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Discovery of 6-[4-(6-nitroxyhexanoyl)piperazin-1-yl)]-9*H*-purine, as pharmacological post-conditioning agent

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ABSTRACT

Novel purine analogues bearing nitrate esters were designed and synthesized in an effort to develop compounds triggering endogenous cardioprotective mechanisms such as ischemic preconditioning (IPC) or postconditioning (PostC). The majority of the compounds reduced infarct size compared to the control group in anesthetized rabbits, whereas administration of the most active analogue **16** at a dose of 3.8 μmol/kg resulted on a significant reduction of infarct size, compared to PostC group (13.4 ± 1.9% vs 26.4 ± 2.3%). These findings introduce a novel class of promising pharmacological compounds that could be used as mimics or enhancers of PostC.

1. Introduction

Acute myocardial infarction (AMI) is a serious and often fatal consequence of coronary artery disease (CAD). Although restoration of blood flow to the jeopardized myocardium is a prerequisite for myocardial salvage, reperfusion itself may lead to additional tissue injury, known as reperfusion injury.[1]

To solve this problem and to improve clinical outcome and prevention of the lethal reperfusion injury, an intervention is needed that would make the heart muscle resistant to necrosis. During the last two decades, scientific efforts focus on improving means for limiting infarct size. However, the morbidity and mortality of CAD remain significant worldwide and lay the ground for the development of novel cardioprotective therapies.

Protection of the ischemic myocardium is known to occur as a result of ischemic preconditioning (IPC), in which repetitive brief periods of ischemia protect the heart from a subsequent prolong ischemic insult.[2] Although IPC is a powerful form of protection, it is of limited clinical application for obvious ethical and practical reasons.[3] Another endogenous form of cardioprotection, similar to IPC but applicable at the time of reperfusion, termed postconditioning (PostC)[4] has been recently described. Short series of repetitive cycles of brief reperfusion and re-occlusion of the coronary artery applied at the onset of reperfusion, reduce the infarct size and coronary artery endothelial dysfunction.

Major signalling pathways and mediators involved in IPC and PostC are: ATP-sensitive potassium mitochondrial channels (mito-K_{ATP}), nitric oxide (NO), cyclic GMP (cGMP), activation of specific RISK kinases (Reperfusion Injury Salvage Kinases) such as PI3K, Akt, and ERKs, inhibition of GSK-3β, which preserve the mitochondrial function by preventing the mitochondrial permeability transition pore (mPTP) opening which is considered the end point of cardioprotection.[5]

Since K_{ATP} channels, nitric oxide (NO) and adenosine receptor activation are involved in IPC, pharmacological interventions, which have only recently been identified, involve nicorandil and adenosine derivatives. A variety of known compounds such as adenosine A_{2B}-selective agonists, cyclosporin, volatile anesthetics, natriuretic peptides, applied in the early minutes of reperfusion, seem to trigger PostC.[3b,5h,6]

However, no accepted adjunctive drug or therapy to acutely further limit myocardial infarct size above and beyond reperfusion exists. Thus, there is an unmet need for a safe and effective adjunctive therapy, which can be administered together with reperfusion, to further reduce the size of a myocardial infarction and improve clinical outcomes such as reducing mortality and decreasing heart failure.

Our group has been involved in the design and synthesis of novel molecules, which may confer their protection by using intracellular pathways involved in IPC. Our preliminary in vivo data in an

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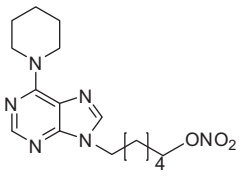


Figure 1. Compound **A**.

experimental model of ischemia/reperfusion in rabbits showed that the presence of a purine moiety and of a nitrate ester (NO donor) is able to confer cardioprotection by means of infarct size reduction, when the compounds were administered before ischemia at very low doses (3.8 μmol/kg). Among the tested compounds, we identified a 6-piperidinyl purine derivative bearing a nitroxyhexyl chain at position 9, compound **A** (Fig. 1) as a pharmacological tool able to induce IPC in vivo.[7]

The aim of the present study was (i) the design and synthesis of analogues of the lead compound **A**, (ii) evaluation of the new derivatives as pharmacological IPC agents and (iii) evaluation of the cardioprotective activity of the lead compound and the new analogues by means of infarct size reduction, when the compounds were administered before reperfusion at a dose of 3.8 μmol/kg. Thus we synthesized the 6-piperidinyl purine analogues in which the nitroxyhexyl chain has been replaced by the nitroxyethyl carboxamide group or a 5-nitroxy-ribosyl moiety, as well as 6-piperidinyl purine bearing a nitrate ester at position 8. In addition 6-piperazinyl-purine analogues containing nitrate esters have been synthesized.

2. Chemistry

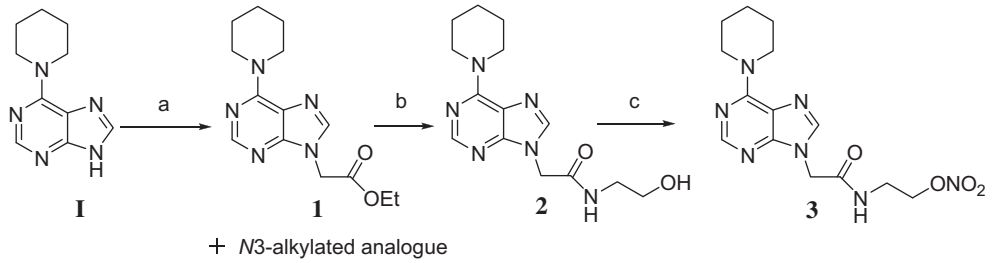
N-acetylation of 6-piperidinyl-purine with ethyl bromoacetate afforded the 9-substituted purine analogue **1** as major isomer. In the NMR spectrum of this compound H2 and H8 appear at 8.13 and 7.70 ppm, respectively, while for 3-isomer at 7.97 and 7.93 ppm. Treatment of **1** with ethanolamine, followed by nitration using acetyl nitrate gave analogue **3** (Scheme 1)

The acetyl protected nucleoside **4** was obtained by condensation of 1,2,3,5-tetra-*O*-acetyl-β-D-ribofuranose with the silylated 6-piperidinyl-purine, under Vorbrüggen conditions, as shown in Scheme 2. The reaction was performed at room temperature in dichloroethane (DCE) in the presence of trimethylsilyl triflate (TMSOTf) affording **4** in 85% yield. NOESY spectrum of **4** showed a correlation between the anomeric H-1′ (6.15 ppm) and H-4′ (4.38 ppm) protons indicating the formation of β-isomer.[8] Moreover, HMBC spectrum of **4** confirmed the N-9 substitution of the purine ring (correlation between H-1′ and C-8 and C-4).[9] N-7 analogue was obtained in 15% yield. After removal of acetyl groups and protection of 2′ and 3′ hydroxyls of compound **5**, the resulting isopropylidene derivative **6** was nitrated and subsequently deprotected to afford **7**.

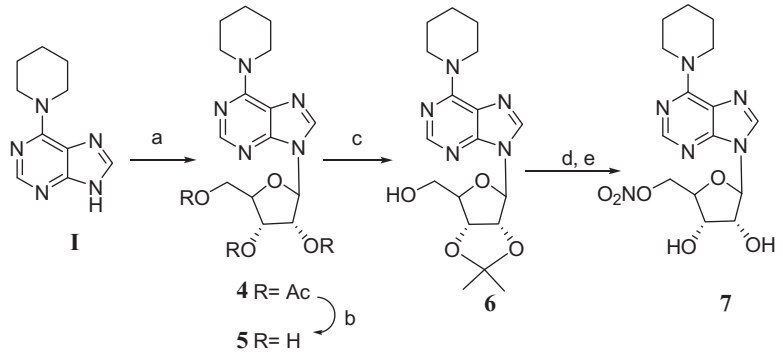
Compound **9**, was synthesized starting from the THP protected 6-piperidinyl-purine **II** (Scheme 3) which was lithiated and subsequently reacted with methylchloroformate to furnish ester **8**. Condensation of **8** with ethanolamine followed by nitration, using 100% HNO_3 and H_2SO_4[10] afforded **9**.

The synthesis of *N*-(nitroxyethylamino)carbonylmethyl analogue **12** is depicted in Scheme 4. Reaction of THP-protected purine **III** with glycine methyl ester, gave **10** which converted to **12** upon condensation with ethanolamine, nitration and deprotection.

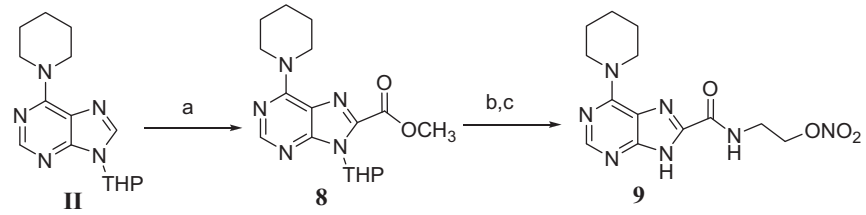
Purine analogue **III** was also used as starting material for the synthesis of 6-piperazinyl-purine analogues (Scheme 5). Amination of **III** with excess of piperazine, followed by acylation with bromoacetyl bromide or activated bromohexanoic acid gave **13** and **14**, respectively, which upon nitration using $AgNO_3$ and deprotection produced **15** and **16**.



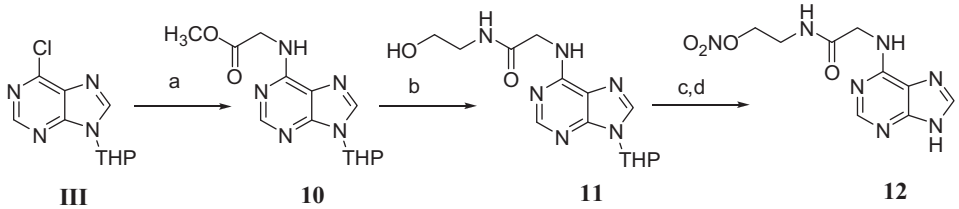
Scheme 1. Synthesis of compound **3**. Reagents and conditions: (a) $BrCH_2CO_2CH_2CH_3$, Cs_2CO_3, anh DMF, 87%; (b) $H_2N(CH_2)_2OH$, 100 °C, 98%; (c) 65% HNO_3, Ac_2O, AcOH, AcOEt, 88%.



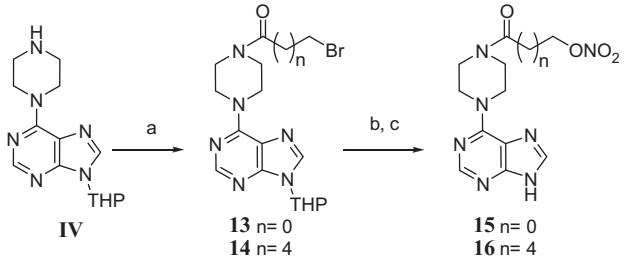
Scheme 2. Synthesis of compound **7**. Reagents and conditions: (a) (i) HMDS, $(NH_4)_2SO_4$, 140 °C, (ii) 1,2,3,5-tetra-*O*-acetyl-β-D-ribofuranose, TMSOTf, DCE, 85%; (b) NaOMe, MeOH, 95%; (c) *p*-TsOH, 2,2-dimethoxypropane, acetone, 91%; (d) 65% HNO_3, Ac_2O, CH_2Cl_2, 0 °C, 93%; (e) 80% TFA/H_2O, 81%.



Scheme 3. Synthesis of compound **9**. Reagents and conditions: (a) $ClCO_2CH_3$, LDA, THF, −78 °C, 70%; (b) $H_2N(CH_2)_2OH$, 100 °C, 100%; (c) 100% HNO_3, H_2SO_4, CH_2Cl_2, −5 °C, 84%.



Scheme 4. Synthesis of compound **12**. Reagents and conditions: (a) $H_2NCH_2CO_2CH_3$·HCl, Et_3N, MeOH, 70 °C, 97%; (b) $H_2N(CH_2)_2OH$, 100 °C, 95%; (c) 65% HNO_3, Ac_2O, CH_2Cl_2, 0 °C, 67%; (d) 50% TFA/CH_2Cl_2, 55%.



Scheme 5. Synthesis of compounds **15** and **16**. Reagents and conditions: (a) $BrCH_2COBr$, Et_3N, CH_2Cl_2, 0 °C (**13**), $Br(CH_2)_5COOH$, CDI, Et_3N, THF (**14**), 71% and 68%, respectively; (b) $AgNO_3$, CH_3CN, 80 °C, 71% and 85%, respectively; (c) 50% TFA/CH_2Cl_2, 57% and 81%, respectively.

3. Discussion

All the compounds were administered in anesthetized rabbits before ischemia or before reperfusion, at the same dose as the lead compound **A** in our previous study[7] (3.8 μmol/kg). The effect of the tested compounds on infarct size reduction is compared with the control group as well as to the IPC or PostC groups. The schematic presentation of the experimental protocol is shown in Figure 2. Specifically, the rabbits were randomized into groups (n = 6–8 per group), anesthetized and subjected to 30 min of myocardial ischemia and 3 h of reperfusion with the following additional interventions: Control group, no intervention, groups in which the compounds were administered bolus at the 40th min and at the 1st min before sustained ischemia, in a total dose of 3.8 μmol/kg, groups in which the compounds were administered bolus at the 20th min of ischemia and the 1st min of reperfusion in a total dose of 3.8 μmol/kg, a IPC group, consisted of two cycles of preconditioning, each composed of 5 min of regional ischemia-10 min reperfusion and a PostC group, consisted of eight cycles of post conditioning (8 × 30 s) immediately after completion of index ischemia.

After the end of the experiments the infarct size (I) and the area at risk (R) were estimated in % I/R. The hemodynamic variables of the different study groups are presented in Table 1. No significant differences were observed among the groups. The effects of the compounds on infarct size are summarized in Table 2.

Given prior to sustained ischemia, compound **3**, bearing a *N*-(nitroxyethyl) aminocarbonylmethyl group at position 9 of purine moiety, reduced infarct size (28.4 ± 1.2%) compared with the control group, but it was less potent than the lead compound **A**. This suggests that not only the presence of a nitrate ester but also the nature of the alkyl chain influences the cardioprotective activity. Replacement of the nitroxyalkyl chain by 5′-nitro-ribosyl group (compound **7**) results in significant improvement of the protection in terms of infarct size (17.9 ± 2.6%). This adenosine analogue is not expected to interact with adenosine receptors. According to literature data, the presence of a secondary amine, such as piperidine, at position 6 of the purine abolishes the activity at the human adenosine receptors (ARs), indicating that, at least one hydrogen atom at the N6-position is essential for hydrogen bonding in the binding site of the AR.[11] The analogue **9** which has the same structural characteristics that nicorandil and its indole and quinoline derivatives (heteroaromatic compound substituted by a *N*-(nitroxyethyl) aminocarbonyl group) was less active (34.4 ± 4.3%) than its indole and quinoline counterparts previously tested.[7]

The analogues **12** and **15** bearing the nitrate ester at position 6 of purine skeleton, administered before ischemia did not reduce infarct size. Compound **16** bearing the nitroxyhexyl chain, was slightly less potent than **A**.

An additional goal of this study was to compare the activity of the new analogues with the effect of PostC. Thus, the compounds were administered bolus at the 20th min of ischemia and the 1st min of reperfusion. Administration of the lead compound **A** did not result on significant reduction of infarct size (36.8 ± 2.0%) compared to the control group.

Administration of compounds **3**, **9** and **12**, bearing the *N*-(nitroxyethyl) aminocarbonyl group, resulted in the death of animals at the 10th and 15th min of reperfusion. The death was due to development of intractable ventricular arrhythmias at the time of reperfusion and thus animals were excluded from the study and these analogues were not further tested.

The effect of compound **7** on infarct size reduction (22.7 ± 3.6%) was comparable to that of PostC (26.4 ± 2.3%) indicating that the replacement of the nitroxyhexyl chain of **A** or the *N*-(nitroxyethyl)aminocarbonylmethyl group of **3** by a 5′nitro-ribosyl moiety results in significant improvement of the cardioprotective activity. As mentioned above, compound **7** is not expected to act at the

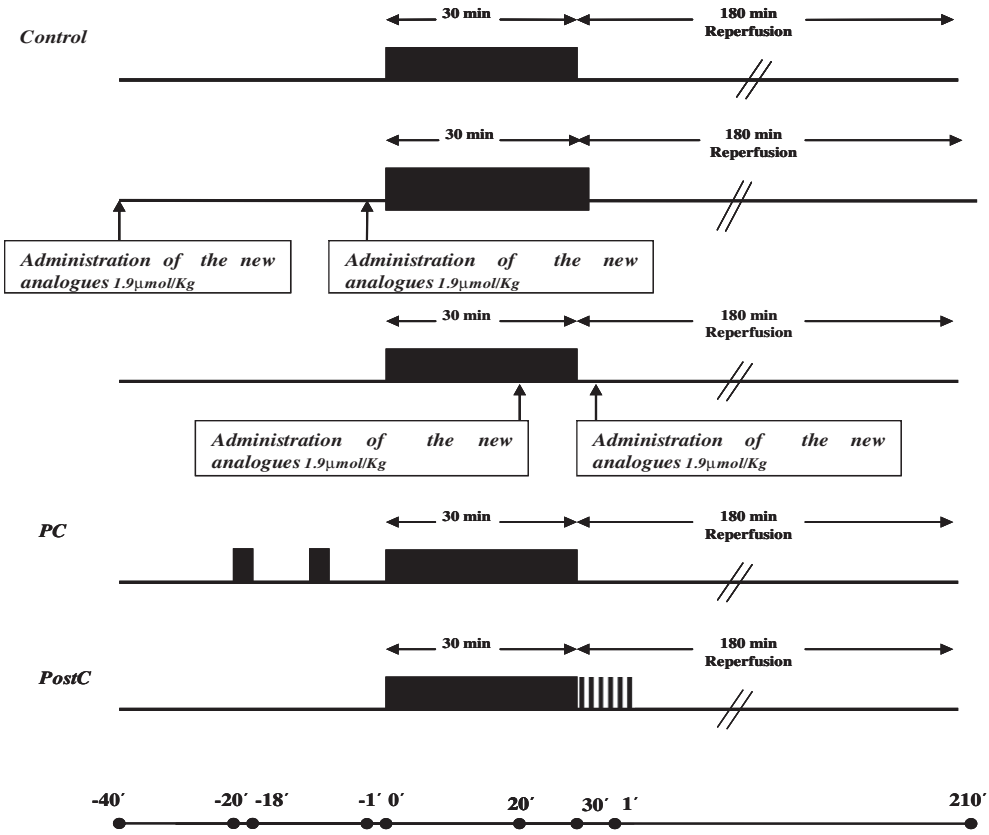


Figure 2. Schematic presentation of the study protocol.

Table 1
Hemodynamic variables of the different study groups.

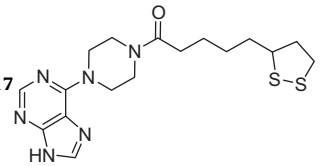
Study group	Baseline		20-min Ischemia		180-min Reperfusion	
	HR	BP	HR	BP	HR	BP
Control	286 ± 12	114 ± 3	284 ± 13	104 ± 6	280 ± 14	100 ± 4
A	272 ± 10	122 ± 5	276 ± 12	112 ± 4	270 ± 13	104 ± 3
IPC	283 ± 13	126 ± 4	284 ± 10	121 ± 6	280 ± 15	116 ± 5
PostC	276 ± 11	119 ± 3	279 ± 12	114 ± 3	275 ± 11	107 ± 4
3	283 ± 14	127 ± 2	280 ± 14	115 ± 5	276 ± 12	102 ± 5
7	275 ± 13	125 ± 4	279 ± 12	113 ± 3	271 ± 15	110 ± 4
9	277 ± 14	117 ± 5	274 ± 15	112 ± 6	269 ± 10	103 ± 7
12	279 ± 12	119 ± 6	282 ± 11	110 ± 5	273 ± 12	104 ± 6
15	280 ± 10	123 ± 3	278 ± 13	114 ± 4	274 ± 13	109 ± 5
16	274 ± 12	128 ± 5	278 ± 15	117 ± 7	269 ± 11	112 ± 3
17	276 ± 14	113 ± 4	279 ± 10	106 ± 5	271 ± 14	101 ± 6
18	281 ± 13	110 ± 6	284 ± 12	102 ± 3	275 ± 12	98 ± 5

HR: mean heart rate in beats/min; BP: mean blood pressure in mm Hg.
20-min ischemia is the period of the initial ischemic insult, 180-min reperfusion is the period of the reperfusion.

adenosine receptors. The known adenosine receptor agonist 5′-nitro-adenosine[12] (analogue **18**, Table 2) did not reduce infarct size (40.2 ± 2.9%).

The 6-piperazinyl-purine derivatives were active when administered before reperfusion. The effect of the analogue **15** (26.2 ± 4.6%) was similar to PostC (26.4 ± 2.3%) while the most active compound **16** reduced infarct size statistically significant compared to PostC (13.4 ± 1.9% vs 26.4 ± 2.3%, $P < 0.05$). According to our previous findings, the proper choice of timing and number of cycles of PostC is important for success. Thus, a PostC protocol consisted of eight cycles of ischemia-reperfusion (8 × 30 s) immediately after completion of index ischemia, offered the maximum protection to the rabbits.[13] In order to investigate an additional benefit of compound **16**, this analogue was administered before reperfusion to the PostC protocol and the infarct size was determined. The I/R was found to be 12.3 ± 0.8% ($P < 0.05$ vs PostC group), indicating that this compound produced a stronger stimulus that PostC in reducing the infarct size and that PostC can achieve maximal protection when a pharmacological agent is co-administered. Cyclosporine, a mPTP inhibitor has been shown to reduce the I/R with an average of 24 ± 4% when administered (10 mg/kg) 10 min before coronary occlusion and of 25 ± 3% when administered 1 min before the reperfusion period in anesthetized rabbits (the same surgical procedure than ours) suggesting that specific inhibition of mPTP opening at reperfusion following acute myocardial infarction provides a powerful antinecrotic

Table 2
Effect of the compounds on infarct size reduction

Compound	Given before ischemia (%)	Given before reperfusion (%)
A	19.8 ± 3.1*	36.8 ± 2.0
3	28.4 ± 1.2*	Not tested[a]
7	17.9 ± 2.6*	22.7 ± 3.6*
9	34.4 ± 4.3	Not tested[a]
12	34.8 ± 4.0	Not tested[a]
15	37.3 ± 4.2	26.2 ± 4.6*
16	24.9 ± 4.1*	13.4 ± 1.9**
17 		28.6 ± 3.2*
5-Nitro-adenosine **18**		40.2 ± 2.9
control	48.1 ± 2.0	
IPC	15.7 ± 2.3*	
PostC		26.4 ± 2.3*

* P <0.05 versus Control.
** P <0.05 versus postC.
[a] These analogues were not further tested due to the death of animals at the 10th and 15th min of reperfusion.

protection.[14] Many studies have failed to show an enhanced cardioprotection when IPC and PostC are combined,[15] probably because both IPC and PostC protect against myocardial reperfusion injury by the same mechanisms, including the PI3K–Akt signal transduction pathway, NO-PKG pathway and mitochondrial ATP sensitive channels, as well as adenosine, oxygen free radicals and the mPTP.[16] Compound **16** exhibited more significant cardioprotection than PostC and cyclosporine in terms of infarct size limiting effect and this may be due to alternative intracellular mechanisms that need to be explored. Further work to uncover the specific mode of action of this compound is currently under investigation.

Based on our experience on lipoic acid analogues[17] and the literature data on the ability of lipoic acid and the piperazinyl-purine derivatives to modulate various kinases,[18] we synthesized and tested the analogue **17** bearing 1,2-dithiolane group instead of nitrate ester. This compound was equipotent (28.6 ± 3.2%) with **15** and less active than **16** indicating that both the length of the alkyl chain and the presence-of the nitrate ester (NO donor) influences the ability of these compounds to reduce infarct size.

It has been shown that the protective effect of IPC can be mimicked pharmacologically with NO donors.[5a] However, the effect of NO donors in PostC, has not been determined in vivo. Nitroglycerin (NTG), given at reperfusion in isolated hearts, failed to reduce the infarct size; the authors suggested that this may occur because of the surge of superoxide radical that is generated at the onset of reperfusion and forms the peroxynitrite anion via NO from NTG.[19] NO is an important mediator in PostC-mediated cardioprotection,[20] while the reduction of peroxynitrite formation plays a significant role in the PostC protective effect.[21] However, NO may react with superoxide anions, whose production is increased during post-ischemic reperfusion to form the toxic nitrating and oxidant agent peroxynitrite ($ONOO^-$). $ONOO^-$ is one of the major triggers of cardiomyocyte apoptosis.[22] Peroxynitrite is formed after NO reaction with superoxide anion at a very fast rate; this causes an irreversible inhibition of the mitochondrial respiratory chain. It also disturbs the cellular function, modifies iron/sulfur centers as well as protein thiol and tyrosine residues. The latter leads to the formation of nitrotyrosine (NT), which serves as a surrogate

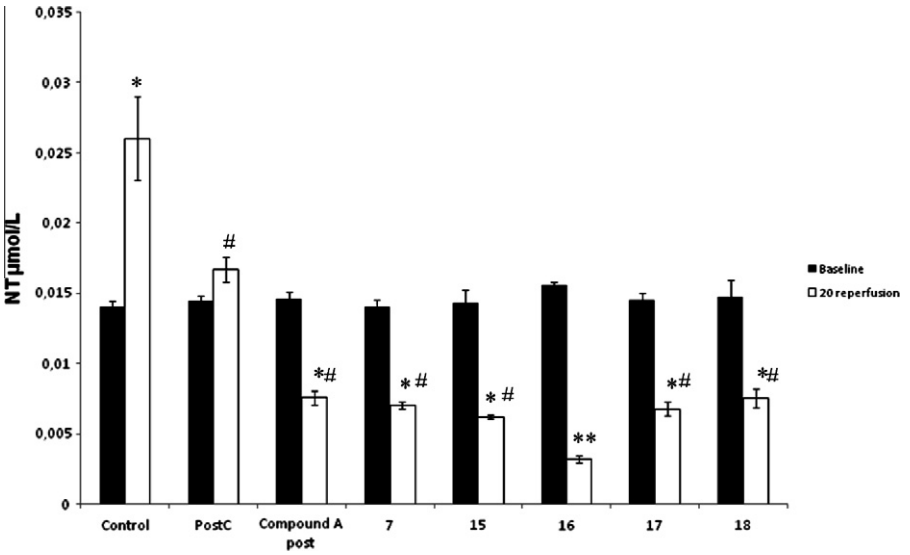


Figure 3. Nitrotyrosine (NT) formation at baseline, and reperfusion (*P <0.05 vs baseline, #P <0.05 vs control, **P <0.05 vs all the other groups).

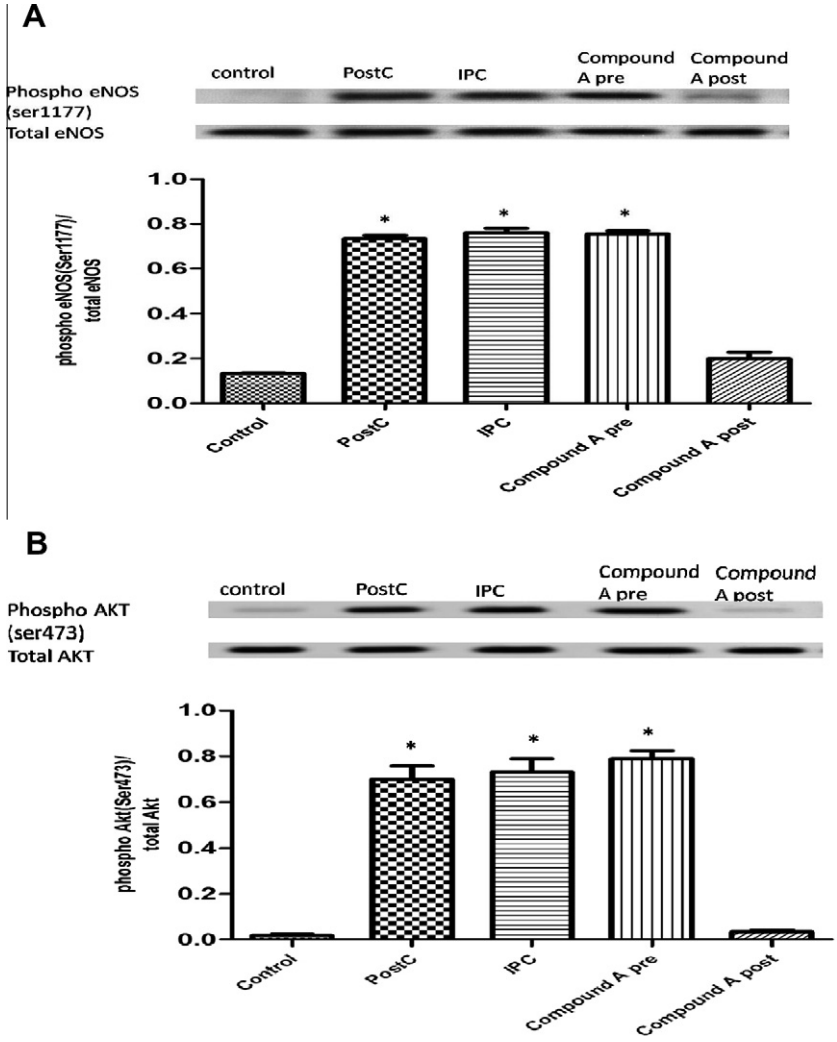


Figure 4. Western Blot analyses: total endothelial nitric oxide synthase (eNOS) and phosphorylated eNOS (A, $^{*}p$ <0.05 vs Control and compound A post); total Akt and phosphorylated Akt (B, $^{*}p$ <0.05 vs Control and compound A post).

marker of the reactive nitrogen species.[23] We have previously shown a significant reduction in myocardial and circulating NT in PostC compared to control group.[13a] In order to investigate the effect of the novel compounds on nitrosative stress, we determined the NT concentration in circulation at the 10th min of reperfusion, when the compounds administered prior to reperfusion and we compared the levels of NT with those of control and PostC groups (Fig. 3). All the tested compounds significantly reduced the NT levels independently of the infarct limiting results. However, a further significant decrease was noted in the group treated with compound **16** compared to the other groups. Thus, the effect of compound **16** on nitrosative stress may be involved in the mechanism by which the drug reduces the infarct size in a larger extent that PostC.

Moreover, we performed additional experiments in order to investigate the difference observed between pre-ischemic and reperfusion treatment with compound **A** and to study the intracellular alterations. In a second series of experiments 20 additional animals (4 for each group) from groups control, PostC, IPC, compound **A** pre (administration of compound A in two doses, 40 min before ischemia and 1st min of ischemia) and compound **A** post (administration of compound A in two doses, 20 min of ischemia and 1st min of reperfusion) were subjected in 30 min ischemia followed by 10 min of reperfusion with the same interventions previously described (Fig. 2). The tissue from ischemic area of myocardium was quickly excised and stored in −80 °C for Akt and eNOS assessment with western blot analysis as previously described.[13] In both endogenous cardioprotective mechanisms IPC and PostC, activation of proapoptotic kinase Akt by phosphorylation exerts infract limiting results.[24] Additionally, subsequent activation of eNOs in ser 1177, activates further signal transduction and plays a crucial role in cell survival.[15c] Induction of PostC and IPC as well as administration of compound **A** before and in the beginning of ischemia raised the phosphorylation level of both eNOs (Fig. 4A) and Akt (Fig. 4B) in contrast to control group and the administration of compound **A** during ischemia and in the beginning of reperfusion. These data, which are in agreement with *I*/*R* results, suggest that the observed difference in infract size limitation, in terms of time administration of the new compounds, might be due to molecular mechanism differentiation and to the pharmacokinetic properties as the activation time of half-life. However specific pharmacokinetic studies are required.

4. Conclusion

Our data suggest that administration of the new analogues induces cardioprotection activating different intracellular signaling pathways depending on the timing of the administration (before

ischemia or before reperfusion). Thus 6-piperidinyl-purine analogues **A**, **3** and **7** are effective when given before ischemia, while 6-piperazinyl-purine derivatives **15** and **16** are active when administered before reperfusion. The administration of compound **16** at reperfusion reduced significantly the infarct size in anesthetized rabbits compared to PostC. Moreover, a significant decrease of nitrotyrosine (NT) levels, at the 10th min of reperfusion, was observed in the group treated with compound **16** compared to the other groups. These findings introduce a novel class of promising pharmacological compounds that could be used as mimics or enhancers of PostC. In order to further investigate the molecular pathways by which compound **16** offers cardioprotection, pharmacological studies are currently in progress.

5. Experimental section

5.1. Chemistry

All starting materials and common laboratory chemicals were purchased from commercial sources and used without further purification. All reactions involving air- or moisture-sensitive reagents were performed under an argon atmosphere. Melting points were determined on a Buchi 510 apparatus and are uncorrected. ^{1}H NMR spectra were recorded on Varian spectrometers operating at 300 MHz or 600 MHz and ^{13}C spectra were recorded at 75 MHz using $CDCl_3$ as solvent. Silica gel plates Macherey-Nagel Sil G-25 UV_{254} were used for thin layer chromatography. Chromatographic purification was performed with silica gel (200–400 mesh). The purity of the tested compounds was determined by HPLC (Thermo Scientific HPLC Spectra System) column Nucleosil 100-5 150 × 4.6 mm C18 5μ, Macherey-Nagel. Mass spectra were obtained on HPLC–MS^n Fleet-Thermo, in the ESI mode. HRMS were recorded, in the ESI mode, on UPLC-MS^n Orbitrap Velos-Thermo.

5.1.1. *N*-(2-Nitroxyethyl)-6-(piperidin-1-yl)-9*H*-purine acetamide (3)

Nitric acid (0.1 mL, 65%), acetic acid (0.5 mL) and acetic anhydride (0.5 mL) were stirred for 10 min at 0 °C and this mixture was added dropwise to a solution of **2** (0.1 g, 0.32 mmol) in CH_2Cl_2. The reaction mixture was stirred at 0 °C for an additional 90 min. After completion of the reaction, 10% aqueous solution of sodium bicarbonate was added and then extracted with ethyl acetate. The organic layer was washed with brine, dried over Na_2SO_4, concentrated in vacuo. The crude residue was purified by column chromatography (CH_2Cl_2/CH_3OH, 97:3) to provide **3** (0.1 g, 88%) as a white solid (mp 172–174 °C). HPLC: gradient elution 90/10 H_2O_0.1%TFA/CH_3CN_0.1%TFA to 20/80 H_2O_0.1%TFA/CH_3CN_0.1%TFA over 30 min, flow rate: 1.5 mL/min, t_R: 6.53 min, detection at 216 nm, purity 100%. ^{1}H NMR (300 MHz, $CDCl_3$) δ: 8.33 (s, 1H, *H*-2), 7.81 (s, 1H, *H*-8), 7.58 (br s, 1H, –CON*H*–), 4.87 (s, 2H, –NCH_2CONH–), 4.52 (t, J = 5.2 Hz, 2H, –CH_2ONO$_2$), 4.37–4.13 (br s, 4H, CH_2), 3.60 (t, J = 5.2 Hz, 2H, –NHCH_2CH$_2$–), 1.82–1.58 (br s, 6H, CH_2). ^{13}C NMR (75 MHz, $CDCl_3$) δ: 166.9, 153.9, 152.4, 150.1, 138.1, 119.8, 71.0, 47.6, 37.1, 26.1, 24.7. HRMS: calcd for $C_{14}H_{20}O_4N_7$ (M+H$^+$) 350.1571; found: 350.1564.

5.1.2. 9-(5-*O*-Nitro-1β-D-ribofuranosyl)-6-(piperidin-1-yl)-9*H*-purine (7)

A suspension of 9-(2,3-*O*-isopropylidene-5-*O*-nitro-1β-D-ribofuranosyl)-6-(piperidin-1-yl)-9*H*-purine (0.12 g, 0.29 mmol) in a mixture of TFA (1.6 mL) and water (4:1) was stirred at room temperature for 1 h. The resulting reaction mixture was concentrated in vacuo and the residue was coevaporated several times with MeOH. The crude residue was purified by column chromatography (CH_2Cl_2/CH_3OH, 95:5 to 90:10) affording **7** (0.09 g, 81%) as a white solid (mp 166–168 °C). HPLC: gradient elution 90/10 H_2O_0.1%TFA/CH_3CN_0.1%TFA to 20/80 H_2O_0.1%TFA/CH_3CN_0.1%TFA over 30 min, flow rate: 1.5 mL/min, t_R: 7.32 min, detection at 216 nm, purity 100%. ^{1}H NMR (600 MHz, $CDCl_3$) δ: 8.15 (s, 1H, *H*-2), 7.86 (s, 1H, *H*-8), 5.88 (d, J = 3.2 Hz, 1H, 1′-H), 4.81 (dd, J = 11.9, 2.6 Hz, 1H, 5′-H), 4.67 (dd, J = 11.9, 4.4 Hz, 1H, 5′-H), 4.37 (d, J = 3.6 Hz, 1H, 4′-H), 4.29–4.22 (m, 2H, 2′-H and 3′-H), 4.13 (br s, 4H, –NCH_2), 3.40–3.27 (m, 2H, 2′-OH and 3′-OH), 1.64 (br s, 6H, –CH_2). ^{13}C NMR (75 MHz, $CDCl_3$) δ: 153.7, 152.0, 149.4, 135.9, 120.2, 89.8, 80.3, 74.1, 71.3, 69.9, 26.0, 24.6. MS m/z: 381.06 (M+H$^+$, 100%), 403.04 (M+Na$^+$, 15%), 782.77 (2 M+Na$^+$, 40%). HRMS: calcd for $C_{15}H_{21}O_6N_6$ (M+H$^+$) 381.1517; found: 381.1507

5.1.3. *N*-(2-Nitroxyethyl)-6-(piperidine-1-yl)-purine-8-carboxamide (9)

Concentrated H_2SO_4 (2 equiv) was slowly and dropwise added at −5 °C to 100% HNO_3 (2 equiv) and the mixture was stirred for additional 10 min. *N*-(2-Hydroxyethyl)-6-(piperidin-1-yl)-9-(tetrahydropyran-2-yl)-purine-8-carboxamide (0.04 g, 0.09 mmol) in CH_2Cl_2 was then dropped slowly to the reaction solution and the reaction mixture was stirred at 0 °C for 30 min. When the reaction was complete, 10% aqueous solution of sodium bicarbonate was added, dried over Na_2SO_4 and concentrated in vacuo. The resulting residue was purified by column chromatography (CH_2Cl_2/CH_3OH, 95:5) to provide **9** (0.03 g, 84%) as a white solid (mp 180–182 °C). HPLC: gradient elution 90/10 H_2O_0.1%TFA/CH_3CN_0.1%TFA to 20/80 H_2O_0.1%TFA/CH_3CN_0.1%TFA over 30 min, flow rate: 1.5 mL/min, t_R: 7.93 min, detection at 216 nm, purity 100%. ^{1}H NMR (600 MHz, $CDCl_3$) δ: 8.49 (s, 1H, *H*-2), 7.61 (t, J = 6.0 Hz, 1H, –CON*H*CH$_2$–), 4.70 (t, J = 5.2 Hz, 2H, –CH_2ONO$_2$), 4.42–4.10 (br s, 4H, –NCH_2 piperidine), 3.87 (t, J = 5.2 Hz, 2H, –NHCH_2CH$_2$–), 1.80–1.69 (m, 6H, –CH_2 piperidine). ^{13}C NMR (75 MHz, $CDCl_3$) δ: 158.7, 154.0, 153.1, 151.2, 140.0, 119.7, 71.2, 36.5, 25.9, 24.4. HRMS: calcd for $C_{13}H_{18}O_4N_7$ (M+H$^+$) 336.1415; found: 336.1407.

5.1.4. *N*-(2-Nitroxyethyl)-2-(9*H*-purin-6-ylamin)-acetamide (12)

To a solution of **11** (0.10 g, 0.27 mmol) in CH_2Cl_2 (1.1 mL) was added trifluoroacetic acid (TFA) (1.1 mL, 13.7 mmol) and then was stirred for 30 min. When the reaction was complete, the mixture was neutralized by addition 10% aqueous $NaHCO_3$, dried over Na_2SO_4 and concentrated in vacuo. The resulting residue was purified by column chromatography (CH_2Cl_2/CH_3OH, 95:5 to 90:10) to provide **12** (0.04 g, 55%) as a white solid (mp 158–160 °C). HPLC: gradient elution 95/5 H_2O_0.1%TFA/CH_3CN_0.1%TFA to 50/50 H_2O_0.1%TFA/CH_3CN_0.1%TFA over 30 min, flow rate: 0.8 mL/min, t_R: 2.56 min, detection at 216 nm, purity 100%. ^{1}H NMR (300 MHz CD_3OD) δ: 8.82 (s, 1H, *H*-2), 8.40 (s, 1H, *H*-8), 4.55 (t, J = 5.1 Hz, 2H, –CH_2ONO$_2$), 4.24 (s, 2H, –NHCH_2–), 3.55 (t, J = 5.1 Hz, 2H, –CH_2CH$_2$ONO$_2$). ^{13}C NMR (75 MHz, $CDCl_3$) δ: 170.3, 155.8, 154.1, 152.6, 138.2, 134.5, 81.9, 71.1, 68.8. MS m/z: 282.08 (M+H$^+$, 100%), 304.08 (M+Na$^+$, 95%). HRMS: calcd for $C_9H_{12}O_4N_7$ (M+H$^+$) 282.0945; found: 282.0942.

5.1.5. 6-(4-Nitroxyacetylpiperazin-1-yl)-9*H*-purine (15)

White solid, yield 57%, mp 166–168 °C. HPLC: gradient elution 95/5 H_2O_0.1%TFA/CH_3CN_0.1%TFA to 50/50 H_2O_0.1%TFA/CH_3CN_0.1%TFA over 30 min, flow rate: 0.8 mL/min, t_R: 2.49 min, detection at 216 nm, purity 100%. ^{1}H NMR (300 MHz $CDCl_3$) δ: 8.29 (s, 1H, *H*-2), 7.88 (s, 1H, *H*-8), 5.08 (s, 2H, –COCH_2ONO$_2$), 4.37 (d, J = 17.2 Hz, 4H, –CH_2), 3.76 (s, 2H, –CH_2), 3.56 (s, 2H, –CH_2). ^{13}C NMR (75 MHz, $CDCl_3$) δ: 163.4, 153.6, 151.8, 150.8, 137.1, 119.1, 67.6, 44.6, 41.6. MS m/z: 308.14 (M+H$^+$, 100%), 330.19 (M+Na$^+$, 25%). HRMS: calcd for $C_{11}H_{14}O_4N_7$ (M+H$^+$) 308.1102; found: 308.1100.

5.1.6. 6-[4-(6-Nitroxyhexanoyl)piperazin-1-yl]-9*H*-purine (16)

White solid, yield 81%, mp 161–163 °C. HPLC: gradient elution 90/10 H_2O_0.1%TFA/CH_3CN_0.1%TFA to 20/80 H_2O_0.1%TFA/CH_3CN_0.1%TFA over 30 min, flow rate: 1.5 mL/min, t_R: 7.65 min, detection at 216 nm, purity 100%. ^{1}H NMR (300 MHz $CDCl_3$) δ: 8.41 (br s, 1H, *H*-2), 7.98 (s, 1H, *H*-8), 4.44 (t, J = 6.5 Hz, 2H, CH_2O-NO_2), 4.39–4.21 (br s, 4H), 3.89–3.72 (br s, 2H), 3.71–3.54 (br s, 2H), 2.40 (t, J = 7.2 Hz, 2H, $NCOCH_2$), 1.81–1.66 (m, 4H), 1.56–1.41 (m, 2H). ^{13}C NMR (75 MHz, $CDCl_3$) δ: 171.3, 155.2, 153.8, 151.3, 137.3, 119.9, 73.1, 45.5, 41.6, 32.9, 26.7, 25.5, 24.6. MS *m*/*z*: 364.17 ($M+H^+$, 30%), 386.08 ($M+Na^+$, 50%). HRMS: calcd for $C_{15}H_{22}O_4N_7$ ($M+H^+$) 364.1728; found: 364.1725.

5.1.7. 6-[4-(1,2-Dithiolan-3-pentanoyl)piperazin-1-yl]-9*H*-purine (17)

6-(Piperazin-1-yl)-9-(tetrahydropyran-2-yl)-9*H*-purine **IV** (0.08 g, 0.27 mmol) and the activated with CDI lipoic acid (LA) (0.11 g, 0.54 mmol) were treated as described for **14**. After removal of the THP group **17** was obtained as a white solid (0.03 g, 60%; mp 152–154 °C). HPLC: gradient elution 90/10 H_2O_0.1%TFA/CH_3CN_0.1%TFA to 20/80 H_2O_0.1%TFA/CH_3CN_0.1%TFA over 30 min, flow rate: 1.5 mL/min, t_R: 9.0 min, detection at 216 nm, purity 100%. ^{1}H NMR (600 MHz $CDCl_3$) δ: 8.40 (s, 1H, *H*-2), 7.97 (s, 1H, *H*-8), 4.36 (br s, 4H, –CH_2 piperazine), 3.78 (s, 2H, –CH_2 piperazine), 3.63 (s, 2H, –CH_2 piperazine), 3.57 (dd, J = 8.4, 6.2 Hz, 1H, –*CH*SS–), 3.13 (ddd, J = 17.9, 8.9, 4.8 Hz, 2H, –CH_2SS–), 2.48–2.43 (m, 1H, –HC*H*CH_2SS), 2.40 (t, 2H, J = 7.5 Hz, –NHCOCH_2-), 1.92–1.87 (m, 1H, –*H*CHCH_2SS–), 1.76–1.66 (m, 4H, –CH_2–), 1.54–1.44 (m, 2H, –CH_2–). ^{13}C NMR (75 MHz, $CDCl_3$) δ: 171.7, 154.0, 151.2, 150.9, 137.4, 119.7, 56.6, 45.7, 40.4, 38.6, 34.9, 33.2, 29.2, 25.1. MS *m*/*z*: 393.22 ($M+H^+$, 40%), 806.86 ($2M+Na^+$, 100%). HRMS: calcd for $C_{17}H_{25}ON_6S_2$ ($M+H^+$) 393.1526; found: 393.1514.

5.1.8. 5′-*O*-Nitro adenosine (18)

Nitro adenosine **18** was obtained as described in WO 2005/117910 A2 with minor modifications. White solid, yield 63%, mp 72–74 °C. HPLC: gradient elution 95/5 H_2O_0.1%TFA/CH_3CN_0.1%TFA to 50/50 H_2O_0.1%TFA/CH_3CN_0.1%TFA over 30 min, flow rate: 0.8 mL/min, t_R: 2.5 min, detection at 216 nm, purity 100%. ^{1}H NMR (300 MHz $CDCl_3$) δ: 8.20 (s, 1H, *H*-2), 7.98 (s, 1H, *H*-8), 5.93 (d, J = 3.4 Hz, 1H, 1′-H), 4.86 (d, J = 2.8 Hz, 1H, 5′-H), 4.75–4.68 (m, 1H, 5′-H), 4.57–4.45 (m, 1H, 4′-H), 4.34 (m, 2H, 2′-H and 3′-H). ^{13}C NMR (75 MHz, $(CD_3)_2CO$) δ: 156.2, 152.7, 149.6, 139.9, 119.9, 89.4, 80.4, 73.5, 73.00, 71.0, 54.1. MS *m*/*z*: 313.11 ($M+H^+$, 100%), 335.00 ($M+Na^+$, 16%). HRMS: calcd for $C_{10}H_{13}O_6N_6$ ($M+H^+$) 313.0891; found: 313.0885.

5.2. In vivo experiments

5.2.1. Surgical preparation

New Zealand White male rabbits with a weight between 2.3 and 3.1 kg received proper care in compliance with the Principles of Laboratory Animal Care formulated by the National Society for Medical Research and the Guide for the Care and Use of Laboratory Animals prepared by the National Academy of Sciences and published by the National Institute of Health (NIH Publication No. 85-23, revised 1996).

Approval from the Ethical Committee and the veterinary authorities of East Attica prefecture was obtained before the study was started.

All animals were anesthetized by injecting 30 mg/kg sodium thiopentone (Pentothal, Abbott) into an ear vein, intubated and mechanically ventilated with a respirator for small animals (MD Industries, Mobile, AL, USA) at a rate adjusted to keep blood gases within the normal range. Two catheters were inserted; one in the jugular vein for fluids and anesthetic and one in the carotid artery for blood pressure monitoring via a transducer attached to a multichannel recorder. The chest was opened via a left thoracotomy and after pericardiotomy the beating heart was exposed. A 3-0 silk thread was passed through the myocardium around a prominent branch of the left coronary artery. Ischemia was induced by pulling the ends of the suture through a small segment of a soft tube, which was firmly attached against the artery with a clamp. The successful induction of ischemia was verified by ST segment elevation on the electrocardiogram and by visual inspection (cyanosis) of the heart. Reperfusion was achieved by releasing the clamp and was verified by the refilling of the artery.

5.2.2. Experimental protocol

The rabbits were randomized into groups consisted of male rabbits (n = 6–8 per group), which were anesthetized and subjected to 30 min of myocardial ischemia and 3 h of reperfusion with the following additional interventions:

(1) Control group, no intervention.
(2) Groups in which compounds were administered bolus at the 40th min and 1st min before sustained ischemia, in a total dose of 3.8 μmol/kg. The dose was defined according to our previous experiments.[7]
(3) Groups in which compounds were administered bolus at the 20th min of ischemia and the 1st min of reperfusion in a total dose of 3.8 μmol/kg. Administration of compounds **3** and **9**, and **12**, resulted in the death of animals at reperfusion. Since these analogues seem to be toxic using this experimental protocol, they were not further tested.
(4) PC group, consisted of two cycles of preconditioning, each composed of 5 min of regional ischemia—10 min reperfusion.
(5) postC group consisted of eight cycles of post conditioning (8 × 30 s) immediately after completion of index ischemia.

Heart rate and blood pressure were measured immediately before sustained ischemia (baseline), at the 20th minute of sustained ischemia and at the end of long reperfusion. Blood samples from all groups were collected at baseline, and at the 10th min of reperfusion for analysis of NT.

After the end of the experiments the infarct size (I) and the area at risk (R) were estimated in % I/R.

5.2.3. Risk area and infarct size measurement

After the end of reperfusion, the hearts were harvested, mounted on an apparatus, and perfused retrogradely via the aorta with normal saline for 2 min. When all residual blood had been removed from the coronary arteries, the coronary ligature was retightened at the same site and 10 ml of green fluorescent microspheres (1–10 μm diameter, Duke Scientific Corp., Palo Alto, CA, USA, suspended in saline) was infused for the delineation of the normally perfused tissue from the risk zone. Hearts were then frozen for 24 h at −10 °C and later sliced into 3-mm-thick sections from the apex to the base. The slices were then incubated in 1% triphenyl tetrazolium chloride (TTC) in isotonic phosphate buffer, pH 7.4 for 20 min at 37 °C. The heart slices were immersed in 10% formaldehyde solution to delineate the infarcted areas more clearly. To identify the borders between the risk zone and the normal area, slices were examined under UV light (wavelength 366 nm). The infarcted, the risk, and the normal areas were traced onto an acetate sheet, which had been placed over the top glass plate. The tracings were subsequently scanned with the Abode Photoshop 6.0 and measured with the Scion Image program. The areas of myocardial tissue at risk and that infarcted were automatically transformed into volumes. Infarct- and risk area volumes were expressed in cm^3 and the percent of infarct to risk area ratio (%I/R) was calculated.[13a,25]

5.2.4. Measurement of circulating nitrotyrosine levels

Blood samples at the 10th min of reperfusion after administration of compounds **A**, **7**, **15**, **16**, **17** and **18** during and after sustained ischaemia as well as from PostC and control groups were centrifuged in 4000 rpm in 4 °C for 10 min. Nitrotyrosine concentration was determined using a commercially available enzyme-linked immunosorbent assay kit according to the manufacture's specifications (Hycult Biotechnology b.v., The Netherlands). For this measurement the antigen present in samples is captured by a solid phase monoclonal antibody and then detected with a biotin-labeled goat polyclonal anti-nitrotyrosine. A streptavidin peroxidase conjugate then binds to the biotinylated antibody. A tetramethylbenzidine (TBM) substrate is added and the yellow product is measured at 450 nm. This kit has a minimum detection level of 2 nM and measurable concentration range of 2–1500 nM.

5.2.5. Western blot analysis

In a second series of experiments twenty additional animals (4 for each group) from groups control, PostC, IPC, Compound **A** pre (administration of compound A in two doses, 40 min before ischemia and 1st min of ischemia) and Compound **A** post (administration of compound **A** in two doses, 20 min of ischemia and 1st min of reperfusion) were subjected in 30 min ischemia followed by 10 min of reperfusion with the same interventions previously described where tissue from ischemic area of myocardium was quickly excised and stored in −80 °C for Akt and eNOS assessment with Western blot analysis as previously described.[13b] In brief tissue-samples were lysated (1% Triton X100, 20 mM Tris pH 7.4–7.6, 150 mM NaCl, 50 mM NaF, 1 mM EDTA, 1 mM EGTA, 1 mM Glycerolphosphatase, 1% SDS, 100 mM PMSF, 0,1% Protease phosphatase inhibitor cocktail) and homogenized. After centrifugation at 11,000*g* for 15 min at 4 °C, supernatants were collected, mixed with Dave's buffer (4% SDS, 10% 2-mercaptoethanol, 20% glycerol, 0.004% bromophenyl blue, 0.125 M Tris/HCl) and heated in 100 °C for 10 min and stored at −80 °C. The protein concentration was determined based on the Bradford dye-binding procedure (Biorad, Protein Assay).

An equal amount of protein was loaded in each well and then was separated by sodium dodecylsulfate–polyacrylamide gel electrophoresis 7.5–11% and transferred onto a polyvinylidene difluoride membrane. After blocking with 5% non-fat dry milk, membranes were incubated overnight at 4 °C with the following primary antibodies: phospho-eNOS (Ser1177), eNOS, phospho-Akt (Ser473) and Akt (Cell Signaling Technology, Beverly, MA, USA). Membranes were then incubated with secondary antibodies for 1–2 h at room temperature (Biorad goat anti-mouse and goat anti-rabbit HRP) and developed using the GE Healthcare ECL Western Blotting Detection Reagents (Thermo Scientific Technologies). Relative densitometry was determined using a computerized software package (NIH Image) and the values for phosphorylated eNOS and Akt were normalized to the values for total eNOS and Akt.

5.2.6. Data analysis and statistics

All results are presented as means ± standard error (SEM). Data of myocardial infarct size (%*I*/*R*), were compared by one-way analysis of variance (ANOVA) with Bonferroni correction and Tukey post-hoc analysis. A calculated *P* value of less than 0.05 was considered to be statistically significant.

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A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.bmc.2012.07.037.

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