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# Impact of a novel cardioprotective agent on the ischaemia-reperfusion-induced Akt kinase activation

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**Abstract**

Cardioprotective effect of a free radical-scavenging compound (HO-3073) was examined during ischaemia-reperfusion (IR) in isolated heart perfusion system and its influence on the pro-survival Akt signalling pathway was addressed. Rat hearts were perfused according to the Langendorff method and subjected to a global 25-min ischaemia and 15, 45 and 90-min reperfusion either untreated or treated with HO-3073 (2, 5 and 10 μM) and/or wortmannin (100 nM, inhibitor of phosphatidylinositol-3-kinase). HO-3073 facilitated the recovery of myocardial energy metabolism as assessed by $^{31}$P NMR spectroscopy (creatine phosphate recovery in reperfusion was 76 ± 5%, while in untreated hearts 32 ± 4%). Functional performance of the hearts followed by a left ventricular balloon manometer was also markedly improved by HO-3073 administration (recovery of rate–pressure product related to normoxia was 47 ± 3%, while in untreated hearts 12 ± 3%). HO-3073 diminished the infarct size measured by TTC staining (29 ± 6% as opposed to 64 ± 7% in untreated ischaemia-reperfusion). HO-3073 also significantly attenuated lipid peroxidation (thiobarbituric acid reactive substances) and protein oxidation (protein carbonyl content) compared to untreated hearts. HO-3073 enhanced the ischaemia-reperfusion-triggered phosphorylation of Akt-1 (activation) and glycogen synthase kinase-3β (inactivation) as evidenced by Western blot analysis. Wortmannin co-administration neutralised the beneficial effects of HO-3073 on cardiac energetics, contractile function, infarct size, as well as Akt signalling. Our results first display that a radical-scavenging molecule possesses the ability to intensify the pro-survival functioning of phosphatidylinositol-3-kinase/Akt pathway, which is presumed to play an additive role in the cardioprotective properties of HO-3073.
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*Keywords:* Akt kinase; Cardioprotection; Free radicals; Energy metabolism; Ischaemia-reperfusion; Signal transduction

## 1. Introduction

Oxygen free radicals are extremely reactive species that are able to exert deleterious effects on cellular components, such as lipids, proteins, enzymes and DNA. Furthermore, numerous protein kinase cascades and inflammatory reactions have recently become established as part of any external stress-related tissue injury, such as heat, ischaemia-reoxygenation and other oxidative, metabolic, toxic, as well as infectious insults. Oxidative challenge of the myocardium influences among others the functioning of the mitogen-activated protein kinases (MAPKs), phospholipase C, protein kinase C (PKC), p53, ATM (ataxia-telangiectasia mutated) kinase, nuclear factor-κB and heat shock proteins [1–3]. Moreover, according to the literature, ischaemia-reperfusion in cardiomyocytes expedites the phosphorylation of the growth-factor-associated kinase Akt (also termed as protein kinase B) in a phosphatidylinositol-3-kinase (PI3-kinase)-dependent manner. Activation

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*Abbreviations:* ASK1, apoptosis signal-regulating kinase-1; eNOS, endothelial nitric oxide synthase; ERK, extracellular signal-regulated kinase; GSK-3β, glycogen synthase kinase-3β; IKKα, IκB kinase-α; IR, ischaemia-reperfusion; JNK, c-*jun* N-terminal kinase; LVDP, left ventricular developed pressure; MAPK, mitogen-activated protein kinase; PDK1, phosphatidylinositol-dependent kinase-1; PI3-kinase, phosphatidylinositol-3-kinase; ROS, reactive oxygen species; RPP, rate–pressure product; TBARS, thiobarbituric acid reactive substances; TTC, triphenyl tetrazolium chloride.

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of the PI3-kinase/Akt pathway occurs when growth factors (such as insulin, insulin-like growth factor, EGF, or PDGF) bind to receptor tyrosine kinases inducing their autophosphorylation. This pathway basically transmits mitogen signals towards its intracellular targets, but is also brought into connection with cell survival upon oxidative insults [4–7] and plays a definite role in cardioprotection during ischaemia-reperfusion injury in mice and rats [7–11]. Peroxynitrite can presumably ignite this signalling machinery under conditions of oxidative stress [1,10,11]. Downstream substrates of Akt kinase are abundant and comprise the pro-apoptotic protein Bad, caspase-9, the apoptotic Forkhead transcription factor, as well as glycogen synthase kinase-3 (GSK-3) [6]. Their phosphorylation leads to inactivation, thus apoptotic cell death is suppressed, while glycogen synthesis and cell cycle progression are initiated. On the other hand, Akt is able to activate p70 ribosomal S6 kinase, IKKα [12] and endothelial nitric oxide synthase (eNOS) [13,14].

Our previous findings and other investigators' data indicated that heterocyclic nitroxide precursor compounds containing a 2,2,5,5-tetramethylpyrroline ring were able to scavenge reactive oxygen species (ROS) and proved to be protective during ischaemia-reperfusion in an isolated heart perfusion system [15–17]. The amine moiety of these molecules is oxidised into nitroxide form both *in vivo* and *in vitro*, which is further transformed to hydroxylamine (this transformation is exemplified in Fig. 1). The cyclic nitroxide-hydroxylamine reaction enables the entrapping of superoxide anion, as well as hydroxyl radical in each cycle [16–20]. Scavenger molecules are capable of reducing the oxidative damage of myocardium, which is manifested in the attenuation of free radical-induced lipid peroxidation, protein oxidation, enzyme inactivation, as well as DNA break formation [15]. Our examined heterocyclic compound containing a six-membered ring (HO-3073) exerted cardioprotection in a fairly low concentration, i.e. in micromolar range. We hypothesised that this efficacy derived not exclusively from its ability to collect the majority of ROS, but there could be additional changes in the cellular homeostasis facilitating the more complete defence against the oxidative challenge.

We analysed the impact of HO-3073 on the cardiac pathophysiology under conditions of ischaemia-reperfusion in an isolated heart perfusion system, including the monitoring of myocardial energy metabolism, cardiac contractile function, as well as measuring the infarct size. In addition, we determined how this compound affected the indicative parameters of oxidative myocardial injury, i.e. lipid and protein damage. In order to reveal to a greater extent the molecular mechanisms of its action, we investigated the influence of HO-3073 on the PI3-kinase/Akt intracellular signal transduction pathway by immunoblot analysis.

## 2. Materials and methods

### 2.1. Chemicals

HO-3073 was synthesised at the Institute of Organic and Medicinal Chemistry and will be published elsewhere (molecular structures of HO-3073 and its metabolites are shown in Fig. 1). All other reagents were of the highest purity commercially available.

### 2.2. Myocardial pathophysiology

#### 2.2.1. Heart perfusion

Isolated hearts of adult male Wistar rats weighing 300–350 g were perfused according to the Langendorff method at a constant pressure of 70 mmHg and at 37° as described previously [21,22]. The investigation conformed with the *Guide for the Care and Use of Laboratory Animals* published by the US National Institutes of Health (NIH Publication No. 85-23, revised 1996), and was approved by the Animal Research Review Committee of the University of Pecs Medical School. Rats were anaesthetised with 200 mg/kg ketamine intraperitoneally and heparinised with sodium heparin (100 IU/rat i.p.). The perfusion medium was a modified phosphate-free Krebs–Henseleit buffer

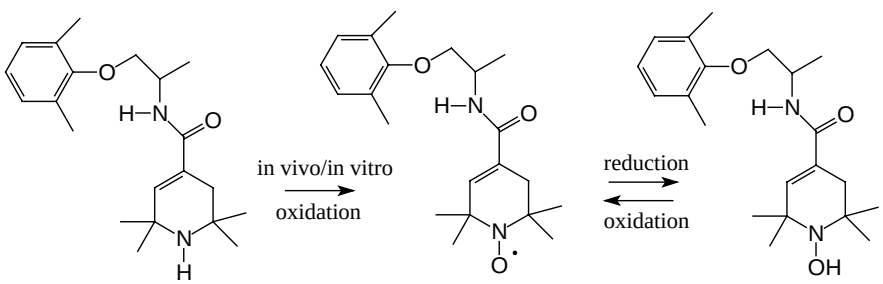


Fig. 1. Chemical structure and possible conversions of HO-3073. HO-3073: 2,2,6,6-tetramethyl-*N*-[1-methyl-2-(2,6-dimethylphenoxy)ethyl]-1,2,3,6-tetrahydropyridin-4-carboxamide.

consisting of 118 mM NaCl, 5 mM KCl, 1.25 mM $CaCl_2$, 1.2 mM $MgSO_4$, 25 mM $NaHCO_3$, 11 mM glucose and 0.6 mM octanoic acid and, in the treated group, plus HO-3073 (in 2, 5 and 10 μM) and/or wortmannin (100 nM or 1 μM) or LY 294002 (10 or 50 μM; commonly used non-specific inhibitors of PI3-kinase [23]). Octanoic acid as a middle-chain fatty acid not requiring the carnitine system for its mitochondrial transport was included in the perfusate because fatty acid oxidation is responsible for about 80% of the energy supply in normal beating hearts. The perfusate was adjusted to pH 7.40 and bubbled with 95% $O_2$ and 5% $CO_2$ through a glass oxygenator. After a washout, non-recirculating period of 15 min, hearts were perfused for 10 min (baseline) and either freeze-clamped to obtain "normoxic" hearts (by avoiding the ischaemic preconditioning of these hearts), or were then subjected to a 25-min global ischaemia by closing the aortic influx and reperfused for either 15, 45, or 90 min. HO-3073 and/or wortmannin were administered into the medium at the beginning of baseline perfusion and were present in the entire perfusion period (reaching the heart for 10 min before the ischaemia and 15, 45, or 90 min of reperfusion). During ischaemia, hearts were submerged into perfusion buffer at 37°. Hearts were freeze-clamped at the end of each perfusion.

#### 2.2.2. Myocardial energy metabolism

Nuclear Magnetic Resonance (NMR) spectroscopy was applied to monitor the levels of creatine phosphate, ATP and inorganic phosphate during the entire perfusion period. NMR spectra were recorded with a Varian $^{UNITY}$INOVA 400 WB instrument. $^{31}$P measurements (161.90 MHz) of perfused hearts were run at 37° in a Z●SPEC® 20 mm broadband probe, applying GARP-1 proton decoupling ($\gamma B_2 = 1.2$ kHz) during acquisition. Field homogeneity was adjusted by following the $^1$H signal ($w_{1/2} = 10$–15 Hz). Spectra were collected with a time resolution of 3 min by accumulating 120 transients in each free induction decay (FID). 45° flip angle pulses were employed after a 1.25-s recycle delay, and transients were acquired over a 10 kHz spectral width in 0.25 s, and the acquired data points (5000) were zero-filled to 16,000. Under the above circumstances, the relative concentrations of the species can be taken proportional to the peak areas, because interpulse delays provided sufficient amount of time for metabolites to be analysed for virtually full recovery of magnetisation. The pH value in the myocardium was calculated by the inorganic phosphate chemical shift from the creatine phosphate peak according to the following equation: $\mathrm{pH} = 6.77 + \log[(\delta - 3.23)/(5.70 - \delta)]$ [24].

#### 2.2.3. Heart function

A latex balloon was inserted into the left ventricle through the mitral valve and filled to achieve an end-diastolic pressure of 8–12 mmHg. All measurements were performed at the same balloon volume. Hearts were selected on the basis of the stability of high-energy phosphates during a control period of 15 min before the experiment (less than 10% fall in creatine phosphate level and no appreciable evolution of inorganic phosphate as assessed by $^{31}$P NMR; based on these criteria less than 10% of hearts were excluded considering all examined groups). The length of baseline perfusion, ischaemia and reperfusion were 10, 25 and 45 min, respectively. The experimental compounds (HO-3073 and/or wortmannin) were added to the perfusion medium after the 15-min control period. Left ventricular developed pressure (LVDP), rate–pressure product (RPP), heart rate (HR) and d$P$/d$t$ were monitored during the entire perfusion period by means of a Haemosys manometer device.

#### 2.2.4. Infarct size

For infarct size measurements 90-min post-ischaemic reperfusion was employed either untreated or treated with 5 μM HO-3073 and/or 100 nM wortmannin. After removing from the Langendorff perfusion apparatus, ventricles were cut out and kept overnight at −4°. Frozen ventricles were sliced into 2–3 mm thick sections, then incubated in 1% TTC at 37° in 0.2 M Tris buffer (pH 7.4) for 30 min. While the normal myocardium was stained brick red, the infarcted areas remained unstained. Size of the infarcted area was estimated by the volume and weight method [25].

### 2.3. Oxidative damage

#### 2.3.1. Lipid peroxidation

Formation of thiobarbituric acid reactive substances (TBARS) was measured to assess lipid peroxidation. Cardiac tissue (reperfused for 15 min) was homogenised in 6.5% trichloroacetic acid (TCA) and a reagent containing 15% TCA, 0.375% thiobarbituric acid and 0.25% HCl was added, mixed thoroughly, heated for 15 min in a boiling water bath, cooled, centrifuged and the extinction of the supernatant was measured at 535 nm against a blank that contained all the reagents except the tissue homogenate. Using malondialdehyde standard, TBARS were calculated as nmol/g wet tissue [26,27].

#### 2.3.2. Protein oxidation

Fifty milligrams of freeze-clamped perfused heart tissue (reperfused for 15 min) was homogenised with 1 mL 4% perchloric acid and the protein content was collected by centrifugation. The protein carbonyl content was determined by means of the 2,4-dinitrophenylhydrazine method [28].

### 2.4. Intracellular signalling

#### 2.4.1. Western blot analysis

Fifty milligrams of heart samples were homogenised in ice-cold Tris buffer (50 mM, pH 8.0) and harvested in two

times concentrated SDS–polyacrylamide gel electrophoretic sample buffer. Proteins were separated on 12% SDS–polyacrylamide gel and transferred to nitrocellulose membranes. After 2-hr blocking (with 3% non-fat milk in Tris-buffered saline), membranes were probed overnight at 4° with antibodies against phospho-specific Akt-1/protein kinase B-α $Ser^{473}$ (1:1000 dilution), non-phosphorylated C-terminal domain of Akt/PKB (1:1000) and phospho-specific GSK-3β $Ser^{9}$ (1:1000). Those amounts of protein samples were employed that contained equal amount of non-phosphorylated Akt/PKB, which allowed the assessment of differences in the phosphorylation states of Akt-1 and GSK-3β. Membranes were washed six times for 5 min in Tris-buffered saline (pH 7.5) containing 0.2% Tween (TBST) prior to addition of goat anti-rabbit horseradish peroxidase-conjugated secondary antibody (1:3000 dilution). Membranes were washed six times for 5 min in TBST and the antibody–antigen complexes were visualised by means of enhanced chemiluminescence on conventional films. After scanning, results were quantified by means of Scion Image Beta 4.02 program.

### 2.5. Statistical analysis

Statistical analysis was performed by analysis of variance and all of the data were expressed as the mean ± SEM. Significant differences were evaluated by use of unpaired Student's *t* test and *P* values below 0.05 were considered to be significant.

## 3. Results

### 3.1. Energy metabolism

$^{31}$P NMR spectroscopic measurement of high-energy phosphate intermediates revealed that ischaemia induced the rapid reduction of creatine phosphate and ATP levels and the fast evolution of inorganic phosphate. Under our experimental conditions, although high-energy phosphates recovered only partially in untreated hearts, HO-3073 promoted the re-establishment of creatine phosphate and ATP levels in each applied concentration (2, 5, 10 μM). As Fig. 2 demonstrates, the maximal post-ischaemic recovery of creatine phosphate in HO-3073-treated hearts (5 μM) significantly surpassed that of untreated and wortmannin-treated hearts ($P < 0.01$). In case of co-administration, wortmannin completely neutralised the protective impact of HO-3073 (Fig. 2A). The time course of ATP followed a similar pattern to that of creatine phosphate (maximal post-ischaemic recovery after 9-min reperfusion was 22 ± 3%, 21 ± 3%, 47 ± 4% and 25 ± 4% of the baseline values for untreated ischaemia-reperfusion, wortmannin-treated, HO-3073-treated, and HO-3073 plus wortmannin-treated hearts, respectively; ATP recovery of HO-3073-treated hearts significantly differed from that of the other three conditions, $P < 0.01$). HO-3073 also brought about the faster and more complete re-utilisation of inorganic phosphate during reperfusion compared to the untreated ischaemia-reperfusion, wortmannin-treated, and HO-3073 plus wortmannin-treated hearts ($P < 0.01$) (Fig. 2B). The intracellular pH fell rapidly during ischaemia from 7.43 ± 0.04 to 5.85 ± 0.05. In untreated hearts during reperfusion a limited, but statistically significant ($P < 0.05$) increase could be observed to 5.99 ± 0.04. In the presence of HO-3073 the myocardial pH markedly rose up to 6.72 ± 0.08 ($P < 0.01$). In case of administration of wortmannin alone or along with HO-3073, the calculated values of intracellular pH during reperfusion were not significantly different from that of untreated hearts.

### 3.2. Cardiac function

At the end of the equilibration period, LVDP was 135 ± 12 mmHg, RPP was 3.5 ± 0.11 × $10^4$ mmHg/min, d*P*/d*t* was 1292 ± 182 mmHg/s and the average heart rate was 245 ± 15 beats/min. Figure 3 shows the evolution of LVDP and RPP during reperfusion as a percentage of the initial values. The recovery of both parameters proved to be significantly better in case of HO-3073 administration compared to untreated, wortmannin-treated, and HO-3073 plus wortmannin-treated hearts ($P < 0.01$). The recovery of d*P*/d*t*, expressed as a percentage of the corresponding pre-ischaemic value, was 14.1 ± 2.8% for untreated and 58.3 ± 5.1% for HO-3073-treated hearts ($P < 0.01$). The post-ischaemic recovery of d*P*/d*t* in wortmannin-treated and HO-3073 plus wortmannin-treated hearts did not significantly differed from that of untreated ischaemia-reperfusion hearts.

### 3.3. Infarct size

TTC staining in five consecutive samples demonstrated that the ischaemia followed by 90-min reperfusion in untreated cases brought about the infarction of 64 ± 7% of the ventricles. In the meantime, HO-3073 administration significantly reduced the infarct size to 29 ± 6% of the heart samples ($P < 0.01$). The PI3-kinase inhibitor wortmannin did not alter the size of infarcted area when administered alone, on the other hand, it abrogated the beneficial influence of HO-3073 on infarct size in the case of co-treatment. Control staining of ''normoxic'' hearts perfused for 10 min (baseline) rendered no appreciable infarcted area.

### 3.4. Lipid peroxidation

Under our experimental conditions, ischaemia-reperfusion increased the amount of TBARS compared to the normoxic hearts ($P < 0.01$) (Table 1). When ischaemia-reperfusion occurred in the presence of 2, 5 and 10 μM

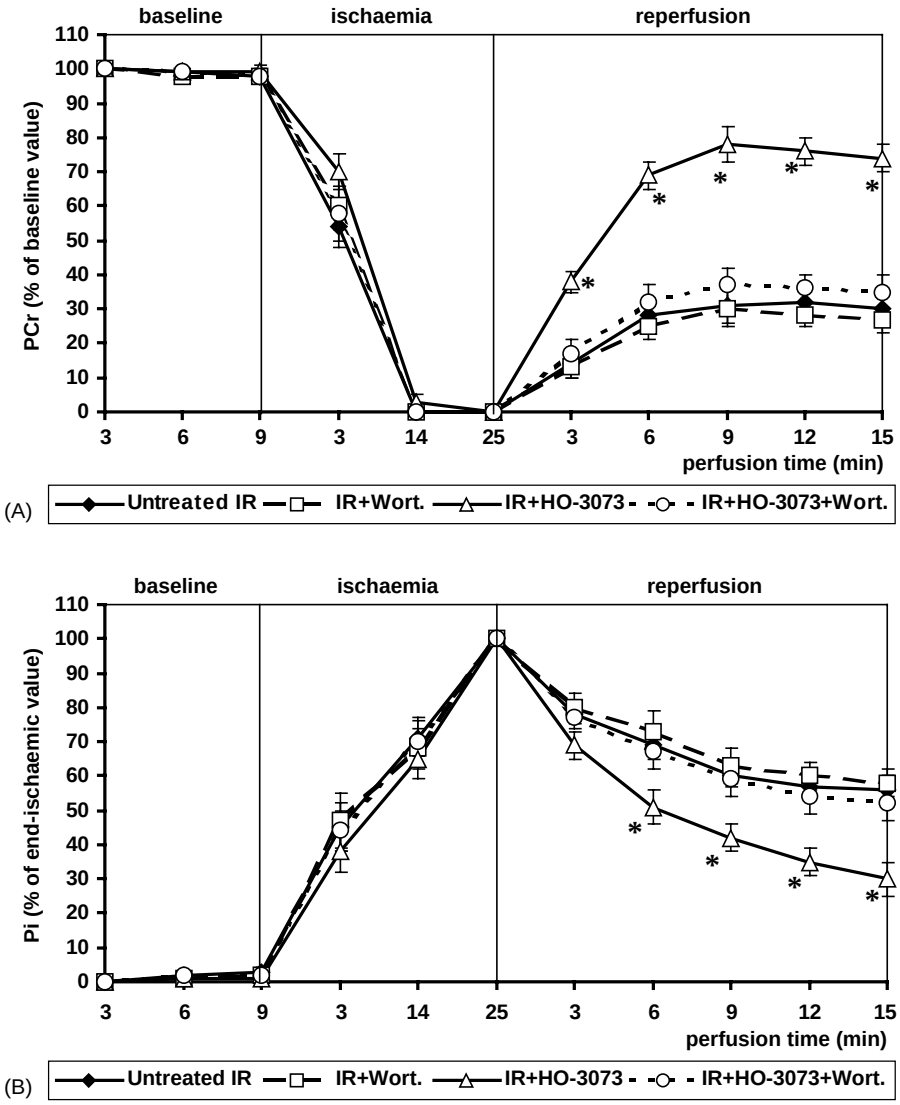


Fig. 2. Time course of creatine phosphate (A) and inorganic phosphate (B) levels during baseline perfusion, ischaemia and reperfusion in Langendorff perfused hearts. Conditions for heart perfusion and NMR measurements were described under "Section 2". IR + Wort.: ischaemia-reperfusion in the presence of 100 nM wortmannin; IR + HO-3073: ischaemia-reperfusion in the presence of 5 μM HO-3073; IR + HO-3073 + Wort.: ischaemia-reperfusion in the presence of 5 μM HO-3073 plus 100 nM wortmannin. Values are given as mean ± SEM for five experiments. *The marked levels of creatine phosphate, as well as inorganic phosphate, in HO-3073-treated hearts were significantly different from the remaining conditions ($P < 0.01$).

Table 1
Effect of HO-3073 on ischaemia-reperfusion-induced lipid peroxidation and protein oxidation in Langendorff perfused hearts

| | TBARS (nmol/g wet tissue) | Protein carbonyl content (μmol/g wet tissue) |
|---|---|---|
| "Normoxic" hearts | 39 ± 3.1 | 1.12 ± 0.14 |
| Ischaemia-reperfusion | 73 ± 5.4[a] | 2.51 ± 0.11[a] |
| IR + HO-3073 (2 μM) | 52 ± 4.2[b] | 1.72 ± 0.10[b] |
| IR + HO-3073 (5 μM) | 41 ± 2.7[b] | 1.43 ± 0.12[b] |
| IR + HO-3073 (10 μM) | 40 ± 2.5[b] | 1.38 ± 0.08[b] |

Conditions for heart perfusion and lipid peroxidation (TBARS) and protein oxidation (protein carbonyl content) measurements were detailed under "Section 2". Values are given as means ± SEM for five experiments.
[a] Difference from "normoxic" heart samples ($P < 0.01$).
[b] Difference from untreated ischaemia-reperfusion values ($P < 0.01$).

HO-3073, the formation of TBARS was significantly diminished ($P < 0.01$) compared to the untreated hearts in a concentration-dependent manner (Table 1), indicating that HO-3073 prevented the ischaemia-reperfusion-induced lipid peroxidation.

### 3.5. Protein oxidation

ROS formation in ischaemia-reperfusion cycle can also trigger the oxidation of proteins in cardiomyocytes, which can be characterised by the quantity of protein-bound aldehyde groups. Table 1 shows that ischaemia-reperfusion markedly elevated the level of protein oxidation, which was significantly restricted ($P < 0.01$) in the presence of HO-3073 during ischaemia-reperfusion.

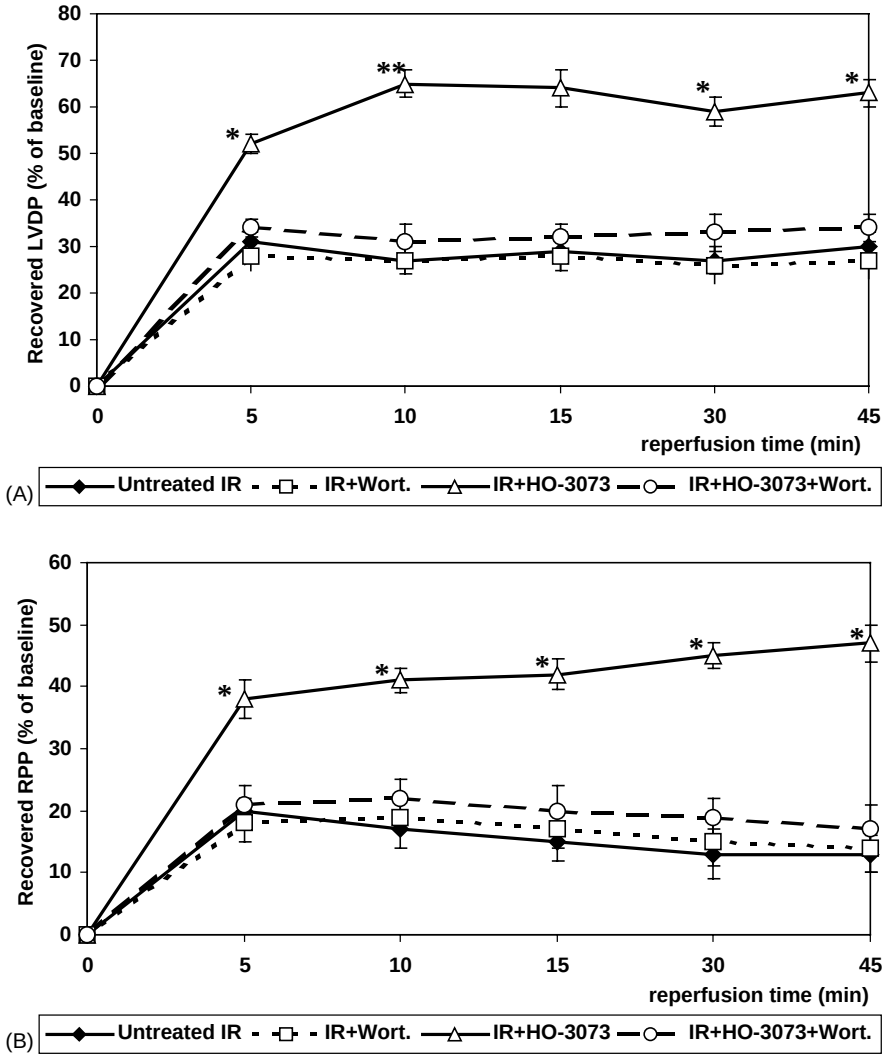


Fig. 3. Recovery of left ventricular developed pressure (LVDP) (A) and rate–pressure product (RPP) (B) during the 45-min post-ischaemic reperfusion as a percentage of the baseline values. IR + Wort.: ischaemia-reperfusion in the presence of 100 nM wortmannin; IR + HO-3073: ischaemia-reperfusion in the presence of 5 μM HO-3073; IR + HO-3073 + Wort.: ischaemia-reperfusion in the presence of 5 μM HO-3073 plus 100 nM wortmannin. Values are given as mean ± SEM for five experiments. *Recovery of HO-3073-treated hearts was significantly higher than under the remaining conditions for both LVDP and RPP ($P < 0.01$).

### 3.6. Phosphorylation state of Akt-1 and GSK-3β

Ischaemia-reperfusion triggered the moderate phosphorylation of Akt-1, which was not influenced by the sole administration of wortmannin. HO-3073 markedly enhanced the phosphorylation of Akt-1, which was hindered by the parallel application of wortmannin (Fig. 4). The similar phosphorylation pattern could be observed at the level of GSK-3β, the downstream substrate of Akt, i.e. wortmannin treatment alone did not affect the ischaemia-reperfusion-induced phosphorylation, but prevented the increased phosphorylation on HO-3073 administration (Fig. 4). Interestingly, HO-3073 also brought about Akt, as well as GSK-3β phosphorylation, when given only for 10 min in baseline perfusion prior to freeze-clamping, a phenomenon not present in untreated baseline (“normoxic”) heart samples (Fig. 4). While wortmannin alone had no impact on the phosphorylation state of either of the kinases during the aforementioned baseline perfusion (data not shown), it did prevent the phosphorylation when was co-administered with HO-3073 (Fig. 4). Administration of 10 μM LY 294002 had similar impact on the phosphorylation state of both kinases in each experimental setting (data not shown).

Since we were unable to completely block Akt, as well as GSK-3β phosphorylation, with 100 nM wortmannin or 10 μM LY 294002, we employed higher concentrations of both inhibitors (1 μM wortmannin and 50 μM LY 294002 as indicated in Ref. [23]). Figure 5 depicts that these higher concentrations still proved to be insufficient for the total blockade of Akt kinase pathway both with and without parallel HO-3073 treatment. These inhibitor concentrations had the same effect on cardiac energy metabolism as it was in the case of using the

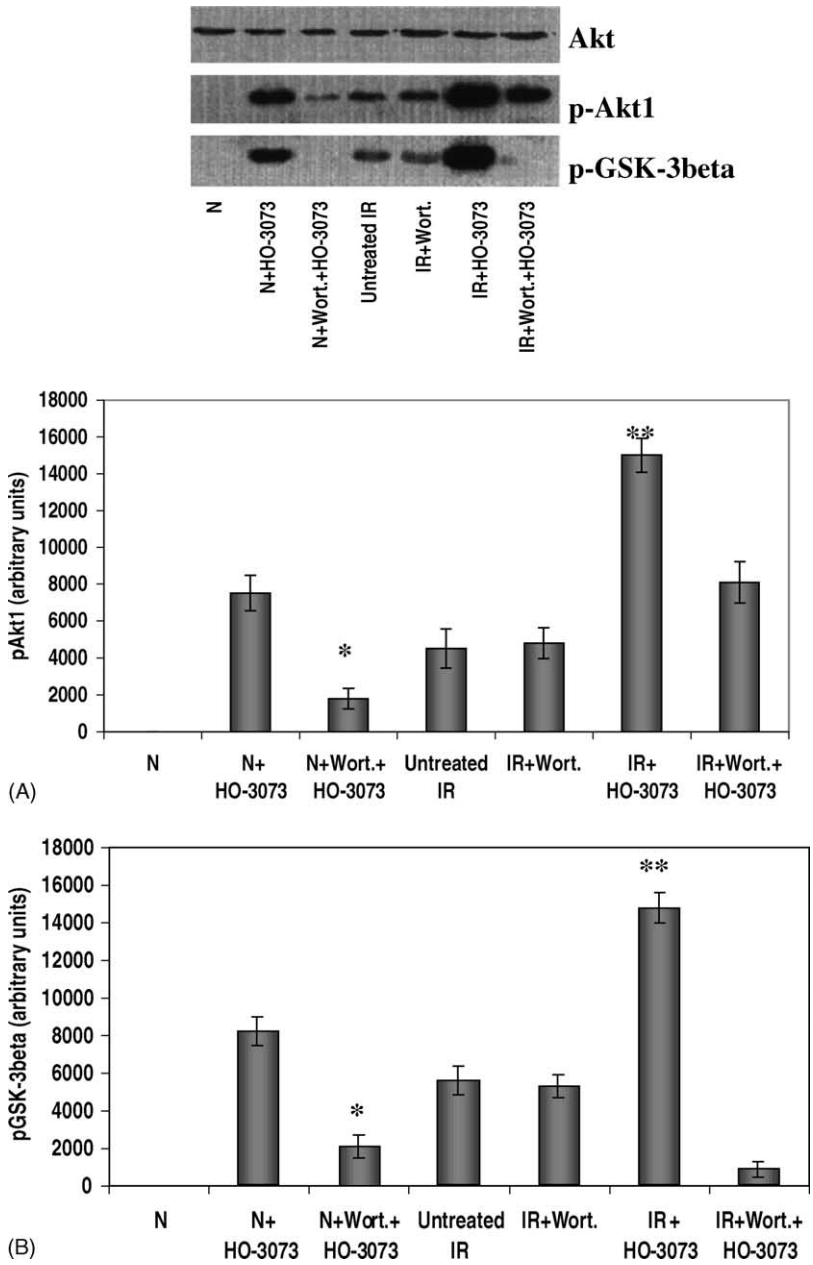


Fig. 4. Effect of HO-3073 and wortmannin on the phosphorylation state of Akt-1, as well as GSK-3β. Representative immunoblots from three experiments with similar results and densitometric evaluations are shown. Akt: non-phosphorylated Akt; p-Akt1: Akt-1 phosphorylated on $Ser^{473}$; p-GSK-3beta: glycogen synthase kinase-3β phosphorylated on $Ser^{9}$. N: ''normoxia'', i.e. baseline perfusion for 10 min; N + HO-3073: baseline perfusion for 10 min in the presence of 5 μM HO-3073; N + Wort. + HO-3073: baseline perfusion for 10 min in the presence of 100 nM wortmannin and 5 μM HO-3073; Untreated IR: ischaemia-reperfusion in the absence of any agent; IR + Wort.: ischaemia-reperfusion in the presence of 100 nM wortmannin; IR + HO-3073: ischaemia-reperfusion in the presence of 5 μM HO-3073; IR + Wort. + HO-3073: ischaemia-reperfusion in the presence of 100 nM wortmannin and 5 μM HO-3073. *Difference of N + Wort. + HO-3073 hearts from N + HO-3073 samples ( $P < 0.01$). **Difference of IR + HO-3073 hearts from the remaining samples (except for N) ($P < 0.01$).

above discussed lower amounts of the agents (data not shown).

## 4. Discussion

Hereby, we demonstrated an interesting aspect of the mode of action of a cardioprotective molecule capable of entrapping free radicals. HO-3073 was able not only to promote the recovery of myocardial energy metabolism and contractile function, mitigate the infarct size and cardiac oxidative damage during post-ischaemic reperfusion in a fairly low concentration (2–10 μM), but also beneficially influenced the intracellular Akt signalling route.

Treatment with HO-3073 furthered the preservation of high-energy phosphate intermediates during reperfusion as evidenced by $^{31}P$ NMR spectroscopic studies, as well as substantially attenuated the extent of cellular oxidative

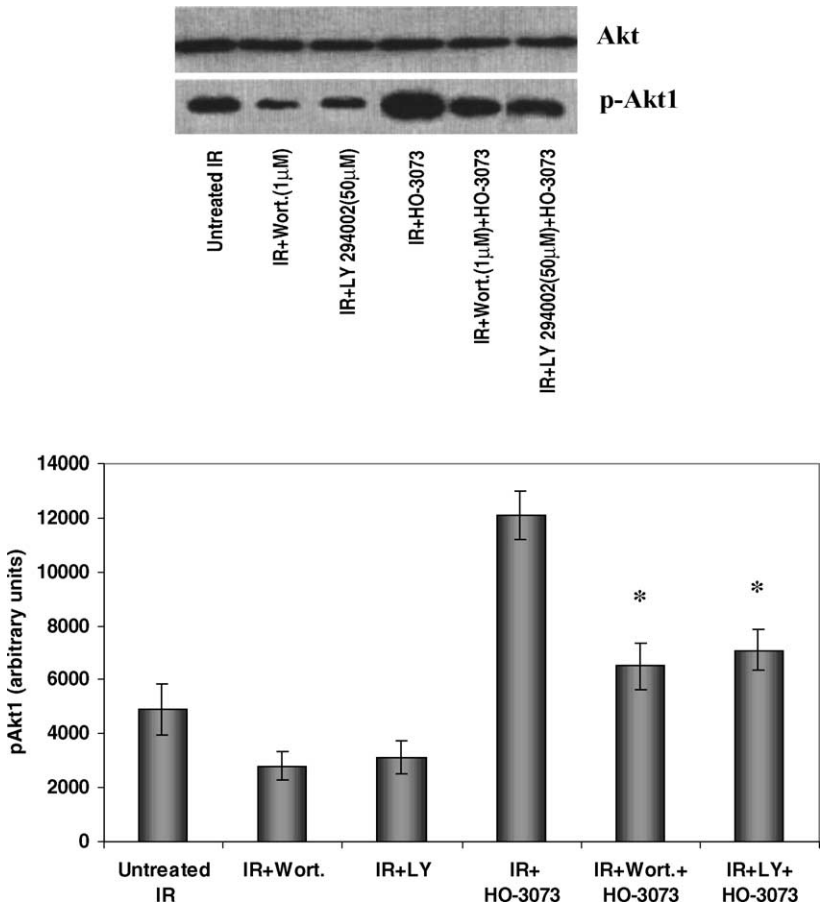


Fig. 5. Effect of 1 μM wortmannin and 50 μM LY 294002 on the ischaemia-reperfusion- and HO-3073-induced Akt-1 phosphorylation. Representative immunoblots from three experiments with similar results and densitometric evaluations are shown. Akt: non-phosphorylated Akt; p-Akt1: Akt-1 phosphorylated on $Ser^{473}$. Untreated IR: ischaemia-reperfusion in the absence of any agent; IR + Wort. (1 μM): ischaemia-reperfusion in the presence of 1 μM wortmannin; IR + LY 294002 (50 μM): ischaemia-reperfusion in the presence of 50 μM LY 294002; IR + HO-3073: ischaemia-reperfusion in the presence of 5 μM HO-3073; IR + Wort. (1 μM) + HO-3073: ischaemia-reperfusion in the presence of 1 μM wortmannin and 5 μM HO-3073; IR + LY 294002 (50 μM) + HO-3073: ischaemia-reperfusion in the presence of 50 μM LY 294002 and 5 μM HO-3073. *Difference from IR + HO-3073 samples ($P < 0.01$).

injury as displayed by decreased lipid peroxidation and protein oxidation. The apparent improvement in creatine phosphate and ATP levels during reperfusion was supported by the rapid and more complete consumption of inorganic phosphate. Re-utilisation of the latter is of crucial importance because elevated concentrations of inorganic phosphate and calcium together with ROS are the most potent triggers of mitochondrial permeability transition. This process leads to the permeabilisation of the outer and inner mitochondrial membranes and the subsequent release of cytochrome *c* into the cytoplasm, which launches the apoptotic cell death cascade [29,30].

Corresponding to the observed restoration of cardiac energetics, the functional performance of HO-3073-treated hearts also exhibited significant improvement. This was obvious in the post-ischaemic evolution of both LVDP and RPP, timely following the recovery of high-energy phosphates. An additional index of cardiac integrity was favourably affected by HO-3073 administration, as well. The extent of infarcted myocardial tissue diminished in case HO-3073 was used during ischaemia-reperfusion. The investigation of these three aspects of myocardial functioning convincingly supported the remarkable cardioprotective properties of the examined mexiletine derivative, which data are in accordance with the findings of other studies employing structurally resembling radical-scavenging molecules [15–17]. This notion and the rather low effective concentration of HO-3073 turned our attention toward the stress-related pro-survival intracellular signal transmission pathways.

Autophosphorylation of receptor tyrosine residues of the PI3-kinase enzyme is induced by growth factors and oxidative stress. Substrate of PI3-kinase (phosphatidylinositol-3,4,5-triphosphate) recruits Akt (also known as protein kinase B) to the membrane and elicits its phosphorylation by PDK1 and another, yet unknown kinase [31,32]. Under conditions of ischaemia and reperfusion, nitric oxide and superoxide anion can form peroxynitrite, which features as a ''ligand'' of receptor tyrosine kinases and leads to the nitration of tyrosine residues eventually igniting the above-mentioned machinery [10,11]. Akt targets a wide variety of substrates by phosphorylation: inactivates the pro-apoptotic

Bcl-2 family member Bad protein, the apoptotic effector enzyme caspase-9 (upstream protease of caspase-3) and Forkhead transcription factor; this may result in reduced apoptosis [10,31]. In addition, p70 ribosomal S6 kinase is activated promoting mRNA translation and cell cycle progression, as well as the phosphorylation of Bad [8]. Akt also induces eNOS activity [13,14], as well as IKKα, the upstream inductor for the nuclear translocation of nuclear factor-κB [12], but blocks glycogen synthase kinase [6,31].

In our current study, the administration of the scavenger compound HO-3073 during ischaemia-reperfusion was associated with more explicit Akt-1 (activation) and GSK-3β phosphorylation (inactivation) than those observed in untreated ischaemia-reperfusion cases. Decreased GSK-3β activity can augment glycogen synthesis (through glycogen synthase), and relieve the inhibition on eukaryotic initiation factor 2b and cyclin $D_1$ favouring cell cycle progression [6,33]. Precise effectors of this HO-3073-related Akt activation, yet, remain to be elucidated.

Nevertheless, the known PI3-kinase inhibitor wortmannin [23] interfered with the myocardial energy metabolism-protecting effects of HO-3073, prevented the restoration of post-ischaemic cardiac contractile function by HO-3073, and also extended the size of infarcted myocardium that was markedly shrunk by the application of HO-3073. These pathophysiological findings were accompanied by the corresponding changes in the phosphorylation of Akt-1 and GSK-3β, i.e. wortmannin could neutralise the Akt-1-activating impact of HO-3073. The cardioprotective compound also brought about Akt-1 and GSK-3β phosphorylation during a 10-min baseline (''normoxic'') perfusion, which was inhibited by wortmannin, strengthening our belief in the capability of HO-3073 to somehow influence the PI3-kinase/Akt signalling.

In the meantime, wortmannin was not able to completely block the ischaemia-reperfusion- as well as HO-3073-induced Akt and GSK-3β phosphorylation. This may be attributed to the eventual restricted delivery of wortmannin into all areas of myocardium and its known limited specificity to PI3-kinase. In order to address this latter notion, the perfusion and immunoblot experiments were repeated in the presence of higher concentration of wortmannin (1 μM), as well as another commonly used PI3-kinase inhibitor LY 294002 (applied in 10 and 50 μM), but they still could not totally repress the phosphorylation of the two kinases under conditions of ischaemia-reperfusion. This context of results may raise another possible explanation for Akt phosphorylation, namely, Akt can be activated partially by a yet undetermined PI3-kinase-independent manner in ischaemia-reperfusion cycle, which is apparently not hindered by PI3-kinase inhibitors.

Insulin treatment-induced Akt and eNOS-mediated low concentrations of nitric oxide have been reported to exhibit anti-apoptotic impacts by nitrosating caspases 3, 6, 7 and 8, inhibiting caspase-dependent Bcl-2 cleavage and eventually downregulating MAPK phosphatase-3 mRNA levels resulting in prolonged phosphorylation of ERK, another pro-survival factor, or by NO inhibition of neutrophil infiltration [9,14,34,35]. Akt also influences glucose uptake by recruiting GLUT-4 to the cell membrane, which propagates the more favourable bioenergetics of glycolytic metabolism [9]. PI3-kinase can also regulate PKC during ischaemic preconditioning, namely through initiating its phosphorylation by PDK1 and PI3-kinase lipid products, which altogether will potentiate the allosteric regulation of PKC by diacylglycerol and NO (formed by eNOS) giving rise to the remarkable finding called second window of protection [36]. In addition, phosphorylation by Akt was also shown to negatively regulate apoptosis signal-regulating kinase-1 (ASK1). ASK1 is believed to be the mediator of oxygen free radical-associated activation of c-*jun* N-terminal kinase (JNK) and p38-MAPK. JNK and p38-MAPK can precipitate apoptosis by mitochondria-dependent caspase activation [37–39]. As a consequence, Akt may also suppress apoptosis related to JNK and p38-MAPK activation through the inhibition of ASK1 [40].

Our data provide the first insight into how a free radical-scavenging cardioprotective sterically hindered amine may interfere with the intracellular signal transduction pathways. HO-3073 preserved cardiac energy metabolism and contractile function during ischaemia-reperfusion and, additionally, attenuated the oxidative injury and the infarct size of the myocardium. Moreover, HO-3073 administration facilitated the activation of PI3-kinase/Akt pro-survival signalling route in a wortmannin-dependent manner. Wortmannin co-administration also neutralised the beneficial effects of HO-3073 on cardiac energetics, contractile function, as well as infarct size. Taken together, we propose that the protective impacts of this agent are not only attributed to its scavenger properties, but may also be related to its ability to upregulate the Akt kinase pathway.

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