg sodium pentobarbital was administered, when required). Each
8 experimental group was composed of 6 animals.
9

2.3 Glucose tolerance

10 The experimental protocol has been performed as described in [26], with some modifications. In
11 order to reproduce the same conditions used for the evaluation of the cardioprotective effects, the
12 animals were anaesthetised with sodium pentobarbital (60 mg/Kg). After the induction of
13 anaesthesia, basal glycaemic levels were monitored by means of the usual glucose-titration kit (New
14 Glucocard G sensor and Glucocard Gmeter, Arkray, A. Menarini diagnostics, Italy). Then, rats were
15 treated with an i.p. injection of vehicle, diazoxide 40 mg/Kg or compound A 40 mg/Kg. Twenty
16 minutes after the pharmacological treatment, glycaemia was measured again and then the rats
17 received an i.p. administration of glucose 1g/Kg and glycaemia was regularly monitored for the
18 following 2 hours. Each experimental group was composed of 6 animals.
19

2.4 Cell culture

20 H9c2 subclonal cell line derives from embryonic rat hearts [27]; although it exhibits also some
21 characteristics of skeletal muscle cells, it is widely used as a valuable cellular model for studies of
22 myocardial ischemia/reperfusion [25,28,29]. H9c2 cells (ATTC, Manassas, VA, USA) were
23 cultured, following the usual procedures in Dulbecco’s modified Eagle’s medium (DMEM, Sigma-
24 Aldrich, St. Louis, MO, USA) supplemented with 10% fetal bovine serum (FBS, Sigma-Aldrich, St.
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Louis, MO, USA), 100 units/ml penicillin and 100 μ g/ml streptomycin in tissue culture flasks at 37°C in a humidified atmosphere of 5% CO₂.

H9c2 cells were cultured up to about 80% confluence in a DMEM medium and 24 hours before the experiment, cells were seeded onto 96-well plates at a density of 8 \times 10³ per well. After 24 hours to allow cell attachment, the medium was replaced in each 96-well plate and the cells received different treatments such as: vehicle (that is 0.05% DMSO), diazoxide (30 or 100 μ M), compound A (3 or 10 μ M), 5-HD (100 or 500 μ M) plus diazoxide (30 or 100 μ M), 5-HD (100 or 500 μ M) plus compound A (3 or 10 μ M). The concentrations of diazoxide and 5-HD were selected on the basis of the experimental protocols usually reported in literature [11]. Then, one plate was placed for 16 hours in an airtight AtmosBag (Sigma-Aldrich) which was saturated with 95% N₂ and 5% CO₂ to simulate ischemia, while the twin plate was placed for 16 hours in an airtight AtmosBag which was saturated with 95% air and 5% CO₂. At the end of the hypoxia period all the cells were subjected to reperfusion by replacing the medium with DMEM and incubation for 2 hours in an atmosphere containing 5% CO₂ / 95 % air at 37°C. After reperfusion, cell viability was assessed using the Cell Proliferation Reagent WST-1 (4-[3-(4-iodophenyl)-2-(4-nitrophenyl)-2H-5-tetrazolium]-1,3-benzene disulphonate) (Roche, Mannheim, Germany) based on the cellular cleavage of the WST-1 to formazan. WST-1 was added at 1/10 of the total volume and after 60 min of incubation at 37°C, the absorbance was measured at 450 nm with a microplate reader (Wallac; PerkinElmer, Wellesley, MA, USA). Absorbance values were normalised as a % of the absorbance shown by vehicle-treated cells exposed in the non-hypoxic atmosphere (Viab. %). Three different experiments were performed in six replicates.

2.5 Rat cardiac mitochondria

2.5.1 Isolation procedure

Rat cardiac mitochondria were isolated by differential centrifugation, as described [30], with minor modifications. Male Wistar rats were killed by cervical dislocation, the heart was removed

1 immediately and placed in ice cold isolation buffer (composition: sucrose 250 mM, Tris 5 mM,
2 EGTA 1mM, pH 7.4 adjusted with HCl). The atria were removed and the ventricular tissue was
3
4 finely minced with surgical scissors (about 2 mm³ pieces) and homogenised using an Ultra-Turrax
5
6 homogeniser (20 ml of isolation buffer per heart).
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9 Three 20sec homogenisation cycles were performed on ice, and then the suspension was centrifuged
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11 at 1075xg for 3 min at 4°C. The resulting supernatant was centrifuged at 11950xg for 10 min at
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13 4°C. The pellet containing the mitochondrial fraction was further re-suspended in the isolation
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15 buffer (without EGTA) and centrifuged at 11950xg for 10 min at 4°C, this step was repeated once
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17 more.
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21 The final mitochondrial pellet was re-suspended in a minimal volume of 400 µl of the isolation
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23 buffer (without EGTA) and stored on ice throughout the experiment, which was performed within 2
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25 hours. Mitochondrial protein concentrations were determined using the Bradford reaction.
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29 Previous experiments (data not shown) confirmed the reliability of the isolation procedures, by
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31 measurement of the mitochondrial respiratory function through an ATP bioluminescence assay, in
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33 agreement with the method of Drew and Leeuwenburgh [31]. Briefly, mitochondria (1 mg
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35 protein/ml) were suspended in the isolation buffer (without EGTA) enriched with succinate 20 mM,
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37 KH₂PO₄ 30 mM and ADP 5' diphosphate 200 µM. The reaction was initiated by the addition of a
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39 luciferin-luciferase reagent. ADP 5' diphosphate replaced by bidistilled water was used as a blank.
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41 3 min after the start of reaction, the measurement was performed using a luminometer (Wallac,
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43 Perkin Elmer, Wellesley, MA, USA). In all the tested preparations, the assay really detected the
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45 light produced by the ATP-dependent oxidation of luciferin by means of the luciferase enzyme. The
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47 ATP-synthase inhibitor oligomycin 2 µg/ml (Sigma-Aldrich, St. Louis, MO, USA) or the uncoupler
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49 of oxidative phosphorylation 2,4-dinitrophenol (100 µM, Sigma-Aldrich, St. Louis, MO, USA)
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51 inhibited ATP production.
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57 **2.5.2 Mitochondrial swelling**

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1 Mitochondrial swelling was assessed spectrophotometrically [32] by measuring the change in
2 absorbance of the suspension at 520 nm (Wallac, Perkin Elmer, Wellesley, MA, USA).
3

4 Mitochondria (1mg protein/ml) were suspended in an incubation medium under gently stirring
5 (composition: KCl 120 mM, K₂HPO₄ 5 mM, Hepes 10 mM, succinic acid 10 mM, MgCl₂ 2 mM,
6 ATP 200 μM, pH 7.4 adjusted with KOH). The decrease of absorbance was monitored for 15 min
7 after the addition of A (10 and 100μM), diazoxide (10 and 100 μM), or vehicle (DMSO 1%). Each
8 time-response curve was obtained with mitochondria isolated from the hearts of at least 6 different
9 animals.
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18 **2.5.3 Mitochondrial Ca⁺⁺ - release**

19 Changes of the medium (i.e. extra-mitochondrial) Ca⁺⁺ concentration were continuously measured
20 with a Ca⁺⁺ selective mini-electrode [30], coupled with a reference electrode (WPI, FL, USA),
21 using a data acquisition software (Biopac Inc. California, USA). The selectivity of the electrode for
22 Ca⁺⁺ over other cations, such as Mg²⁺, K⁺ and Na⁺, was > 10⁵. In order to correlate the
23 potentiometric measurements (in mV) with the corresponding concentrations of Ca⁺⁺ ions in the
24 solution, opportune calibration curves were generated before each experiment using known
25 concentrations of CaCl₂. Mitochondria (1 mg protein/ml) were suspended under gently stirring in
26 the incubation medium (composition: KCl 120 mM, K₂HPO₄ 5 mM, Hepes 10 mM, succinic acid
27 10 mM, MgCl₂ 2 mM, CaCl₂ 100 μM, pH 7.4 adjusted with KOH). Mitochondrial Ca⁺⁺ - release was
28 induced 5 min after the rapid Ca⁺⁺ accumulation, by adding, in the incubation medium at 5 min
29 intervals, cumulative increasing concentrations of compound A (3-300 μM) or the reference drug
30 diazoxide (3-300 μM). The potassium ionophore valinomycin (2nM-2μM) (Sigma-Aldrich, St.
31 Louis, MO, USA), was also used as a reference channel-independent drug causing K⁺ influx in
32 mitochondria. When required, ATP (200 μM), used to inhibit the mito-K_{ATP} channels, and
33 cyclosporine A (CsA, 0.2 μM, Sigma-Aldrich, St. Louis, MO, USA), MPTP blocker, were
34 incubated 2 min before the cumulative addition of the K_{ATP}- openers. Each concentration-response
35 curve was obtained with mitochondria isolated from the hearts of at least 6 different animals.
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2.5.4 Mitochondrial Ca⁺⁺- uptake

Mitochondrial Ca⁺⁺- uptake was potentiometrically measured [30] following the above reported protocol, with the following modification: mitochondria (1 mg protein/ml) were added under gently stirring to the incubation medium (composition: KCl 120 mM, K₂HPO₄ 5 mM, Hepes 10 mM, succinic acid 10 mM, MgCl₂ 2 mM, CaCl₂ 100 μM, pH 7.4 adjusted with KOH), medicated with vehicle (DMSO 1%), diazoxide (100 mM) or compound A (300 mM). The maximal variation of concentration of Ca⁺⁺ in the medium, related to its accumulation in the mitochondrial matrix, was measured. Each result was obtained with mitochondria isolated from the hearts of at least 3 different animals.

2.5.5 Mitochondrial membrane potential

Mitochondrial membrane potential ($\Delta\psi$) was potentiometrically measured with tetraphenylphosphonium (TPP⁺)-sensitive mini-electrodes [33], coupled with a reference electrode (WPI, FL, USA), using a data acquisition software (Biopac Inc. California, USA). Electrodes were calibrated before each experiment using known concentrations of TPP⁺Cl⁻ (Sigma-Aldrich). Mitochondria (2 mg protein/ml) were suspended under gently stirring in the incubation medium (composition: KCl 120 mM, K₂HPO₄ 5 mM, Hepes 10 mM, succinic acid 10 mM, MgCl₂ 2 mM, EGTA 1 mM, pH 7.4 adjusted with KOH). The value of the potential was calculated according to the following Nernst-derived experimental equation:

$$\Delta\psi = 60 \times \text{Log} \frac{V_0 \cdot \frac{[TPP^+]_0}{[TPP^+]_t} - V_t - K_0 P}{V_m P + K_i P}$$

Where $\Delta\psi$ is the mitochondrial membrane potential (mV), V_0 is the volume of the incubation medium before the mitochondria addition, V_t is the volume of the incubation medium after the mitochondria addition, V_m is the volume of mitochondrial matrix (μl/mg protein), $[TPP^+]_0$ and $[TPP^+]_t$ are concentrations of TPP⁺ before the addition of mitochondria and at time t, respectively, P

1 is the protein concentration (mg), K_0 and K_i are apparent external and internal partition coefficients
2 of TPP^+ , and were estimated as 14.3 $\mu\text{l}/\text{mg}$ and 7.9 $\mu\text{l}/\text{mg}$, respectively. The volume of
3 mitochondria was taken as 1 $\mu\text{l}/\text{mg}$ of protein [33].
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7 Changes of $\Delta\psi$ were continuously recorded before and after the addition in the incubation medium
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9 of cumulative increasing concentrations of compound **A** (10-300 μM) or the reference drug
10
11 diazoxide (10-300 μM).
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14 The potassium ionophore valinomycin 2nM-2 μM (Sigma-Aldrich) was also used as a reference
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16 channel-independent drug causing K^+ influx in mitochondria. When required, ATP (200 μM), used
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18 to inhibit the mito- K_{ATP} channels, was incubated in the medium, 2 min before the mitochondria
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20 addition. Each concentration-response curve was obtained with mitochondria isolated from the
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22 hearts of at least 6 different animals.
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25 26 27 28 29 **2.6 Data analysis**

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31 All data are expressed as mean \pm standard error. Concentration response curves were analysed by a
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33 non-linear fitting equation by computerised methods (software: GraphPadPrism 4.0). Data were
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35 statistically analysed by ANOVA and P values lower than 0.05 were considered as indicative of
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37 significant differences.
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43 44 **3. Results**

45 46 **3.1 Acute infarct**

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48 Hearts of vehicle-treated animals submitted to LAD ligation showed clear evidence of I/R injury,
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50 with a large amount of damaged tissue ($A_i/A_{LV} = 35 \pm 3\%$). As expected, preconditioning protocol
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52 afforded significant cardioprotective effects, producing about a half-reduction of the damaged areas
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54 ($A_i/A_{LV} = 17 \pm 6\%$) and pre-treatment with diazoxide (40 mg/Kg) led to almost equivalent anti-
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56 ischemic effects ($A_i/A_{LV} = 21 \pm 2\%$).
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1 Pharmacological treatment with increasing doses (2, 10, 40 mg/Kg) of compound A, produced
2 significant and dose-dependent cardioprotective responses ($A_i/A_{LV} = 29 \pm 1\%$, $21 \pm 3\%$ and $18 \pm 2\%$
3 respectively). When tested on 5-HD (5 mg/Kg) pre-treated animals, compound A (40 mg/Kg) failed
4 to exhibit any significant cardioprotective activity (Fig. 2). 5-HD (5 mg/Kg) fully antagonised also
5 the effects of diazoxide (data not shown).
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11 **3.2 Blood pressure**

12 The administration of diazoxide and compound A (both at the dose of 40 mg/Kg i.p.) was followed
13 by a weak and not significant decrease of systolic blood pressure, with a maximal decrease of about
14 15% and without any significant alteration of heart rate (Fig. 3). Cromakalim, a well-known K_{ATP} -
15 activator endowed with heavy hypotensive effects, was also selected for this protocol and
16 administered at the dose of 1 mg/Kg i.p., which exhibited anti-ischemic effects in the previous study
17 [18]. Cromakalim caused an immediate dramatic and long-lasting decrease (about 50%) of systolic
18 blood pressure, which was associated with a significant compensatory tachycardia (Fig. 3).
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36 **3.3 Glucose metabolism**

37 The administration of diazoxide (40 mg/Kg i.p.) determined *per se* an appreciable (but not
38 statistically significant) increase (about 40 %) of non-fasting glycaemic levels; moreover, in the i.p.
39 glucose tolerance test, this benzothiadiazine caused an higher glycaemic peak and delayed the
40 recovery of normoglycaemic levels. Compound A was completely devoid of effects on glucose
41 metabolism (Fig. 4).
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53 **3.4 H9c2 cell viability**

54 The incubation in an anoxic environment (95% N_2 and 5% CO_2) caused a drastic reduction of H9c2
55 cell viability ($Viab.\% = 58 \pm 5$). Pre-treatment of cells with diazoxide (100 μM) and A (10 μM)
56 conferred a strong resistance, producing a full preservation of cell viability ($Viab.\% = 103 \pm 6$ and
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2 99±3, respectively). 5-HD (100 and 500µM) fully antagonised the protective effects of diazoxide
3 100 µM (Viab.% = 67±5 and 55±7, respectively). The protective activity of compound A 10 µM
4 was significantly reduced by 5-HD 100 µM (Viab.% = 78±7) and completely abolished by 5-HD
5 500 µM (Viab.% = 64±5).
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9 Lower concentrations of diazoxide (30 µM) and A (3 µM) still conferred appreciable levels of
10 resistance, causing a significant preservation of cell viability (Viab.% = 86±4 and 81±5,
11 respectively). The effects of both diazoxide and compound A were almost completely abolished by
12 5-HD 100 µM (Viab.% = 63±3 and 55±4, respectively) (Fig. 5). Preliminary experiments showed
13 that 5-HD alone (100 and 500 µM) does not influence the cell viability.
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24 **3.5 Mitochondrial swelling**

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26 The spectrophotometrical evaluation of the influence of the test compounds on the matrix volume
27 showed that both diazoxide and compound A evoked mitochondrial swelling. In particular, the
28 decreases in absorbance at 520 nm recorded 15 min after the addition of diazoxide 100 µM or
29 compound A 100 µM were 0.110±0.013 and 0.123±0.014 (in absorbance arbitrary units),
30 respectively (Fig. 6). Compound A and diazoxide, both tested at 10 µM, did not produce any
31 significant decrease of absorbance (Fig. 6).
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43 **3.6 Ca⁺⁺- release from pre-loaded mitochondria**

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45 Ca⁺⁺ ions (100 µM) were rapidly and almost fully accumulated by mitochondria. The K⁺-ionophore
46 valinomycin (2 nM-2 µM) caused a concentration-dependent massive Ca⁺⁺- release from Ca⁺⁺ pre-
47 loaded mitochondria; an almost complete release of the ion was induced by valinomycin 20 nM
48 (data not shown).
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56 Diazoxide (10-300 µM) and compound A (10-300 µM) induced a concentration-dependent outflow
57 of Ca⁺⁺, and the former exhibited a level of potency (pEC₅₀= 4.78±0.12) about one order of
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2 magnitude higher than the latter ($pEC_{50} = 3.89 \pm 0.04$). The effects of diazoxide and compound A
3 were strongly antagonised by both ATP 200 μ M and CsA 0.2 μ M (Fig. 7).
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6 7 **3.7 Ca^{++} - uptake into Ca^{++} - free mitochondria**

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9 The addition of Ca^{++} - free mitochondria to the Ca^{++} - rich medium medicated with the vehicle led to
10 a rapid and dramatic decrease of the ion concentration in the medium, corresponding to an efficient
11 accumulation of the cation in the mitochondrial matrix. The final concentration of Ca^{++} was stably
12 reached within 1-2 min and was 8 ± 3 μ M.
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16 The addition of Ca^{++} - free mitochondria to the Ca^{++} - rich medium medicated with diazoxide 100
17 μ M (i.e., the concentration evoking an almost maximal effect on Ca^{++} -release from Ca^{++} preoated
18 mitochondria) led to a very modest decrease of the ion concentration in the medium, corresponding
19 to significant abolition of the process of accumulation of the cation in the mitochondrial matrix. The
20 final concentration of Ca^{++} was stably reached within 1-2 min and was 88 ± 6 μ M.
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24 As observed for diazoxide, the addition of Ca^{++} - free mitochondria to the Ca^{++} - rich medium
25 medicated with compound A 300 μ M (i.e., the concentration evoking an almost maximal effect on
26 Ca^{++} -release from Ca^{++} preoated mitochondria) led to a very modest decrease of the ion
27 concentration in the medium, corresponding to significant inhibition of the process of upload of the
28 cation in the mitochondrial matrix. The final concentration of Ca^{++} was stably reached within 1-2
29 min and was 79 ± 7 μ M (Fig. 8).
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32 33 **3.8 Mitochondrial membrane potential**

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35 Isolated heart mitochondria showed basal levels of $\Delta\psi$ ranging around -180 mV. Both diazoxide
36 (10-300 μ M) and compound A (10-300 μ M) determined a partial depolarisation of mitochondria,
37 with maximal effects of about 25 mV. In particular, significant effects of diazoxide started at
38 relatively low concentrations (starting from 30 μ M) and proceeded in a more evident concentration-
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1 dependent fashion. As already observed in the experiments on mitochondrial Ca^{++} - release,
2 compound **A** evoked a marked and significant depolarising response only at the highest
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4 concentration of 300 μM . ATP 200 mM antagonised the effects of both derivatives (Fig. 9).
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6 Valinomycin showed powerful effects (with maximal depolarisation of about 60 mV, produced by
7
8 valinomycin 20 nM), which were not influenced by ATP (data not shown). Moreover, the
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10 relationships between changes in membrane potential and Ca^{++} -release from the matrix were further
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12 examined. In particular, each concentration of the tested drugs (both diazoxide and compound **A**)
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14 produced on isolated mitochondria a couple of data: a value of $\Delta\psi$ modification and a value of
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16 Ca^{++} -release. All these couples of experimental values have been reciprocally plotted (Fig. 10) and
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18 the linear regression analysis clearly confirmed that the two effects are closely correlated ($R^2 =$
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4. Discussion

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In this study, compound **A** was tested in an experimental model of acute infarct, more closely resembling the complex clinical pattern of the heart infarct. The *in vivo* study also addressed the evaluation of the potential impact of the new molecule on blood pressure and glucose metabolism, which are typical undesired effects of non-selective agents, clearly due to the activation of sarc- K_{ATP} channels expressed in vascular smooth muscle and pancreatic beta cells.

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In the model of experimentally-induced infarct, compound **A** showed cardioprotective effects almost comparable to that produced by the exogenous “self-defence” mechanism of IPC. Moreover, the anti-ischemic effects of compound **A** showed a dose-dependent fashion and were equivalent (or slightly superior) to those of the reference drug diazoxide. As already observed in the previous studies on isolated hearts, the cardioprotective effects of compound **A** were inhibited by 5-HD, a putative selective blocker of mito- K_{ATP} channels.

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Both compound **A** and diazoxide (tested at the dose of 40 mg/Kg i.p., that induced clear and significant cardioprotection) caused modest and not significant alterations of systolic blood pressure

1 and heart rate, while the potent and non-selective K_{ATP} -opener cromakalim (at the i.p. dose of 1
2 mg/Kg, i.e. the one which induced significant cardioprotection in the previous work) caused an
3 immediate and dramatic fall of the arterial pressure, associated with a compensatory tachycardic
4 response. As regards the impact on glucose metabolism, in good agreement with other experimental
5 observations [34], diazoxide caused a marked (about 40%), albeit not significant, increase of the
6 basal levels of non-fasting glycaemia. Furthermore, the test of intraperitoneal glucose tolerance
7 demonstrated a strong and significant hyperglycaemic response in diazoxide-treated rats.
8 Compound **A** was completely ineffective on glucose metabolism, indicating that this spiro-cyclic
9 derivative, at the dose inducing cardioprotective effects, is unlikely to interact significantly with
10 potassium channels of pancreatic beta cells.
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24 The probable involvement of mito- K_{ATP} channels emerged also from the experimental protocols on
25 cultured H9c2 cells. In these cells, the anoxic condition caused a dramatic impact, leading to a high
26 level of cell death. Both diazoxide and compound **A** (at concentrations about one order of
27 magnitude lower than the reference benzothiadiazine) produced powerful protective effects against
28 the cell injury induced by the incubation of H9c2 cultures in the anoxic atmosphere. Again, the
29 mito- K_{ATP} blocker 5-HD antagonised the protective effects.
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39 Therefore, the studies were focused on the identification of characteristic effects of compound **A** at
40 the mitochondrial level.
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43 Movements of K^+ ions across the inner mitochondrial membrane, such as their influx into the
44 matrix through electrogenic mechanisms (K^+ channels and K^+ leak) and their extrusion mainly due
45 to K^+/H^+ exchanger, are fundamentally involved in the control of the matrix volume [35-37].
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51 In particular, the opening of K^+ channels leads to a K^+ influx exceeding the K^+ extrusion, which is
52 compensated by increased H^+ outward pumping and accumulation of phosphates in the matrix. This
53 causes osmotic influx of water and mitochondrial swelling [37,38]. These effects on mitochondrial
54 volume are considered the most relevant physiological consequences of mito- K_{ATP} activation [6]; in
55 agreement with its pharmacodynamic feature of a K^+ channel activator, diazoxide caused
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mitochondrial swelling, at the same concentration evoking protective effects on H9c2 cells. A similar effect was produced by compound **A**, which caused mitochondrial swelling, but at a concentration slightly higher (about one order of magnitude) than that exhibiting protective effects on H9c2 cells.

Mitochondria represent a powerful mechanism for buffering excessive increases of cytosolic Ca^{++} , through an avid accumulation into the matrix, mainly mediated by Ca^{++} -uniporter and facilitated by the large electrochemical gradient [39]. Influx of K^{+} ions causes mitochondrial membrane depolarisation thus reducing Ca^{++} -upload and/or promoting Ca^{++} -release from Ca^{++} -preloaded mitochondria. Diazoxide caused a dose-dependent unloading of Ca^{++} from Ca^{++} -charged mitochondria and prevented Ca^{++} upload in Ca^{++} -free mitochondria. The diazoxide-induced Ca^{++} release from mitochondria was completely abolished by ATP, i.e. by the main endogenous blocker of the K_{ATP} channels. These findings are in good agreement with the experimental work of other authors [30].

Moreover, the depolarisation induced by K^{+} channel openers is known to represent a trigger for the transient activation of MPTP [40], which probably prevents a following irreversible and deleterious opening of MPTP at reperfusion [41]. In turn, this transient MPTP opening is the principal pathway for Ca^{++} release from the matrix [42]. Hence, the diazoxide-induced Ca^{++} discharge was also evaluated in the presence of CsA, an inhibitor of MPTP. In these experimental conditions, diazoxide failed to induce Ca^{++} release from mitochondria, in agreement with authoritative data from the literature [30,40,43]. The effects of diazoxide on mitochondrial Ca^{++} movements (release and accumulation) were fully mimicked by compound **A**. As well, the Ca^{++} release induced by compound **A** was abolished by ATP and CsA. Nevertheless, in this pharmacological feature, compound **A** showed a level of potency lower than diazoxide. Moreover, the concentrations of compound **A** inducing Ca^{++} release from mitochondria were dramatically higher (more than two orders of magnitude) than those required for the protective effects on H9c2 cells.

1 In order to evaluate a correlation between the effects on Ca^{++} movements and those on
2 mitochondrial membrane potential, further experiments were made to evaluate the possible
3 depolarising responses and the range of effective concentrations. Both diazoxide and compound A
4 caused a partial depolarisation of the mitochondrial membrane, and such an effect was antagonised
5 by ATP. As shown in Fig. 10, it is evident that the effects evoked by the two compounds on Ca^{++}
6 movements and those induced on membrane potential were well correlated ($R^2 = 0.95$).
7 Valinomycin had more intense depolarising effects, which were not affected by ATP (data not
8 shown). Again, as observed in the experiments on the Ca^{++} release, compound A showed levels of
9 potency lower than the benzothiadiazine diazoxide; and again, as already observed in all the other
10 experimental protocols performed on isolated mitochondria (i.e., the experiments on the Ca^{++}
11 movements and those on mitochondrial swelling), the concentrations of compound A inducing
12 depolarising effects were dramatically higher (more than two orders of magnitude) than those
13 required for the protective effects on H9c2 cells.

14 However, it should be noted that this discrepancy is not particularly surprising, since such a great
15 difference between the concentrations required to depolarise the mitochondrial membrane potential
16 and cause Ca^{++} release in isolated mitochondria and the concentrations required for a
17 pharmacological activation of mito- K_{ATP} channels is a common feature, shared by almost all the
18 mito- K_{ATP} openers; indeed, many authors attribute this particular feature to epiphenomenic effects
19 that are independent of mito- K_{ATP} channels and that are linked to a non-specific inhibition of the
20 electron transport [6]. Together with the above hypothesis on a non-specific toxicity, recent reports
21 seem to offer other alternative or complementary keys of interpretations. In particular, there is
22 convincing evidence about the involvement of multi-molecular cardioprotective signals
23 (signalosomes) in IPC, which derive from non-mitochondrial compartments (caveolae) and are
24 delivered to mitochondrial membranes, where they trigger complex pathways, involving the
25 activation of mito- K_{ATP} channels as a fundamental step [44]. Thus, it cannot be excluded that the
26 interaction between mito- K_{ATP} -activators and the mito- K_{ATP} channel (or the effects deriving from
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1 such an interaction) might be facilitated and/or strengthened by the presence of other factors, which
2 are present in the whole cells but are lost in the isolated mitochondrial preparations.
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4 Moreover, it should be noted that compound **A** shows a higher level of lipophilicity
5 (LogP=3.34±0.77) than diazoxide (LogP=1.07±0.23). Given the intracellular localisation of the
6 pharmacological target, this physico-chemical factor might represent (at least in part) a further
7 “pharmacokinetic factor” affecting the different behaviour of the molecules, when tested *in vivo*, in
8 isolated hearts, in intact cells or in all the different protocols performed on isolated mitochondria.
9 These aspects will be investigated in future experimental works, more specifically aimed to
10 characterise the pharmacokinetic feature of the new molecule.
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12 In conclusion, this study confirmed the anti-ischemic properties of compound **A**, indicating that this
13 new derivative is more potent than diazoxide in the *in vivo* model of heart infarct and, more
14 importantly, its cardiac effects are not accompanied by significant influences on blood pressure and
15 glucose metabolism, which represent serious limits for the use of non-selective agents. On the basis
16 of the actual knowledge on mito-K_{ATP} channels and on the pharmacological tools for studying them,
17 the experimental results reasonably suggest that the pharmacodynamic mechanistic basis of cardio-
18 protection resides in the activation of mito-K_{ATP} channels. Therefore, compound **A** represents in
19 itself an innovative anti-ischemic spiro-benzopyranic molecule and an interesting prototype for the
20 development of improved analogues showing a more favourable therapeutic window.
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25 cultures.
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27 **6. Conflict of interest**

28 The authors declare no conflict of interest.
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Legends

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5 **Figure 1.** Chemical structure of compound **A**. The synthesis of this spiro-morpholone derivative
6 **A** and analogous molecules is described in Breschi et al., 2006.

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10 **Figure 2.** Infarct size, expressed as a percentage of the whole area of the left ventricle
11 ($A_i/A_{LV}\%$), after different *in vivo* treatments in a model of coronary occlusion-reperfusion on rat
12 hearts. Bars represent the $A_i/A_{LV}\%$ of the hearts of rats that 2 hours before the experimental
13 procedures received vehicle or respectively an i.p. injection of: diazoxide 40 mg/Kg, compound
14 **A** (2 mg/Kg, 10 mg/Kg, 40 mg/Kg) and 5-HD 5 mg/Kg plus compound **A** 40 mg/Kg. The
15 effects of IPC are also shown. The asterisks indicate a value significantly different from the
16 vehicle (* = $P < 0.05$; ** = $P < 0.01$).

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21 **Figure 3.** Values of systolic blood pressure (expressed in mmHg; A) and heart rate (expressed
22 as beats/min; B), sphygmomanometrically recorded in anaesthetised rats, before and after the
23 i.p. administration (arrow) of vehicle, diazoxide 40 mg/Kg, compound **A** 40 mg/Kg or
24 cromakalim 1 mg/Kg. The asterisks indicate a value significantly different from the vehicle (* =
25 $P < 0.05$; ** = $P < 0.01$; *** = $P < 0.001$).

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31 **Figure 4.** Changes of glycaemic levels, recorded in anaesthetised rats after the i.p.
32 administration (arrow) of vehicle, diazoxide 40 mg/Kg or compound **A** 40 mg/Kg, followed by
33 the i.p. administration of glucose 1g/Kg (at time 0). The asterisks indicate a value significantly
34 different from the vehicle (*** = $P < 0.001$).

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41 **Figure 5.** Cell viability normalised as a percentage of the reference values recorded in the non-
42 ischemic vehicle-treated cells. The bars represent the H9c2 viability after the indicated
43 treatments. The symbol \square indicates a value of viability significantly different from the reference
44 value N.I. (## = $P < 0.01$). The symbol * means a value of viability significantly different from
45 the vehicle-treated ischemic cells (* = $P < 0.05$; ** = $P < 0.01$). The symbol # means statistical
46 differences from the corresponding value obtained in the absence of 5-HD (# = $P < 0.05$; ## = $P <$
47 0.01).

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54 **Figure 6.** Rat heart mitochondrial swelling. A) The histograms report the change in absorbance
55 arbitrary units (520 nm) in the mitochondrial suspension, at the 15th minute after the addition of

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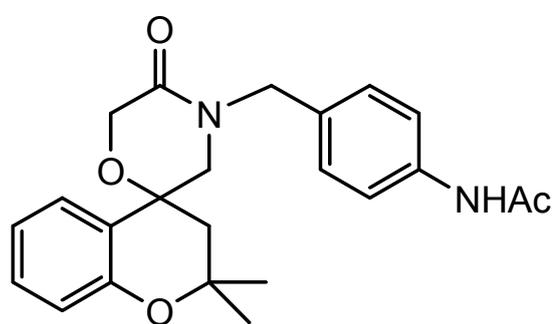
vehicle, diazoxide 10 or 100 μM and compound A 10 and 100 μM . B) Time-course curve of the decrease in absorbance at 520nm recorded for 15 minutes after the addition (arrow) of vehicle, diazoxide 100 μM and compound A 100 μM . ANOVA proved that the curves obtained after treatment with diazoxide or compound A are significantly different from that recorded in vehicle-treated mitochondria.

Figure 7. Concentration-response curves of the Ca^{++} releasing effects evoked by increasing concentrations of diazoxide (A) and compound A (B) in the absence and in the presence of ATP 200 μM or CsA 0.2 μM .

Figure 8. The bars represent the Ca^{++} concentrations measured in the medium, after the addition of Ca^{++} -free mitochondria and their consequent Ca^{++} -uptake, in the presence of the vehicle, diazoxide or compound A. in the absence and in the presence of ATP 200 μM or CsA 0.2 μM . The asterisks indicate a value significantly different from the vehicle (***) = $P < 0.001$).

Figure 9. Concentration-response curves for the mitochondrial membrane depolarising effects evoked by increasing concentrations of diazoxide (A) and compound A (B) in the absence and in the presence of ATP 200 μM .

Figure 10. The figure shows the reciprocal plotting of the effects produced by each tested concentration of drugs (diazoxide and compound A) on mitochondrial membrane potential vs the effects evoked by the same concentrations of the two molecules on Ca^{++} release from Ca^{++} -preloaded mitochondria. The linear regression analysis allowed to find a good level of correlation ($R^2 = 0.95$).



Compound **A**

Figure 1

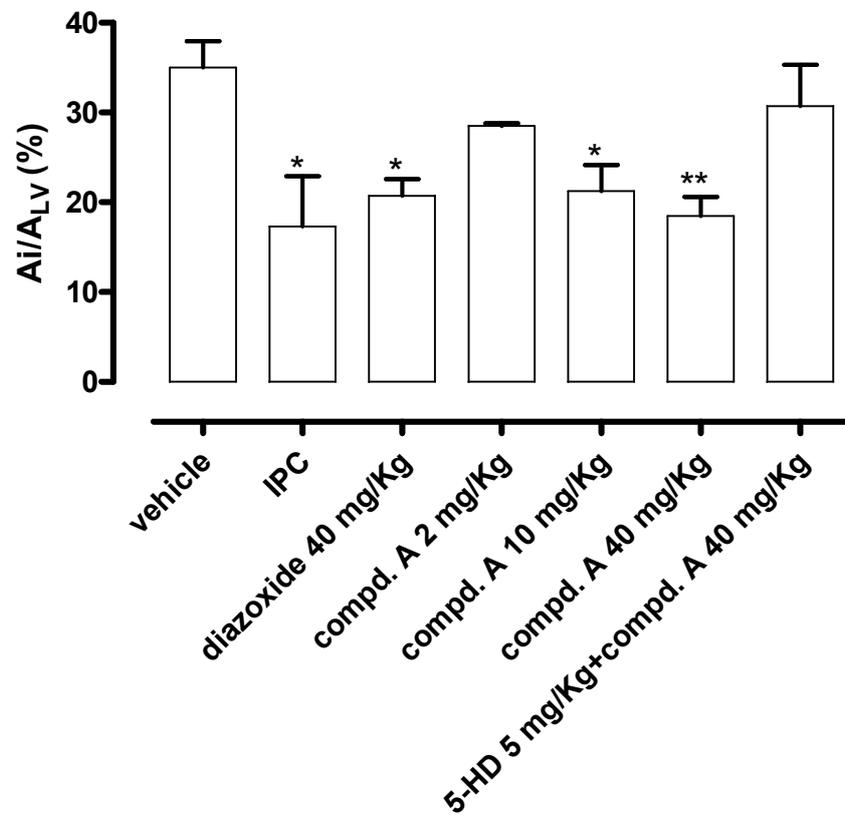


Figure 2

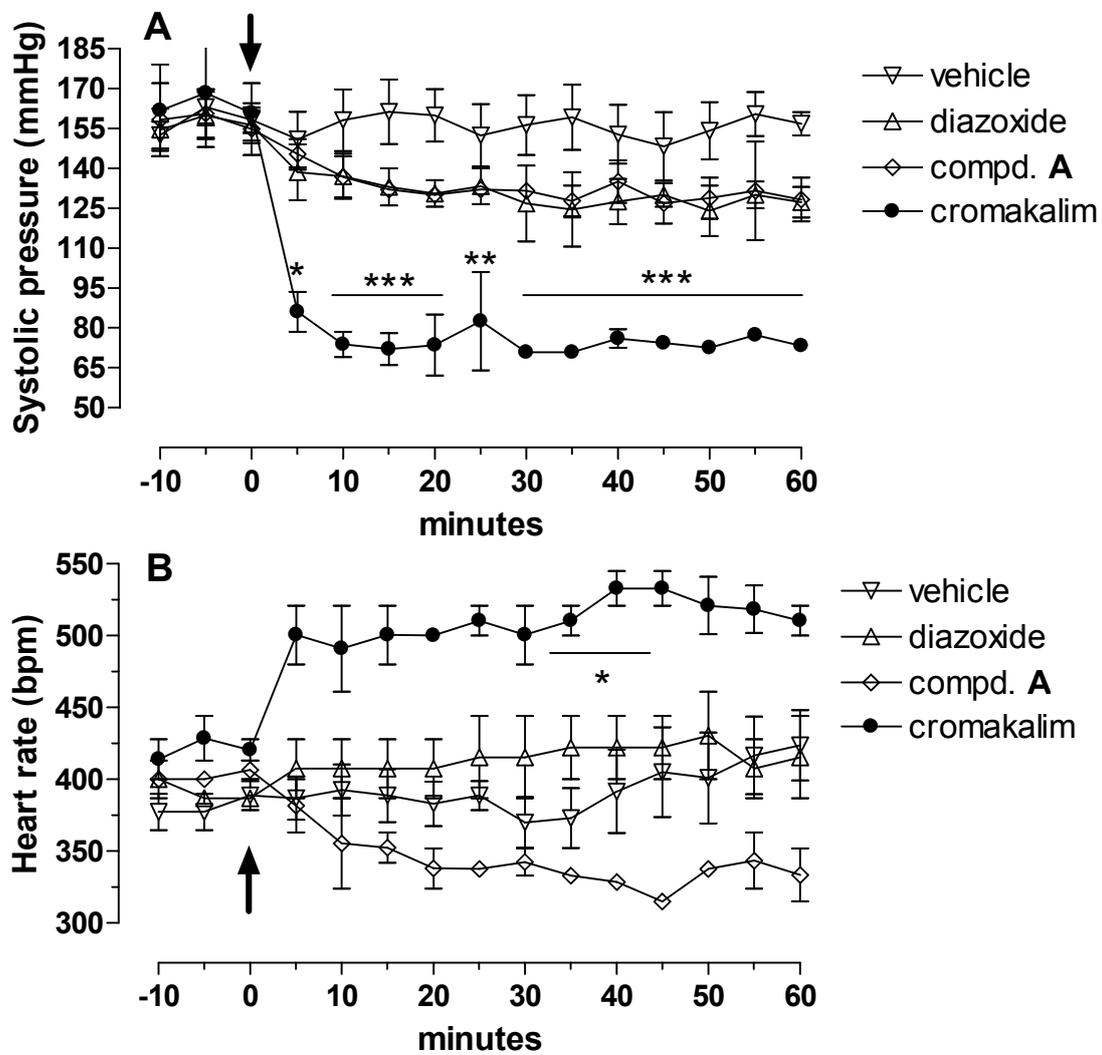


Figure 3

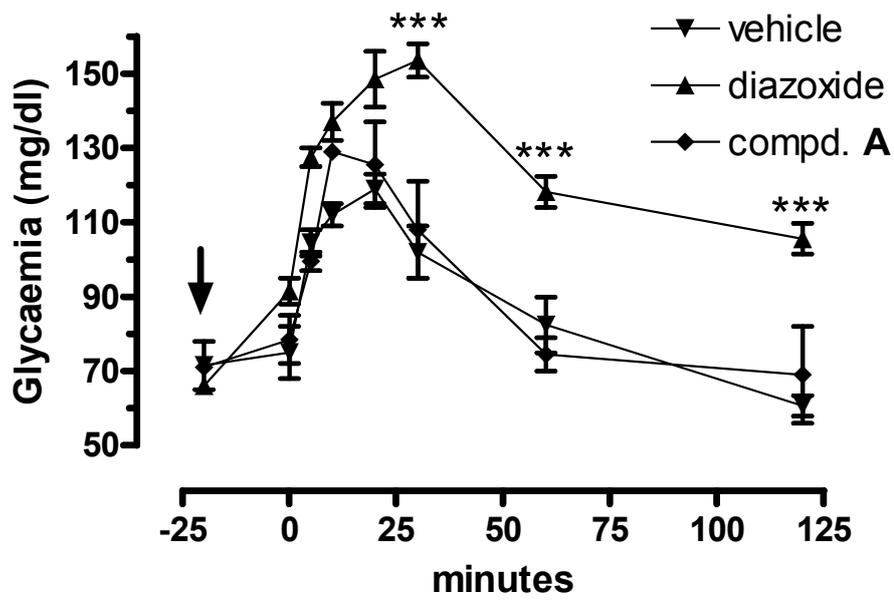


Figure 4

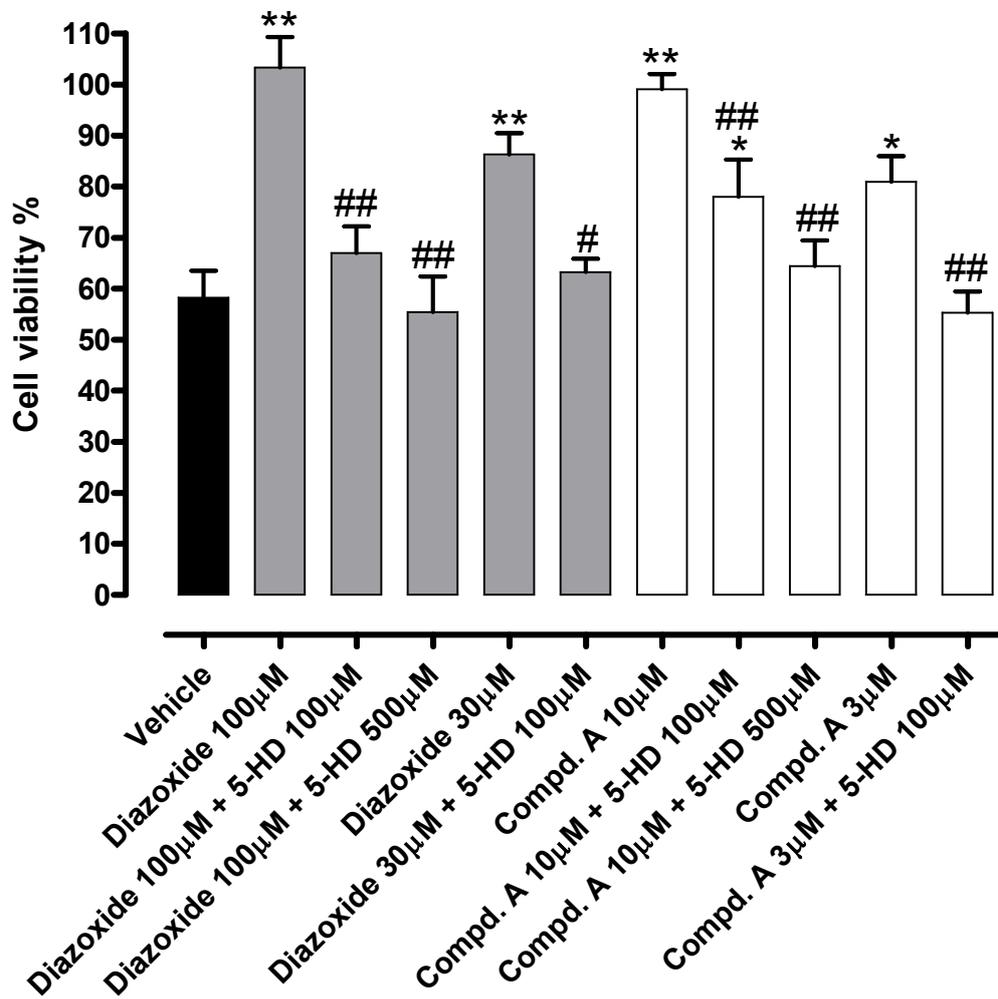


Figure 5

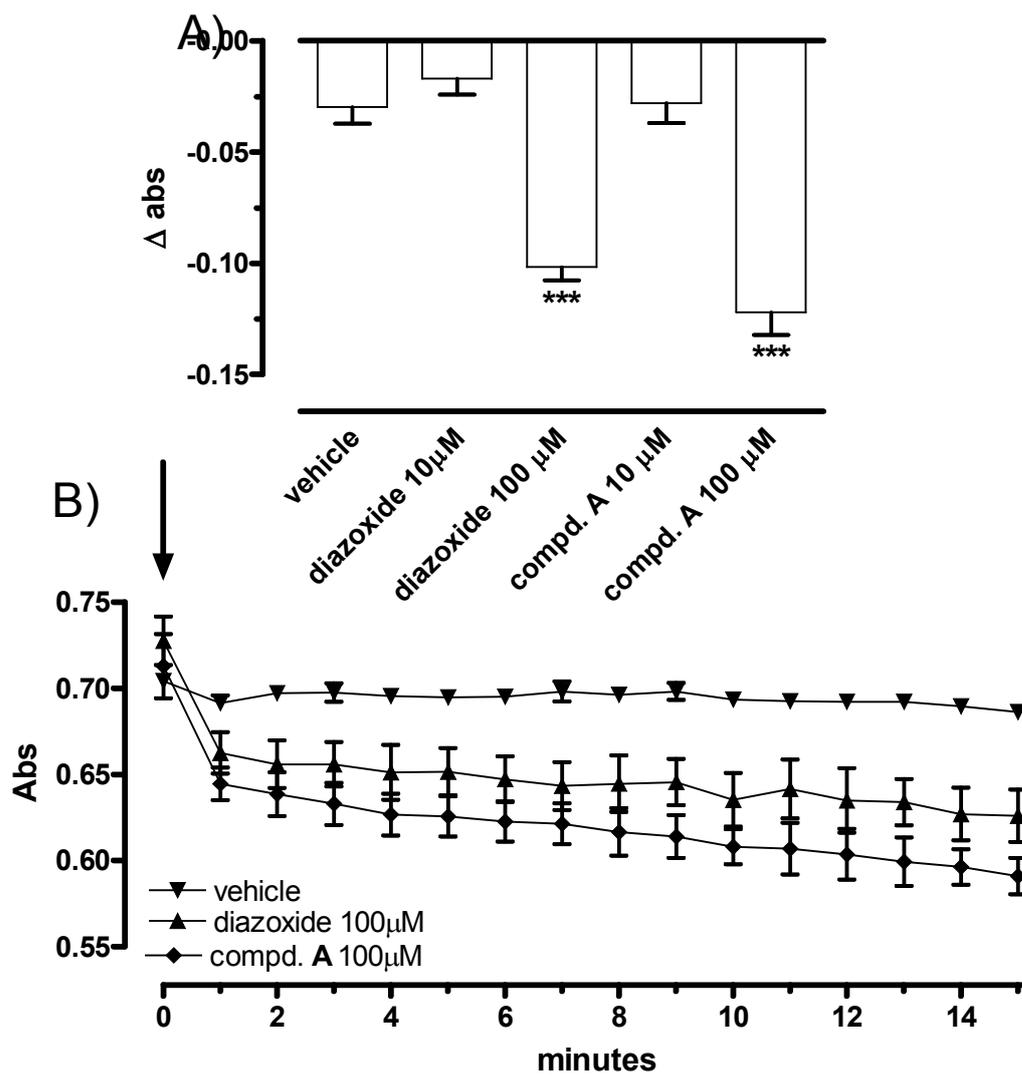


Figure 6

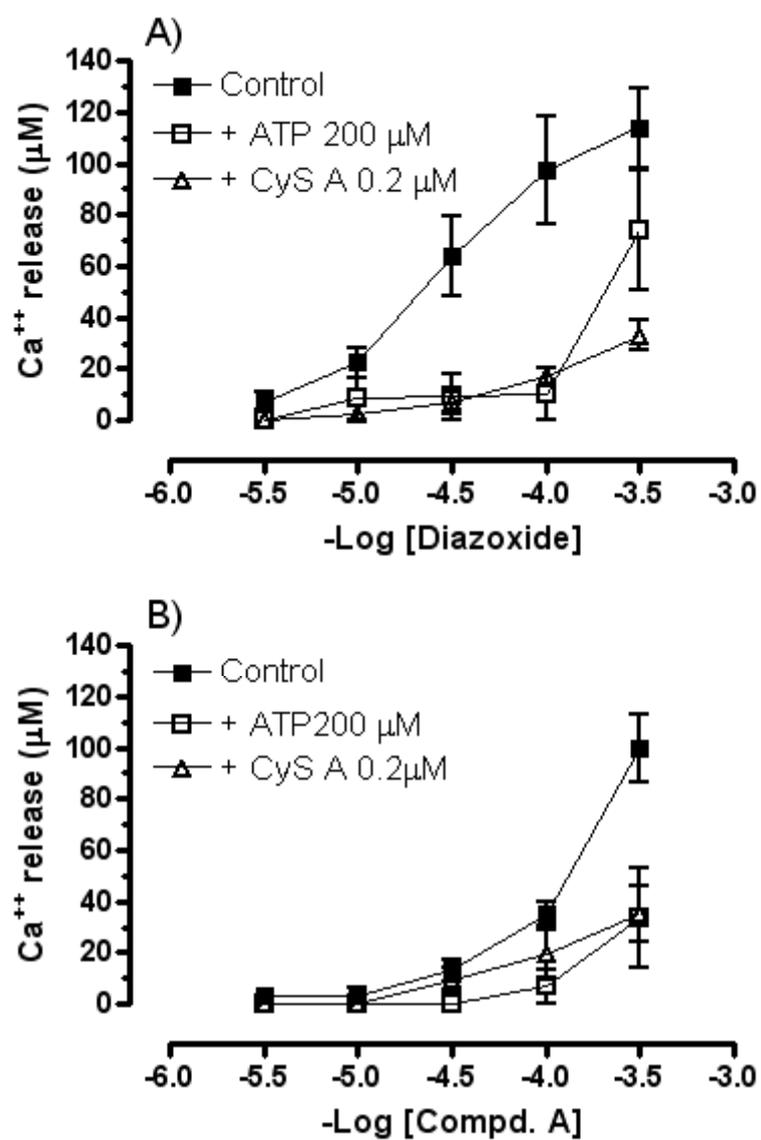


Figure 7

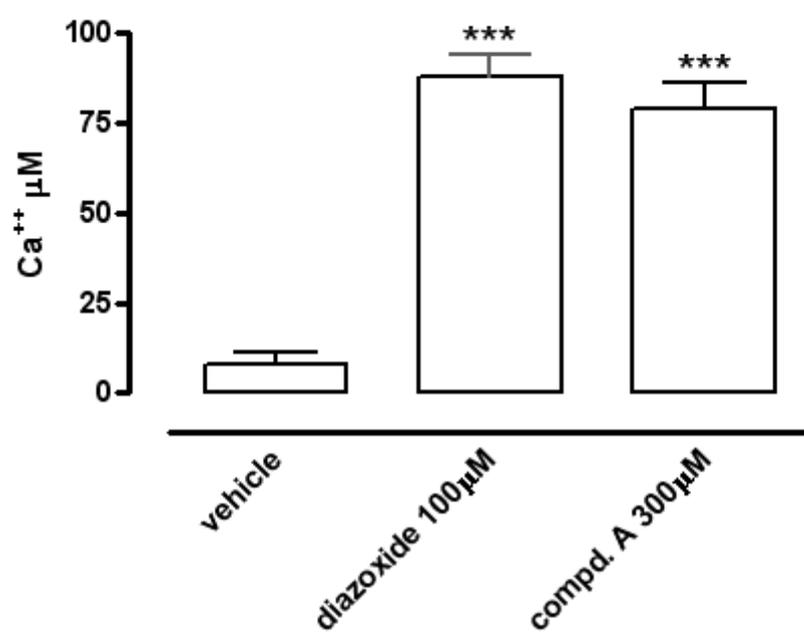


Figure 8

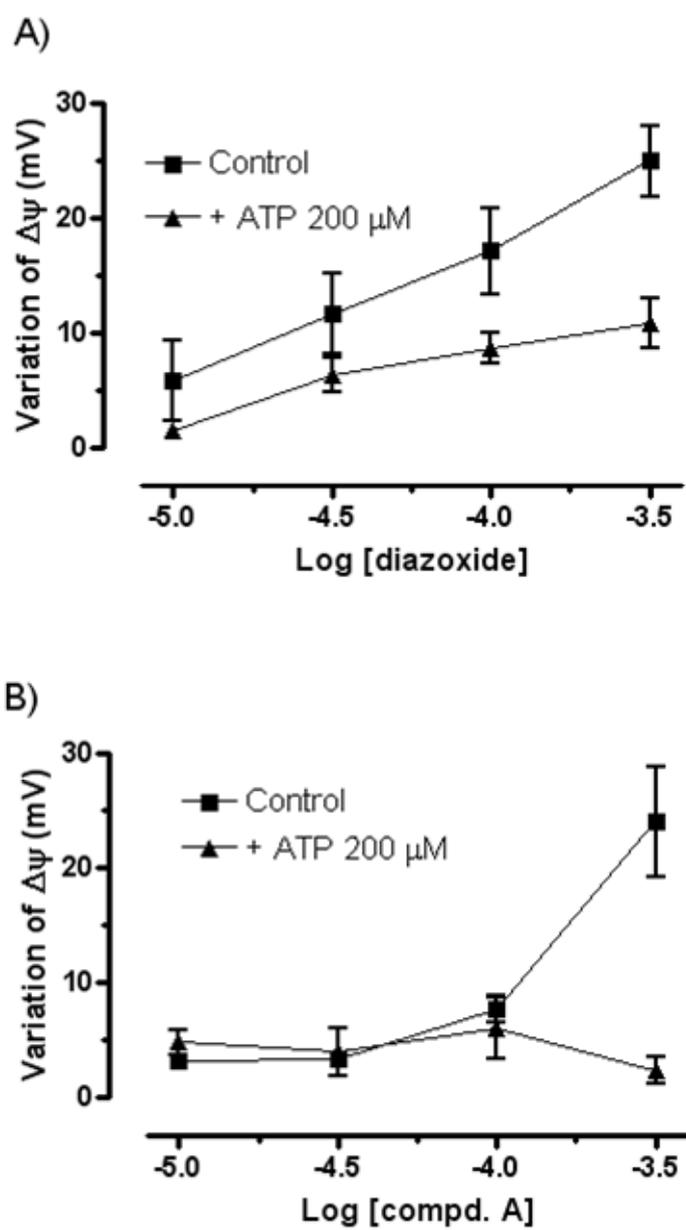


Figure 9

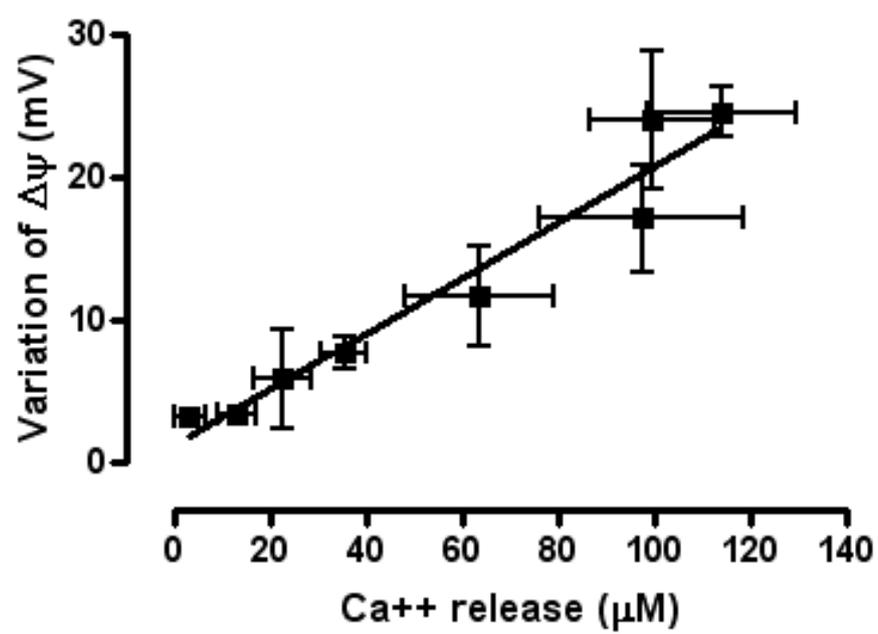


Figure 10