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Design and synthesis of nitrate esters of aromatic heterocyclic compounds as pharmacological preconditioning agents

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Abstract—Ischemic preconditioning (IPC) constitutes an endogenous protective mechanism in which one or more brief periods of myocardial ischemia and reperfusion render the myocardium resistant to a subsequent more-sustained ischemic insult. Pharmacological preconditioning represents an ideal alternative of IPC. We now describe the design and synthesis of indole, quinoline, and purine systems with an attached pharmacophoric nitrate ester group. The indole and quinoline derivatives **4** and **5** possess structural features of the nitrate containing K_{ATP} channel openers. Purine analogues **11** and **12**, substituted at the position 6 by a piperidine moiety and at position 9 by an alkyl nitrate, could combine the effects of the nitrate containing K_{ATP} channel openers and those of adenosine. Compound **13** bears the nicotinamide moiety of nicorandil instead of nitrate ester. Compounds **4**, **5**, and **11** reduced infarction and the levels of malondialdehyde (MDA) at reperfusion in anesthetized rabbits. Compounds **12** and **13** did not significantly reduce the infarct size. Analogues **4** and **5** increased cGMP and MDA during ischemia, while combined analogue **4** and mito-K_{ATP} blocker 5-hydroxydecanoic acid (5-HD) abrogated this benefit suggesting an action through mitoK_{ATP} channel opening. Treatment with derivative **11** combined with 5-HD as well as treatment with **11** and adenosine receptor blocker 8-(*p*-sulfophenyl)theophylline (SPT) did not abrogate cardioprotection. Compound **11** is a lead molecule for the synthesis of novel analogues possessing a dual mode of action through cGMP–mitoK_{ATP} channel opening-free radicals and through adenosine receptors.

1. Introduction

Ischemic preconditioning (IPC) has been well recognized as an endogenous mechanism which protects the heart in all species studied, including man.[1] In brief, short cycles of ischemia prior to a prolonged ischemic insult render the heart more tolerant against a potentially lethal episode of ischemia and lead to a reduction in the size of the infarct.[2] Although the hard end-point of myocardial protection is the limitation of infarct size after appropriate triggering,[3] the mechanism of preconditioning is rather obscure with several unrecognized issues regarding the intracellular signaling pathways.[4]

The phenomenon of preconditioning was first described in 1986,[2] and has been reported in various species[5–7] as well as in human isolated myocytes[8] and muscle tissue.[9] A promising finding is that it has also been reported in human in-vivo hearts.[1]

Although preconditioning is a powerful endogenous form of myocardial protection, the application of high-grade ischemic stress as a preconditioning stimulus is limited in the clinical setting of myocardial protection. This is because the optimal preconditioning protocols are not allowed in clinical trials because of ethical and practical reasons. Thus, pharmacological precondition-

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ing represents an ideal alternative to preconditioning and greater effort needs to be exerted to invent pharmacological tools that mimic high-grade preconditioning.[10]

Pharmacological interventions that trigger preconditioning in clinical practice (Fig. 1) are adenosine and N^6-(2-phenylisopropyl)adenosine[11,12] together with K_{ATP} channel openers,[13] such as nicorandil.

Opening of the mitochondrial K_{ATP} channels and subsequent generation of reactive oxygen species (ROS) are considered pivotal steps in the mechanism of preconditioning.[14] Moreover, nitric oxide (NO) has been implicated in this mechanism and it is a requisite cofactor in the preconditioning mediated by the ATP-sensitive potassium channel (K_{ATP}).[15] NO activates cytosolic guanylate cyclase, which in turn elevates the concentration of cyclic GMP (cGMP), leading to vasodilation.

Nicorandil, a hybrid of selective mitochondrial K_{ATP} channel opener and NO donor, has been extensively used in many experimental studies and with very promising results in multicenter clinical trials.[13] Adenosine protects the heart via a signal transduction pathway different from that of other agonists such as acetylcholine, bradykinin, and opioids.[16]

As stated above, one pathway of preconditioning involves opening of the mitochondrial K_{ATP} channels and ROS production, whilst the other one involves adenosine and appears to be independent of free radicals.[16] In order to confer pharmacological protection to the ischemic heart, novel therapeutic strategies, based on the intracellular signaling pathways of preconditioning have emerged. Experimental and clinical studies so far have used various substances with preconditioning-like[16,17] or with anti-preconditioning effect.[18]

Here, we report the design and synthesis of indole, quinoline and purine systems with an attached pharmacophoric nitrate ester group. The indole and quinoline derivatives possess structural features of nitrate containing K_{ATP} channel openers.[19] Purine analogues **11** and **12** substituted at the position 6 by a piperidine moiety and at position 9 by an alkyl nitrate could combine the effects of the nitrate containing K_{ATP} channel openers and those of adenosine. Additionally, the adenine analogue **13** bearing the nicotinamide moiety of nicorandil at position 9 was synthesized in order to investigate if the presence of nicotinamide or the nitrate ester is crucial for the cardioprotective activity. The new compounds were examined (i) for their ability to trigger the beneficial effect of IPC in rabbits by means of myocardial infarct size reduction, (ii) their efficiency on lipid peroxidation, and (iii) their effect on the circulating cGMP levels during ischemia/reperfusion.

We then endeavored to determine the molecular pathway of preconditioning triggered by the new compounds. To this end, the combinations of the indole derivative **4** with the mitoK_{ATP} blocker 5-hydroxydecanoic acid (5-HD), the adenine derivative **11** and 5-HD, and **11** with the adenosine receptor blocker 8-(*p*-sulfophenyl)theophylline (SPT) were investigated for their effect in changing infarct size.

2. Results

2.1. Chemistry

Bromination of amides **1a**,**b** using Ph_3P and CBr_4 gave the bromoamides **2** and **3**, which in turn were converted to the nitrates **4** and **5** by treatment with $AgNO_3$ using the methods described in the literature.[20] (Scheme 1). Compound **5** is reported in the patent literature[21] but it was prepared by another synthetic method, acylating $H_2NCH_2CH_2ONO_2$.

The known 6-piperidine purine **6** (prepared by heating 6-chloropurine with piperidine)[22] was used as a starting material for the synthesis of **11** and **12**. It is known that alkylation of purine analogues is rarely regiospecific,[23–28] with the N9-alkylated derivative normally being the major product but with the formation of significant amounts of N7 or N3. Ratios of regioisomers formed can vary with the nature of the alkylating species. Moreover, it has been observed that N9/N7-alkylation ratios are influenced significantly by the size of the substituent at C6 on purine rings.

N9-Substitution was attempted using two different synthetic routes. Alkylation of compound **6** using 6-bro-

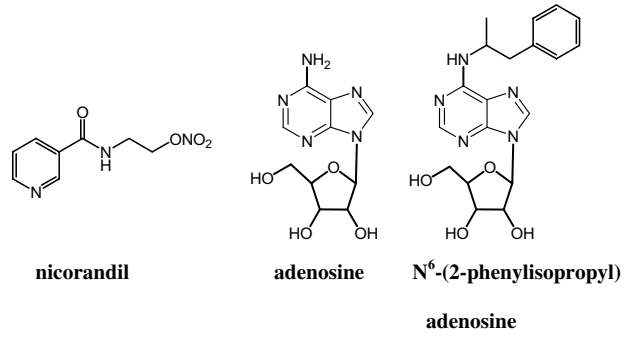
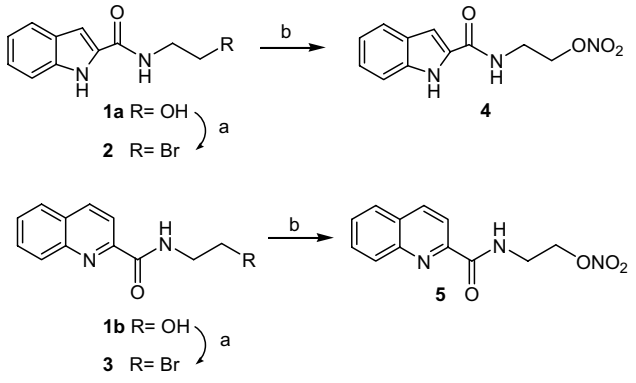
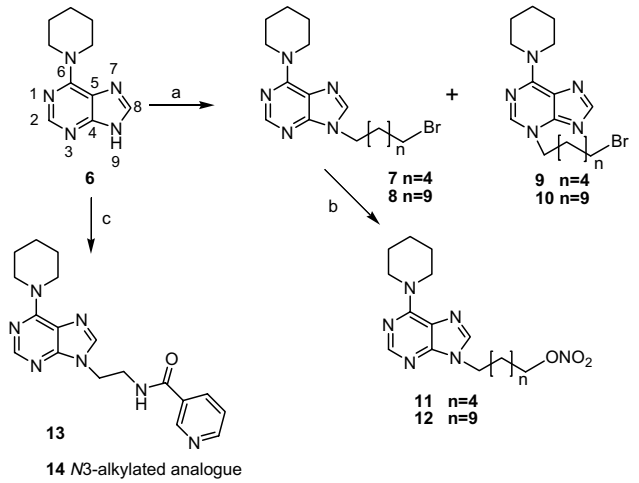


Figure 1. Chemical structures of nicorandil, adenosine, and N^6-(-2-phenylisopropyl) adenosine (PIA).



Scheme 1. Reagents and conditions: (a) CBr_4, Ph_3P, CH_2Cl_2; (b) $AgNO_3$, anhyd CH_3CN, 80 °C.



Scheme 2. Reagents and conditions: (a) 6-bromohexanol or 11-bromoundecanol, Ph_3P, DIAD, THF, (b) $AgNO_3$, anhyd CH_3CN, 80 °C; (c) *N*-(2-hydroxyethyl)-1*H*-pyridin-2-carboxamide, Ph_3P, DIAD, THF.

mohexyl nitrate or 11-bromoundecyl nitrate[29] did not give satisfactory yields. By contrast, the reaction of **6** with the appropriate bromoalkanol under Mitsunobu conditions led to higher selectivity for the N9-alkylated products (Scheme 2). Compound **8** was thus obtained in 46% yield together with 12% of the N3-alkylated product **10**. The chemical shifts of the H2 and H8 protons for the N9-substituted analogues are 8.26 and 7.65 ppm, respectively, while for N3-substituted analogues are 7.97 and 7.87 ppm. The regiochemical assignments were supported by NOESY experiments that showed a substantial correlation between H2 and NCH_2 protons of the alkyl chain of **10** that was not found for the corresponding N9-substituted compound.[24] N7-alkylation is not favoured, probably due to the presence of piperidine group at position 6. Compound **13** was obtained in 42% yield from **6** and (2-hydroxyethyl)-nicotinamide using the Mitsunobu reaction, together with 10% of the N3-alkylated analogue **14**. Finally, nitration of the bromides **7**, **8** using $AgNO_3$ afforded the nitrate derivatives **11** and **12**, respectively.

2.2. Infarct size

Forty four male rabbits were randomly divided into seven groups and were subjected to 30 min of myocardial ischemia and 3 h of reperfusion after the following prior interventions: (1) no intervention (CTL), (2) PC group, two cycles of preconditioning, each composed of 5 min of regional ischemia–10 min reperfusion, (3) group A, administration of compound **4**, (4) group **B**, administration of compound **5**, (5) group **C**, administration of compound **11**, (6) group **D**, administration of compound **12**, and (7) group **E**, administration of compound **13**.

The infarct-to-risk zone ratio, which is the most reliable index of protection,[14] was 47.4 ± 2.6% in the control group and 13.5 ± 3.3% in the PC group ($p < 0.05$ vs control) as shown in Figure 2. Administration of compounds **4**, **5**, and **11** reduced the infarct size to 20.5 ± 5.2%, 22.4 ± 4.7%, and 19.8 ± 3.1%, respectively ($p < 0.01$ vs control). Administration of compounds **12** and **13,** although they reduce the myocardial infarction, did not have statistical significance (30.5 ± 7.3%, and 37.03 ± 6.5% $p = 0.08$, Bonferroni correction).

Combined treatment of **4** and 5-HD abrogated the benefit obtained by treatment with compound **4** (40.12 ± 5.2%, $p < 0.05$ vs group A and NS vs control values), whereas combined treatment of **11** and 5-HD did not abrogate infarct size reduction (21.9 ± 4.6%, $p < 0.05$ vs control values). Based on these results we tested the combined treatment of compound **11** with the adenosine receptor blocker 8-(*p*-sulfophenyl) theophylline (SPT). This combination did not remove the protection afforded by compound **11** in terms of infarct size (28.03 ± 5.2%, $p < 0.05$ vs control) (Fig. 4).

The infarct zone differed between the control group and the groups A, B, and C and between the control group and the groups C + 5HD, and C + SPT. However, there was no difference in the areas at risk after sustained ischemia and 3 h reperfusion in any of the examined groups. The hemodynamic variables (blood pressure and heart rate) at baseline, at 30 min of ischemia and

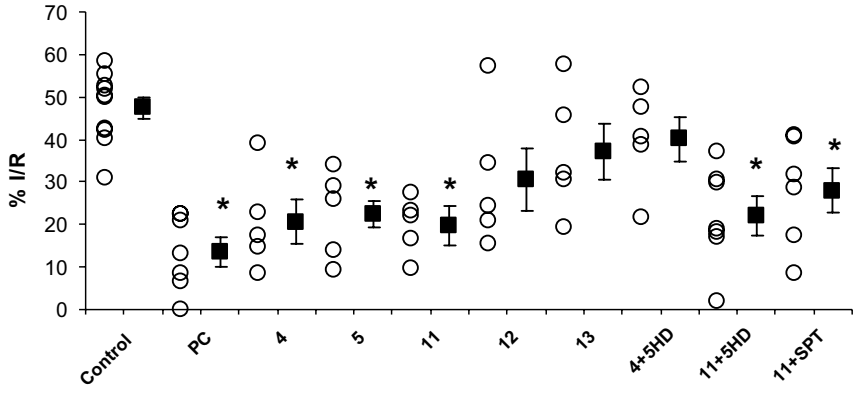


Figure 2. Effects of various study groups on infarct size expressed as a percentage of risk zone, % (*I*/*R*) in rabbit heart following 30 min of ischemia and 180 min of reperfusion. $^*P < 0.05$ versus control group.

at 180 min of reperfusion were not affected by the tested compounds.

2.3. Measurement of MDA

The effect of different treatments on MDA production at different time points of ischemia/reperfusion, as an index of lipid peroxidation and the development of oxidative stress,[30] is shown in Figure 3. Lipid peroxidation is considered a critical mechanism for the injury that occurs during reperfusion of the myocardium.[30] The increase in circulating MDA concentration was found to be significant at the 20th min of reperfusion compared to the baseline ($^{*}p < 0.05$) in the control and PC groups. In all study groups, the MDA levels were significantly decreased compared to the control group at the 20th min of reperfusion ($^{\#}p < 0.05$). Moreover, in groups PC, A, and B, but not in the group receiving the analogues **11** and **12** (groups C and D) the MDA levels were significantly higher at the 20th min of sustained ischemia compared to their own baseline values ($^{**}p < 0.05$).

2.4. Circulating cGMP changes

The effect on circulating cGMP levels for different study groups is shown in Figure 4. Baseline values are the same in all the groups before intervention. At the 20th min of sustained ischemia circulating cGMP levels were significantly elevated in groups PC, A, B, and D compared to the control group ($p < 0.05$).

3. Discussion

The present study demonstrates that the administration of the new analogues bearing a nitrogen containing heterocycle and a nitrate ester group, such as **4**, **5**, and **11**, reduces the infarct size and triggers a pharmacological analogue of preconditioning in vivo. The main purpose of our study was to design and synthesize new molecules that act as cardioprotectants by means of reducing infarct size and therefore compared them with the effect of ischemic PC. Consequently, we did not compare them

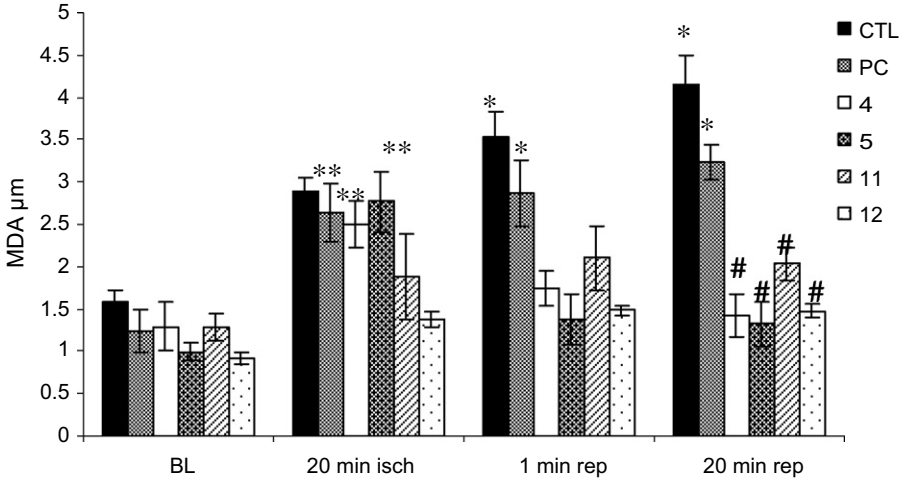


Figure 3. MDA formation during ischemia/reperfusion at baseline, 20th min of ischemia, 1st and 20th min of reperfusion ($^{*}p < 0.05$ vs baseline, $^{**}p < 0.05$ vs baseline, and 20th min of reperfusion, $^{\#}p < 0.05$ vs the control group).

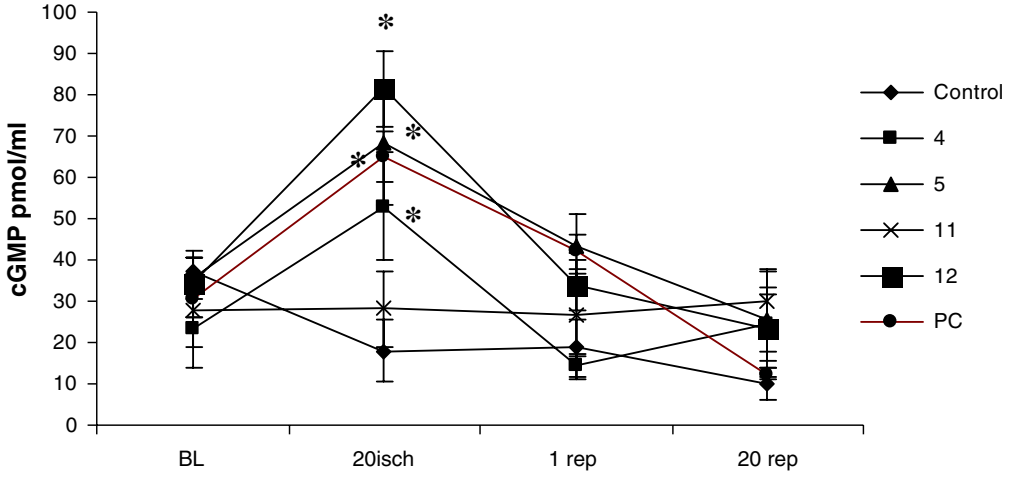


Figure 4. Changes in circulating cGMP during ischemia/reperfusion at baseline, 20th min of ischemia, 1st and 20th min of reperfusion $^{*}p < 0.05$ versus the control group.

with drugs that trigger ischemic PC. Furthermore, our main target was to design new molecules which will offer cardioprotection by a combination of both molecular pathways that trigger IPC.

Compounds **4**, **5**, and **11** reduce the infarct size when administered prior to sustained ischemia in anesthetized rabbits to a similar degree with PC. Compound **13**, bearing the nicotinamide moiety of nicorandil rather than a nitrate ester, did not significantly reduce the infarct size. Since this compound is less cardioprotective than compound **11** containing the nitrate ester, it was not further examined. The dosage used and the time given for the synthesized compounds in our experiments were according to that used for nicorandil in previous studies.[18,31]

All the active compounds bear the pharmacophoric nitrate ester group. It is well established that although endogenous NO is not necessary for ischemia-induced early preconditioning,[32] exogenous NO can elicit a preconditioning-like protection.[33] We and others have tested nitroglycerin (an NO-releasing drug which bears a nitrate ester group), which is extensively used in the treatment of coronary artery disease, as a pharmacological analogue of preconditioning and found that it induces early and late preconditioning in anesthetized rabbits.[34,35] However, the presence of a nitrate ester group does not necessarily confer an NO donor property to a compound. In order to determine if the presence of the nitrate ester group in the compounds **4**, **5**, and **11** is necessary for cardioprotection we synthesized compound **13**, in which the alkylnitrate ester group was replaced by the nicotinamide moiety. The beneficial effect on reducing the infarct size was lost, indicating that the nitrate ester group is crucial for triggering cardioprotection in these compounds.

The mediators involved in the mechanism of preconditioning have been extensively investigated in many studies and various models.[36] It is of interest that NO, guanylate cyclase, cGMP, and PKG are significant mediators, which result in the opening of mitoK_{ATP} channels.[4] We have previously shown that the concentration of circulating cGMP is higher during long ischemia if the rabbit hearts have previously been exposed to one cycle of 5 min ischemia and 10 min reperfusion prior to sustained ischemia.[37] In the present study, derivatives **4** and **5** significantly increased circulating cGMP levels at the 20th min of ischemia in a way similar to the PC group. Furthermore, regardless of the experimental model, there is a great deal of evidence that intracellular free radicals play a pivotal role as triggers of intracellular mediators in preconditioning.[38] Infusion of free radical scavengers, superoxide dismutase, and *N*-2-mercaptopropionyl glycine (MPG) eliminated the protection derived from preconditioning in rabbits.[39] In preconditioning, there is initially a slight increase of oxidative stress, as shown by an increase in MDA formation during subsequent ischemia and reperfusion in isolated rat hearts.[40] We have previously demonstrated the formation of free radicals during preconditioning by the increased levels of circulating lipid peroxidation products (MDA and MDA + 4-hydroxy-nonenal) during ischemia and reperfusion.[7] In the present study, analogues **4** and **5** significantly increased the concentration of MDA at the 20th min of sustained ischemia when compared to their baseline values in a way similar to that found in the PC group. Moreover, these compounds increased the levels of cGMP at the 20th min of ischemia. The elevated levels of MDA and cGMP can be explained by the opening of mitoK_{ATP} channels by analogues **4** and **5**, which may possibly activate the protective mechanism of preconditioning.

In order to investigate further the hypothesis that compounds **4** and **5** act through mitochondrial K_{ATP} channel opening, we included an additional series of experiments where the indole analogue was co-administered with the K_{ATP} channel blocker 5-HD. The addition of 5-HD abrogated the beneficial effect of analogue **4**, indicating that the cardioprotective effect of this molecule is due to the opening of mitoK_{ATP} channels.

It has been shown that protection by adenosine or N^6-(2-phenylisopropyl) adenosine could not be blocked by either MPG or the specific mitoK_{ATP} channel opening blocker 5-hydroxydecanoic acid (5-HD).[16] In order to further investigate the molecular pathway by which compound **11** offers cardioprotection, it was co-administered with the K_{ATP} channel blocker 5-HD. This combination did not abort the protective effect of the compound **11**. In order to investigate whether compound **11** confers its protection via the adenosine receptors alone, we made a further experiment in which compound **11** was co-administered with the adenosine receptor blocker SPT. The protection remained and therefore it seems that this molecule acts on both the adenosine and mitoK_{ATP} pathways, indicating that it involves both signaling cascades.

However, it is well known that antioxidants have generally a beneficial effect against the oxidative damage.[41,42] Oxygen radicals formed following ischemia and reperfusion cause lipid peroxidation that can result in cell membrane breakdown causing cell swelling.[43] All the tested compounds protect the myocardium from oxidative damage by inhibiting lipid peroxidation, as confirmed by the reduction of MDA levels at the 20th min of reperfusion compared to the control group. This result may add an additional benefit for the cardioprotection induced by these compounds.

4. Conclusion

Although all the active compounds contain a heteroaromatic ring and a nitrate ester, they do not act by the same mechanism of cardioprotection. Compounds **4** and **5**, in which the pyridine ring of nicorandil is replaced by indole or quinoline moiety, probably act in the same way as nicorandil. Of note, the addition of the K_{ATP} channel blocker 5-HD abrogated the beneficial effect of analogue **4**, while both compounds increased cGMP and MDA levels at the 20th min of ischemia, indicating that their cardioprotective effect

is due to the opening of mitoK$_{ATP}$ channels. These results along with the literature data[19a,b] on nicorandil analogues suggest that the presence of a nitrooxyethyl carboxamide and a nitrogen containing heteroaromatic ring are a requisite for activity through mitoK$_{ATP}$ channel opening.

Adenine analogue **13** bearing the nicotinamide moiety of nicorandil did not reduce the infarct size. However, combining in one molecular scaffold a nitrooxy alkyl group and a purine moiety led to different mode of action, probably, by combining both mitoK$_{ATP}$ channel opening and adenosine receptors interaction.

Despite optimal therapy, the morbidity and mortality in patients with coronary artery disease remain significantly high and the challenge for the development of novel cardioprotective strategies for the reduction of myocardial injury continues. One such intervention for protecting the ischemic and reperfused heart is ischemic preconditioning.[43]

Compound **11** is a lead molecule for the synthesis of novel analogues possessing a dual mode of action through cGMP–mitoK$_{ATP}$ channel opening-free radicals and through adenosine receptors. Further work to uncover more details on the specific mode of action of compound **11** as well as its derivatives is currently under investigation.

5. Experimental

5.1. Chemistry

Melting points were determined on a Buchi 510 apparatus and are uncorrected. NMR spectra were recorded on a Bruker AC 300 spectrometer operating at 300 MHz for ^{1}H and 75.43 MHz for ^{13}C spectra with $CDCl_3$ as a 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). Mass spectra were recorded on TSQ 7000 Finigan instrument in the ESI mode. Elemental analyses were carried out on a Perkin-Elmer Series II CHNS/O 2400 analyser.

5.1.1. *N*-(2-Bromoethyl)-1*H*-indol-2-carboxamide (2). To a solution of **1a** (0.200 g, 0.98 mmol) in anhyd CH_2Cl_2 (5 ml) at ambient temperature, was added Ph_3P (0.385 g, 1.47 mmol) and CBr_4 (0.487 g, 1.47 mmol). The mixture was stirred for 2 h and then neutralized, at 0 °C, with NaOH 2 N and extracted with AcOEt. The organic layer was washed with satd aqueous NaCl, dried, and concentrated. The residue was purified by column chromatography (petroleumether/AcOEt 70:30). Yield 0.149 g (57%), viscous oil. ^{1}H NMR δ: 9.62 (br s, 1H, ArN*H*), 7.66 (d, 1H, J = 7.3 Hz, Ar*H*), 7.45 (d, 1H, J = 9.0 Hz, Ar*H*), 7.33–7.30 (m, 1H, Ar*H*), 7.17–7.13 (m, 1H, Ar*H*), 6.92 (s, 1H, Ar*H*), 6.64 (br s, 1H, –CON*H*CH$_2$–), 3.94 (t, 2 H, J = 5.8 Hz, –C*H*$_2$Br), 3.61 (t, 2H, J = 5.8 Hz) MS: m/z 187 (100%) $(M-Br)^+$, 79, 81 (Br, 1:1).

5.1.2. *N*-(2-Nitooxyethyl)-1*H*-indol-2-carboxamide (4). To a solution of bromide **2** (0.145 g, 0.54 mmol) in anhyd CH_3CN (5 ml) was added $AgNO_3$ (0.138 g, 0.81 mmol) and the mixture was heated at 80 °C for 2 h. The reaction mixture was then filtered through Celite and washed with CH_2Cl_2 and the filtrate evaporated. The crude product was purified by column chromatography (petroleumether/AcOEt 70:30) yielding 0.067 g (48%), yellowish solid, mp 137–139 °C.

^{1}H NMR δ: 9.29 (br s, 1H, ArN*H*), 7.66 (d, 1H, J = 7.9 Hz, Ar*H*), 7.43 (d, 1H, J = 7.9 Hz, Ar*H*), 7.33–7.28 (m, 1H, Ar*H*), 7.18–7.13 (m, 1H, Ar*H*), 6.90 (s, 1H, Ar*H*), 6.52 (br s, 1H, –CON*H*C H$_2$–) 4.67 (t, 2H, J = 5.5 Hz), 3.86 (t, 2H, J = 5.5 Hz) ^{13}C NMR δ: 162.0, 136.3, 129.7, 127.5, 124.9, 122.1, 120.9, 111.9, 102.7, 71.7, 36.9. Anal ($C_{11}H_{11}N_3O_4$) Calcd C, 53.01; H, 4.45; N, 16.86. Found: C, 52.70; H, 4.47; N, 16.50.

5.1.3. *N*-(2-Nitrooxyethyl)-quinoline-2-carboxamide (5). Compound **3** (0.109 g, 0.39 mmol) was treated as described for **4**. Yield 0.047 g (46%), white solid, mp 85–87 °C. ^{1}H NMR δ: 8.56 (br s, 1H, –CON*H*–), 8.26–8.23 (m, 2H, Ar*H*), 8.09–8.05 (m, 1H, Ar*H*), 7.86–7.78 (m, 1H, Ar*H*), 7.77–7.60 (m, 1H, Ar*H*), 7.62–7.57 (m, 1H, Ar*H*), 4.69 (t, 2H, J = 5.5 Hz, –C H$_2$C*H*$_2$NO$_3$), 3.88 (t, 2H, J = 5.5 Hz, –NHC*H*$_2$CH$_2$–). ^{13}C NMR δ: 164.9, 149.0, 146.4, 137.6, 130.2, 129.7, 129.4, 128.1, 127.7, 118.7, 71.7, 36.9. Anal ($C_{12}H_{11}N_3O_4$) Calcd C, 55.17; H, 4.24; N, 16.08. Found: C, 55.22; H, 4.59; N, 15.81.

5.1.4. Synthesis of N9-substituted 6-piperidinylpurines. To a solution of compound 6-(piperidin-1-yl) purine **6** (0.120 g, 0.59 mmol) in anhyd THF (5 ml) is added the appropriate bromoalkanol (0.59 mmol) and triphenylphosphine (1.2 equiv), followed by the addition of DIAD (1.2 equiv). The mixture was stirred at ambient temperature for 24 h and then diluted by AcOEt and washed with sat. aqueous NaCl. The organic layer was dried and evaporated to dryness and the residue was purified by column chromatography (CH_2Cl_2/CH_3OH 97:3) affording the N9- and N3-alkylated analogues.

5.1.5. 9-(6-Bromohexyl)-6-(piperidin-1-yl) purine (7). Yield: 0.10 g, 46%. Colorless oil. ^{1}H NMR ($CDCl_3$) δ: 8.27 (s, 1H, *H*2), 7.65 (s, 1H, *H*8), 4.25–4.15 (m, 4H, C*H*$_2$), 4.10 (t, 2H, J = 6.7 Hz, –C*H*$_2$N–), 3.31 (t, 2H, J = 6.7 Hz, –C*H*$_2$Br), 1.87–1.65 (m, 10H), 1.47–1.25 (m, 4H). MS: m/z 366, 368 (100%) $(M, M+2)^+$, (relative peak height ratio was 1:1).

5.1.6. 9-(11-Bromoundecyl)-6-(piperidin-1-yl) purine (8). Yield: 0.09 g, 46%. Colorless oil. ^{1}H NMR ($CDCl_3$) δ: 8.32 (s, 1H, *H*2), 7.69 (s, 1H, *H*8), 4.35–4.20 (s, 4H, C*H*$_2$), 4.14 (t, 2H, J = 6.7 Hz, –C*H*$_2$N–), 3.38 (t, 2H, J = 6.7 Hz, –C*H*$_2$Br), 1.87–1.70 (m, 10H), 1.38–1.23 (m, 14H). MS: m/z 436, 438 (10%) $(M, M+2)^+$, (relative peak height ratio was 1:1).

5.1.7. 3-(11-Bromoundecyl)-6-(piperidin-1-yl) purine (10). Yield: 0.02 g, 12%. Colorless oil. ^{1}H NMR ($CDCl_3$) δ: 7.97 (s, 1H, Ar*H*), 7.87 (s, 1H, Ar*H*), 4.72 (br s, 2H,

CH_2), 4.28 (t, 2H, J = 7.3 Hz, –CH_2Br), 4.10 (br s, 2H, CH_2), 3.38 (t, 2H, J = 6.7 Hz, –CH_2N–), 1.84–1.65 (m, 10H), 1.31–1.22 (m, 14H). ^{13}C NMR ($CDCl_3$) δ: 152.5, 151.6, 150.1, 141.1, 120.8, 63.6, 34.0, 32.7, 29.3, 29.0, 28.6, 28.1, 26.5, 26.3, 24.6.

5.1.8. 9-(6-Nitrooxyhexyl)-6-(piperidin-1-yl) purine (11). To a solution of compound **7** (0.10 g, 0.27 mmol) in anhyd acetonitrile was added $AgNO_3$ (1.5 equiv), the mixture was stirred at 80 °C for 2 h and then filtered. The solvent was evaporated and the residue was purified by column chromatography (CH_2Cl_2/CH_3OH 97:3) affording 0.05 g, (54%), of **11** as yellow oil. ^{1}H NMR ($CDCl_3$) δ: 8.25 (s, 1H, *H*2), 7.64 (s, 1H, *H*8), 4.33 (t, 2H, J = 6.7 Hz, –CH_2NO_3), 4.21–4.16 (m, 4H, CH_2), 4.10 (t, 2H, J = 6.7 Hz, –CH_2 N–), 1.86–1.55 (m, 10H), 1.41–1.24 (m, 4H). ^{13}C NMR ($CDCl_3$) δ: 153.7 152.3, 150.7, 137.8, 119.8, 72.9, 46.3, 43.4, 29.8, 26.5, 26.1, 25.2, 24.7. Anal ($C_{15}H_{24}N_6O_3$) Calcd C, 53.56; H, 7.19; N, 24.98. Found: C, 53.33; H, 7.21; N, 24.64.

5.1.9. 9-(11-Nitrooxyundecyl)-6-(piperidin-1-yl) purine (12). A solution of compound **8** (0.08 g, 0.19 mmol) was treated as described for **11**. Yield: 0.05 g, 58%, yellow oil. ^{1}H NMR ($CDCl_3$) δ: 8.32 (s, 1H, *H*2), 7.69 (s, 1H, *H*8), 4.42 (t, 2H, J = 6.7 Hz, –CH_2NO_3), 4.30–4.18 (m, 4H, CH_2), 4.14 (t, 2H, J = 7.3 Hz, –CH_2N–), 1.86–1.67 (m, 10H), 1.30–1.24 (m, 14H). ^{13}C NMR ($CDCl_3$) δ: 153.9, 152.3, 150.7, 137.9, 119.8, 73.4, 46.3, 43.7, 30.0, 29.3, 28.9, 26.6, 26.1, 25.6, 24.8. Anal ($C_{20}H_{34}N_6O_3$) Calcd C, 59.09; H, 8.43; N, 20.67. Found: C, 58.75; H, 8.60; N, 20.31.

5.1.10. *N*-[2-(6-Piperidine-1-yl-purine-9-yl)-ethyl]-nicotinamide (13). Reaction of compound **6** (0.083 g, 0.41 mmol) with *N*-(2-hydroxyethyl)-nicotinamide (0.61 mmol), as described for **7** and **8** afforded the N9- and N3-alkylated analogues.

Yield: 0.060 g, 42%. White solid, mp 162–164 °C. ^{1}H NMR ($CDCl_3$) δ: 9.01 (s, 1H, Ar*H*), 8.75 (s, 1H, Ar*H*), 8.63 (d, 1H, J = 3.6 Hz, Ar*H*), 8.26 (s, 1H, *H*2), 8.11 (d, 1H, J = 8.0 Hz, Ar*H*), 7.68 (s, 1H, *H*8), 7.33–7.31 (m, 1H, –CON*H*–), 4.41 (t, 2H, J = 5.3 Hz, –CH_2N–), 4.17–4.07 (m, 4H, CH_2), 3.83 (t, 2H, J = 2.7 Hz, –NHCH_2CH_2N–), 1.38–1.28 (m, 6H) ^{13}C NMR ($CDCl_3$) δ: 165.8, 153.8, 152.2, 152.1, 150.8, 148.3, 138.2, 135.0, 129.6 123.4, 119.9, 43.8, 41.4, 26.1, 24.7 MS: m/z 352 $(M+H)^+$. Anal ($C_{18}H_{21}N_7O$) Calcd C, 61.52; H, 6.02; N, 27.90. Found: C, 61.24; H, 6.19; N, 27.56.

5.1.11. *N*-[2-(6-Piperidine-1-yl-purine-3-yl)-ethyl]-nicotinamide (14). Yield: 0.013 g, 10%. Yellowish oil. ^{1}H NMR ($CDCl_3$) δ: 9.18 (s, 1H, Ar*H*), 9.10 (s, 1H, Ar*H*), 8.69 (d, 1H, J = 3.4 Hz, Ar*H*), 8.15 (d, 1H, J = 8.0 Hz, Ar*H*), 7.97 (s, 1H, Ar*H*), 7.93 (s, 1H, Ar*H*), 7.38–7.34 (m, 1H, –CON*H*–), 4.68–4.56 (m, 4H, CH_2, –NHCH_2CH_2N–), 3.97–3.95 (m, 4H, CH_2, –CH_2N–), 1.79–1.73 (m, 6H) ^{13}C NMR ($CDCl_3$) δ: 166.0, 152.5, 151.3, 148.8, 141.7, 135.3, 129.7, 123.6, 121.2, 49.9, 41.5, 24.7.

5.2. Surgical preparation

New Zealand white male rabbits weighing between. 2.3 and 3.1 kg were used. All animals received proper care in compliance with the '*Principles of Laboratory Animal Care*', published by the National Society for Medical Research, and the *Guide for the Care and Use of Laboratory Animals*, prepared by the Academy of Sciences and published by the National Institutes of Health (Institute of Laboratory Animal Resources Commission on Life Sciences, 1996).

All animals were anesthetized by slowly injecting pentobarbital (30 mg/kg) into an ear vein. They were then subjected to tracheal incision and intubation for mechanical ventilation with a respirator for small animals (MD Industries, Mobile, AL, USA). The ventilator was properly adjusted at a rate of approximately 35 respirations/min, in order to maintain blood gases and pH within the normal range. Two polyethylene catheters were inserted; one was positioned in the carotid artery for continuous blood pressure monitoring, and the other in the jugular vein for fluid infusion (1 ml of normal saline containing 1000 IU heparin/100 ml was administered every 30 min), drug administration, and additional anesthesia when necessary. A bipolar chest lead was used for continuous electrocardiographic recording. Blood pressure and heart rate were continuously monitored. The chest was opened via a left thoracotomy in the fourth intercostal space 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 and by pulling the ends of the suture through a small segment of a soft tube induced regional ischemia; the tube was then firmly attached against the artery with a clamp. The successful induction of ischemia was verified by visual inspection (cyanosis) and by ST elevation on the electrocardiogram. Reperfusion was achieved by unclamping the tube.[44]

5.3. Experimental protocol

First step. Forty-four rabbits were randomly divided into seven groups; all animals were subjected to the following interventions prior to 30 min regional ischemia of the heart and 3 h reperfusion:

Control group (CTL) (n = 10): no intervention.

PC group (n = 7): Two cycles of preconditioning, each composed of 5 min of regional ischemia–10 min reperfusion.

Group A (n = 5): indole analogue **4**.
Group B (n = 6): quinoline analogue **5**.
Group C group (n = 6): adenine analogue **11**.
Group D (n = 5): adenine analogue **12**.
Group E (n = 5): adenine analogue **13**.

The tested compounds were administered in a total dose of 1.3 mg/kg, 40 min before and 1 min before sustained ischemia. Each dose was dissolved in 0.2 ml DMSO

and 0.8 ml normal saline given intravenously as a bolus. Control and PC groups received 0.2 ml DMSO and 0.8 ml normal saline 40 min before and 1 min before sustained ischemia. Blood samples were taken at different time points (baseline, at 20 min of sustained ischemia, at 1 and 20 min of reperfusion), and plasma was obtained for MDA measurements as a lipid peroxidaton marker and for cGMP determination.

Second step. In a second series of experiments 17 additional rabbits were divided in three groups:

Group (A + 5HD) ($n = 5$): combined treatment with the indole derivative **4** and the mitoK_{ATP} channel blocker 5-hydroxydecanoic acid (5-HD).
Group (C + 5HD) ($n = 6$): combined treatment with the adenine analogue **11** and the mitoK_{ATP} channel blocker 5-hydroxydecanoic acid (5-HD).
Group (C + SPT) ($n = 6$): combined treatment with the adenine analogue **11** and the adenosine receptor blocker 8-(*p*-sulfophenyl) theophylline (SPT).

The tested compounds were administered in a total dose of 1.3 mg/kg, 40 min before and 1 min before sustained ischemia. 5-HD (Sigma–Aldrich Chemical Co.) was dissolved in normal saline and was given intravenously as a bolus dose of 5 mg/kg into the jugular vein 40 min before sustained ischemia, as previously described.[45] SPT (Sigma–Aldrich Chemical Co.) was dissolved in normal saline and was given intravenously as a bolus dose of 10 mg/kg into the jugular vein as previously described.[46,47]

5.4. Risk area and infarct size

At the end of the experiment, hearts were removed, mounted on a perfusion apparatus and perfused for 2 min retrogradely via the aorta with normal saline (20 ml/min, 50 mmHg, room temperature). When all residual blood had been removed from the arteries, the coronary ligature was retightened to the same extent as before and 5 ml of green fluorescent microspheres (2–9 μm diameter; Duke Scientific Corporation, Palo Alto, CA, USA, suspended in saline) were slowly infused over 5 min for the delineation of the normally perfused tissue from the risk zone. Hearts were then frozen for 24 h at −20 °C and sliced into 3 mm thick sections from the apex to the base. The slices were then incubated in 1% triphenyl tetrazolium chloride solution (TTC) for 20 min at 37 °C and the infarcted area was defined as the negatively stained region. The heart slices were then immersed in 10% formaldehyde solution for 24 h to delineate the infarcted (tetrazolium chloride negative) areas more clearly. To clarify the borders between the risk zone and the normal area, slices were examined under ultraviolet light (wavelength 366 nm). Infarcted, risk, and normal areas were traced onto an acetate sheet, photographically enlarged and quantified by planimetry (Scion Image Program, Epson Perfection 1200S scanner, Adobe Photoshop 6.0). The areas of infarction and myocardial tissue at risk for infarction were automatically transformed to volumes by multiplying by the slice thickness (3 mm). Infarct and risk area volumes were expressed in cm^3 and the percent of infarct to risk area (I/R) calculated as previously described.[48]

5.5. Measurement of malondialdehyde (MDA)

MDA plasma concentrations at different time points (in basal conditions, at the 20th min of sustained ischemia, at the 1st and 20th min of reperfusion) were determined spectrophotometrically and expressed as μM using a commercial kit (Oxford Biomedical Research Colorimetric Assay for lipid peroxidation) with some modifications.[45] The method we used is based upon the reaction of the chromogenic reagent *N*-methyl-2-phenyl-indole with MDA. One molecule of free MDA combines with two molecules of the chromogenic agent to produce a stable chromophore with a maximal absorbance at 586 nm.

Briefly, 0.65 ml of 10.3 mM *N*-methyl-2-phenyl-indole in acetonitrile was added to 0.2 ml of plasma. After vortexing for 3–4 s and adding 0.15 ml of HCl 37%, samples were mixed well and incubated at 45 °C for 60 min. The samples were then cooled on ice, centrifuged at 3500*g* for 20 min and the absorbance at 586 nm was measured. A standard curve of an accurately prepared standard MDA solution (from 2 to 20 μmol/ml) was also run for quantitation. Measurements for each group were performed in triplicate.

5.6. cGMP enzyme immunoassay

cGMP was determined using a commercially available enzyme immunoassay kit following the manufacturer's instructions (Assay Designs; Ann Arbor, MI, USA). In brief, plasma samples (or standards) were added in 96-well plates pre-coated with goat antibody specific to rabbit IgG. An alkaline phosphatase solution conjugated to cGMP was then added in all wells (except blank wells) followed by the addition of a polyclonal antibody to capture the cGMP in a competitive manner. After a 2 h incubation on a plate shaker (25 °C, 500 rpm), excess reagents were removed by washing and finally by aspiration. A *p*-nitrophenyl phosphate substrate was then added for 1 h and the enzyme reaction was stopped with the addition of trisodium phosphate. The yellow color generated was read on a microplate reader at 450 nm with correction at 550 nm. The intensity of the bound yellow color is inversely proportional to the concentration of cGMP in either standards or samples. Optical density values were used to calculate the concentration of cGMP with the use of Prism4 V. 4.03 (Graph Pad; USA) software.

5.7. 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. A calculated p value of less than 0.05 was considered to be statistically significant.

A one factor repeated measures ANOVA model was used to compare each variable separately during the

treatment period (baseline until 20th min of reperfusion). Pairwise multiple comparisons were performed using the method of Tukey critical difference.

All tests were two-sided with a 95% significance level. Statistical analysis was carried out using the statistical package SPSS vr 10.00 (Statistical Package for the Social Sciences).

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