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Synthesis of a hypoxia-targeted conjugate of the cardioprotective agent 3′,4′-dihydroxyflavonol and evaluation of its ability to reduce ischaemia/reperfusion injury

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ABSTRACT

3′,4′-Dihydroxyflavonol (DiOHF) is a cardioprotective flavonol that reduces injury associated with myocardial ischaemia and reperfusion. We hypothesized that the efficacy of DiOHF could be enhanced through its targeting to hypoxic regions of partial reperfusion. Copper(I)-catalyzed ligation of an azide-modified DiOHF analogue to 2-propargyl-nitroimidazole afforded a DiOHF–nitroimidazole conjugate (DiOHF–NIm). When incubated with Con8 cells under normoxic conditions DiOHF–NIm could be detected in both the culture supernatant and cell lysate, whereas under hypoxic conditions it was present in substantially reduced amounts consistent with its selective metabolism under hypoxia. DiOHF–NIm possessed antioxidant activity comparable to DiOHF through scavenging of superoxide produced by NADPH/NADPH oxidase, but had significantly attenuated vasorelaxant activity. DiOHF–NIm treatment significantly reduced lactate dehydrogenase release following ischaemia/reperfusion in hindlimbs of anaesthetized rats (p <0.05), to a level similar to DiOHF treatment but also at earlier time points. DiOHF–NIm significantly reduced levels of myeloperoxidase (p <0.05), a biomarker of neutrophil accumulation, whereas the reduction afforded by DiOHF was not significant. DiOHF–NIm therefore represents a promising potential therapeutic for ischaemia/reperfusion injury.

Dihydroxyflavonol (DiOHF **1**, Fig. 1A) is a synthetic flavonol that is a promising therapeutic for the treatment and prevention of myocardial reperfusion injury. DiOHF causes vasorelaxation in rat isolated thoracic aorta and is an effective scavenger of superoxide.[1–4] Administration of intravenous DiOHF affords a significant reduction in myocardial infarct size and injury after ischaemia/reperfusion (I/R) in anaesthetized sheep, with the level of protection being similar to that of the 'gold standard', the surgical intervention ischaemic preconditioning (IPC), which is the most effective protection against I/R injury in use.[5,6] Daily treatment of goats with DiOHF over four weeks reperfusion after myocardial ischaemia significantly reduced infarct size and prevented left ventricular remodeling, and inhibited myocyte apoptosis in the noninfarcted myocardium.[7] Levels of caspase-3, bax and cytochrome c were also significantly reduced in the DiOHF-treated group compared to vehicle, consistent with decreased apoptosis.[7] DiOHF treatment also significantly reduced the extent of myocyte apoptosis identified by TUNEL assay in the noninfarcted zone of the myocardium.[7]

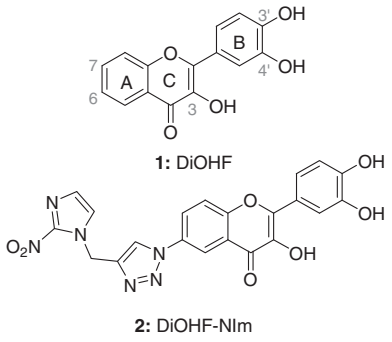

Figure 1. Structure of 3′,4′-dihydroxyflavonol (DiOHF, **1**) and putative hypoxia-targeted flavonol (DiOHF–NIm, **2**).

Ischaemia and reperfusion causes vascular and organ damage as demonstrated by a reduced vasodilator reserve.[8,9] The reduction in the capacity to dilate the vasculature arises from the adhesion of leukocytes to the endothelium and consequent plugging of capillaries in the microvasculature—termed the no-reflow phenomenon.[10] A rapid burst of oxygen radical generation upon reperfusion contrib-

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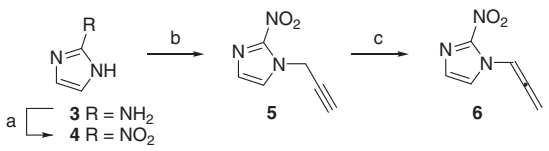
utes to this vascular blockage by enhancing leukocyte activation and adhesion.[10] As a result the reperfused tissue affected by ischaemia remains poorly perfused and hypoxic.

Nitroimidazoles are a class of reductively-activated drugs that include the antibiotics metronidazole (Flagyl, Sanofi-Aventis) and tinidazole (Fasigyn, Pfizer), the radiation sensitizer misonidazole, and the promising antituberculosis drug PA-824.[11,12] Nitroimidazoles undergo differential metabolism in hypoxic versus normoxic cells and enter cells by diffusion wherein they undergo a cascade of reductions[13,14] producing reactive intermediates (e.g., hydroxylamines) that can react with intracellular nucleophiles, resulting in trapping of the molecule within cells as bioconjugates.[14] Alternatively, the nitro group can be reduced to an amine, which upon protonation affords an ionic species that cannot diffuse from the cell.[14] ^{18}F-Labelled nitroimidazoles (e.g., F-MISO) have been used as positron emission tomography markers to visualize hypoxic regions.[15] The hypoxia-targeting property of 2-nitroimidazoles has been exploited in cancer chemotherapy with conjugates of 2-nitroimidazole and alkylating agents used to selectively kill hypoxic tumour cells.[16] Nitroimidazoles also act as hypoxia-selective radiation sensitizers, through their ability to accept an electron from radiation-created DNA radicals, forming the nitro radical anion, which then fragments to form DNA-alkylating agents.[17]

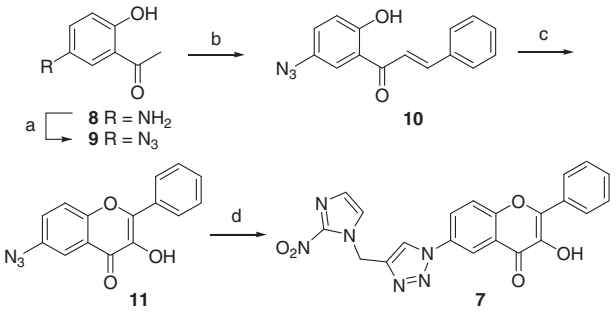
In this study, we investigated whether the pharmacological activity of DiOHF could be enhanced by targeting it to a potential site of action, namely poorly perfused hypoxic tissue where reperfusion leads to high levels of damaging reactive oxygen species. On the basis of previous structure–activity studies we have designed a DiOHF–2-nitroimidazole conjugate (DiOHF–NIm) **2** that should possess antioxidant properties but be attenuated in its vasorelaxant effect (Fig. 1B). We report proof-of-concept studies to determine if DiOHF–NIm is selectively metabolized under hypoxic conditions, and its in vitro antioxidant and vasorelaxant activities. Finally, we compare the ability of DiOHF–NIm and DiOHF to protect against I/R injury in rat hindquarters.

A triazole linker was selected owing to its metabolic stability and ease of introduction from azide- and alkyne-functionalized precursors.[18–20] The site of attachment of the nitroimidazole to the flavonol was identified from previous structure–activity relationship studies. Specifically, the antioxidant activity of DiOHF is optimal when the B ring bears a 3′,4′-catechol.[2–4,21–23] Moreover, the 3-OH group attached to the 2,3-double bond and adjacent to the 4-carbonyl in the C ring is a feature of the most effective flavonoid antioxidants.[22] DiOHF is also vasorelaxant, but substitution at the 6- or 7-positions of the A ring results in attenuation of vasorelaxant activity,[24] which may be useful in therapeutic applications to mitigate unwanted effects upon blood pressure. 2-Nitroimidazole was used as a hypoxia-targeting agent as its reduction potential (−418 mV at pH 7)[25] lies within the range that is believed to be appropriate for selective activation within hypoxic cells (−330 to −450 mV),[16,17] and as it has been used in a range of experimental hypoxia-targeting agents.[16] As a class 2-nitroimidazoles possess relatively weak cytotoxic properties under hypoxia and so should not exacerbate tissue damage caused by I/R.[26]

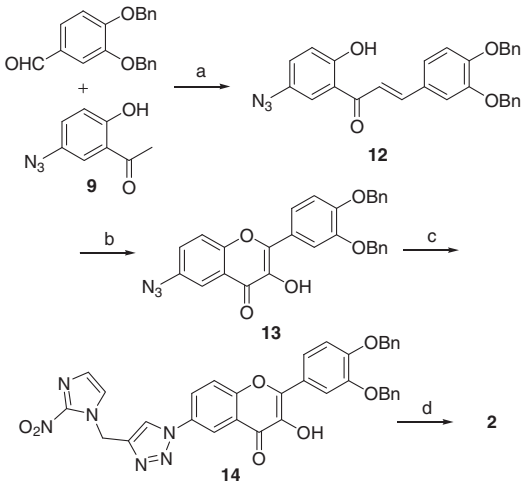
Nitroimidazole **4** was prepared by diazotization of 2-aminoimidazole **3**, followed by nitration using sodium nitrite and $CuSO_4$



Scheme 1. Reagents and conditions: (a) fluoboric acid, $NaNO_2$, $CuSO_4$, 65%; (b) propargyl bromide, K_2CO_3, dry acetone, 5 h, 80%; (c) as for (b) but for overnight.



Scheme 2. Reagents and conditions: (a) $NaNO_2$, concd H_2SO_4, NaN_3, 91%; (b) benzaldehyde, NaOH, EtOH, 57%; (c) H_2O_2, NaOH, EtOH, 46%; (d) **5**, $CuSO_4$, sodium ascorbate, TBTA, DMSO/water, 50%.



Scheme 3. Reagents and conditions: (a) 40% KOH, EtOH, dioxane, 66 h; (b) 5.4% NaOH, H_2O_2, EtOH, dioxane, 29% over two steps; (c) **5**, $CuSO_4$, sodium ascorbate, TBTA, DMSO/water, 75%; (d) TFA, thioanisole, 38%.

(Scheme 1).[27] Treatment of 2-nitroimidazole **4** with propargyl bromide in refluxing acetone in the presence of K_2CO_3 for 5 h afforded the propargylated nitroimidazole **5**. When this reaction was run overnight, the sole product isolated was the allene **6**, suggesting that the latter is thermodynamically favoured (Scheme 1).

The nonhydroxylated flavonol analogue **7** was initially targeted as a model compound upon which to develop a viable synthetic route. Thus, diazotization of **8**,[28] followed by azide substitution furnished 5-azido-2-hydroxyacetophenone **9** in good yield (91%). Sequential Claisen–Schmidt condensation and Algar–Flynn–Oyamada reaction of **9** smoothly afforded the azidoflavonol **11** (Scheme 2). The CuAAC reaction was performed using Cu^I generated in situ from $CuSO_4$ in the presence of the reducing agent sodium ascorbate, and the Cu^I-stabilizing ligand, tris-(benzyltriazolylmethyl)amine (TBTA).[29,30] The TBTA ligand enhances reaction yields through preventing disproportionation of Cu(I) in aqueous solvent.[30] CuAAC reaction of **5** and **11** in a DMSO/water mixture afforded the target compound **7**.

DiOHF–NIm **2** was prepared through a similar route commencing from **9** and 3,4-dibenzyloxybenzaldehyde (Scheme 3). Tandem Claisen–Schmidt condensation and Algar-Flynn-Oyamada oxidation afforded 6-azido-3′,4′-dibenzyloxyflavonol **13**. CuAAC reaction with **5** afforded the triazole **14**. Debenzylation of **14** proved challenging, and investigation of a range of debenzylation conditions ultimately identified TFA/thioanisole as the preferred choice, affording DiOHF–NIm **2** in a 38% yield.

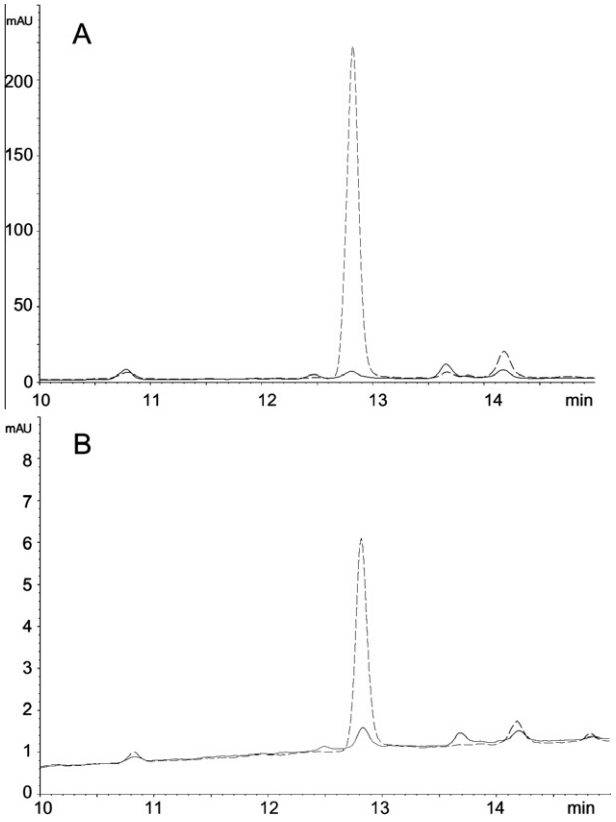


Figure 2. Overlay of HPLC traces of Con8 cells incubated with **2** under normoxic (- - -) and hypoxic (—) conditions: (A) culture supernatant, (B) cell lysate. Equivalent amounts of lysate or supernatant were sampled and after sample preparation were analyzed by HPLC. *Y*-axis represents absorbance units (mAU) at 370 nm; *x*-axis indicates time of HPLC run. R_t (**2**) = 12.9 min. Note difference in vertical scale between panels A and B.

In order to gain evidence for selective metabolism of DiOHF–NIm **2** under hypoxic conditions DiOHF and DiOHF–NIm **2** were incubated with rat adenocarcinoma Con8 cells under normoxic and hypoxic conditions and the presence of drug assessed by HPLC analysis of cell lysate and supernatant (Fig. 2). DiOHF was detected at similar amounts in cell supernatant and cell lysate when incubated under either normoxic or hypoxic conditions (data not shown), demonstrating that this flavonol can penetrate cell membranes. HPLC analysis of cell lysate and supernatant from Con8 cells incubated with DiOHF–NIm **2** under normoxic conditions showed the presence of this flavonol in significant amounts in both fractions. In contrast, in the cell supernatant and lysate of cells incubated with DiOHF–NIm **2** under hypoxic conditions the amount detected was substantially reduced. Interestingly, no new compounds could be identified, which is consistent with its conversion to a reactive intermediate such as a hydroxylamine or a radical anion that can react with cellular constituents such as proteins. This supports the conclusion that DiOHF–NIm **2** undergoes hypoxia-selective metabolism resulting in sequestering of the flavonol within the cell.

Vasorelaxant activity was assessed by measuring inhibition of contraction of rat thoracic aorta induced by phenylephrine (PE), as it has previously been shown that flavonols can act as functional antagonists of PE.[2] As shown in Figure 3A, DiOHF–NIm **2** showed weak inhibition of PE-induced contraction whereas DiOHF was able to significantly inhibit vasocontraction by PE. DiOHF–NIm **2** was also assayed for the ability to cause relaxation in precontracted rat aortic rings. The vascular activity of **2** was compared to that of the parent compound, DiOHF, which is the most potent vasorelaxant flavonol that has been identified to date.[1,2,4] DiOHF, but not **2**, can completely relax precontracted rat aortic rings (pEC_{50} of 5.33 ± 0.07) (Fig. 3B). This finding agrees with our previous study,[24] wherein substituents introduced on the A ring of DiOHF results in attenuation of vascular activity. The mechanism(s) by which flavonols cause vasorelaxation remains an open question, although it has been suggested that it is related to their ability to inhibit the utilization of calcium in vascular smooth muscle contraction.[2,31] It has also been shown that the vasorelaxant activity of DiOHF derives, in part, from inhibition of the Rho/RhoA kinase pathway.[32]

It is argued that flavonol antioxidant activity preserves vascular function in the presence of oxidative stress owing to their ability to scavenge ROS before they can react with the endogenous vasodilator NO.[33] Antioxidant activity was assessed against superoxide produced by NADPH oxidase in rat aortic rings. DiOHF–NIm **2** (at 10^{-6}–10^{-4} M) significantly reduced superoxide levels (ANOVA, Dunnett's post test, $p < 0.01$), from aortic rings (Fig. 4A). Thus, while substitution of the A ring of DiOHF with a nitroimidazole moiety results in attenuation of vascular activity, antioxidant activity is largely unaffected. To test the ability of **2** to prevent oxidant-induced endothelial dysfunction, endothelium-dependent relaxation was assessed in the presence of the autooxidant pyrogallol. Incubation with pyrogallol caused approximately 50% reduction in R_{max} to acetylcholine, whereas **2** at 10^{-5} M was able to prevent endothelial dysfunction significantly ($p < 0.001$, Fig. 4B).

Finally, we examined the effect of DiOHF and DiOHF–NIm **2** on I/R injury in rat hindquarters. Serum lactate dehydrogenase (LDH) levels are a useful biomarker to assess injury resulting from hind-

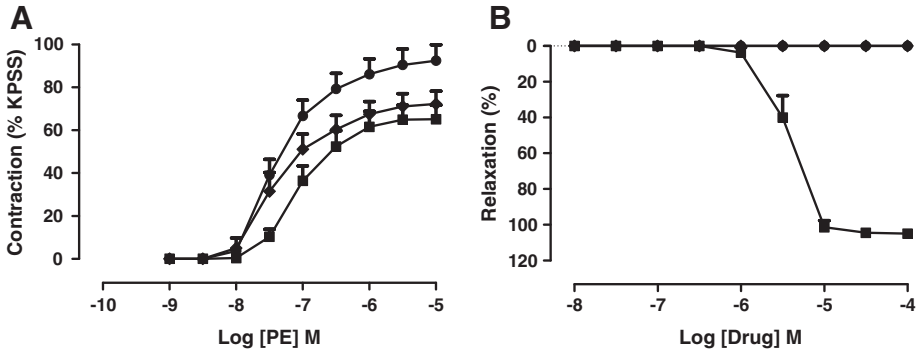


Figure 3. (A) Effect of **2** and DiOHF on PE-induced contraction of isolated rat aortic rings. Concentration–response curve to PE in the presence of DiOHF, 10^{-5} M (■; $n = 4$), **2**, 10^{-5} M (♦; $n = 6$) or vehicle (●; $n = 6$). For DiOHF the maximum response (R_{max}) to PE is significantly different to R_{max} to PE when the aortic ring was incubated with vehicle ($p < 0.001$, one way ANOVA, Dunnett's post test). (B) Effect of **2** and DiOHF on precontracted rat aortic rings. Relaxation of precontracted rat aortic rings to vehicle (●; $n = 4$), DiOHF (■; $n = 4$) and **2** (♦; $n = 3$). Aortic rings were precontracted using a combination of PE and U46619 to a similar level (40–60%).

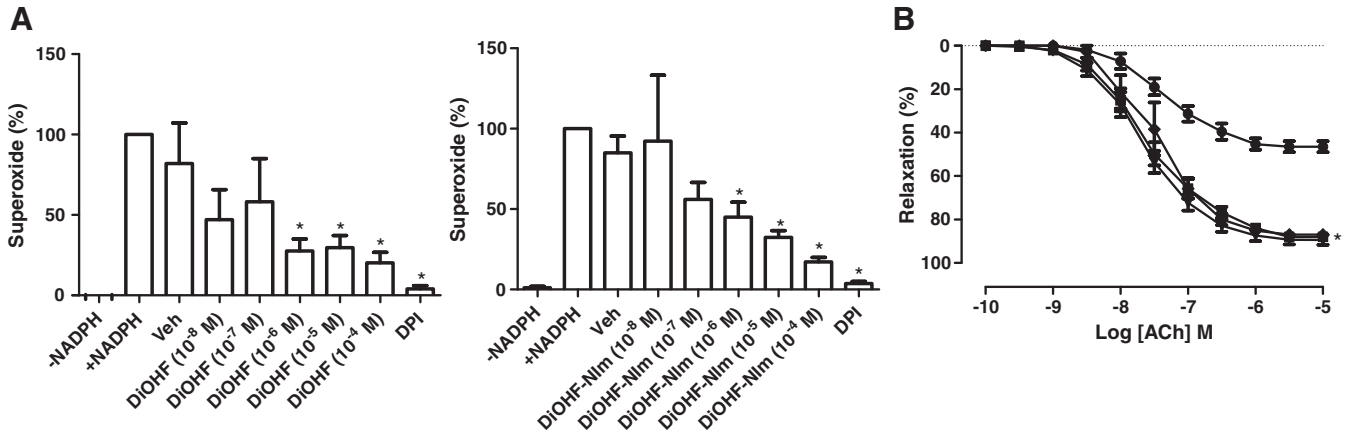


Figure 4. (A) Effect of **2** and DiOHF on NADPH-activated superoxide levels measured by lucigenin-enhanced chemiluminescence. Superoxide production was measured in the presence of a range of flavonol concentrations. DiOHF (at 10^{-6}–10^{-4} M; $n = 6$), and **2** (at 10^{-6}–10^{-4} M; $n = 6$–7) were able to significantly reduce superoxide levels ($p < 0.01$, ANOVA, Dunnett's post test). The non-selective NADPH oxidase inhibitor diphenylene iodonium chloride (DPI; $10^{-4.5}$ M) was used as a positive control. (B) Effect of **2** and DiOHF on acetylcholine-induced relaxation of rat aortic rings in the presence of the superoxide generator, pyrogallol. Concentration–response curve to acetylcholine in the presence of pyrogallol and vehicle (●; $n = 12$), pyrogallol and DiOHF, 10^{-5} M (■; $n = 12$), pyrogallol and **2**, 10^{-5} M (♦; $n = 6$), or vehicle only (▼; $n = 6$). DiOHF–NIm **2** and DiOHF were able to significantly prevent pyrogallol-induced endothelial dysfunction when R_{max} of the flavonol treatments are compared to R_{max} of pyrogallol and vehicle curve ($p < 0.001$, ANOVA, Dunnett's post test). Aortic rings were precontracted using a combination of PE and U46619 to similar levels (40–60%).

quarters I/R in anesthetized rats. Bilateral hindquarters I/R in rats caused elevated circulating levels of LDH after 30 min until 3 h of reperfusion, peaking at 3 h. Blood samples taken after 2 h of ischaemia but before reperfusion did not show elevated levels of LDH (331 ± 37 U/L), whereas reperfusion significantly increased plasma LDH levels. Rats treated with a bolus dose of DiOHF (5 mg/kg) 5 min prior to reperfusion showed a reduction in circulating LDH levels, which was significant only at the 2 and 3 h time points. However, treatment with an equimolar dose of **2** (8.6 mg/kg) significantly reduced plasma LDH levels, which was significant at all time points measured after reperfusion (Fig. 5A).

As MPO is the most abundant protein found in neutrophils,[34] measurement of MPO activity is a useful biomarker for neutrophil accumulation. Neutrophil accumulation has been shown to occur in the hindlimb muscle upon hindquarters I/R.[35] Hindquarters I/R resulted in a significant increase in myeloperoxidase (MPO) levels compared to sham-operated animals (Fig. 5B). The increase in MPO activity indicates neutrophil accumulation at the site of injury, which is a contributor to reperfusion injury. Treatment with DiOHF–NIm **2** 5 min before reperfusion was able to significantly attenuate this increase ($p < 0.05$). The level of MPO after DiOHF treatment was not significantly different to vehicle. The mechanism by which flavonols reduce LDH levels probably relates to their antioxidant and ROS-scavenging activities. ROS are an important contributor to I/R injury with a large burst of ROS being consistently shown to occur upon reperfusion.[36–38]

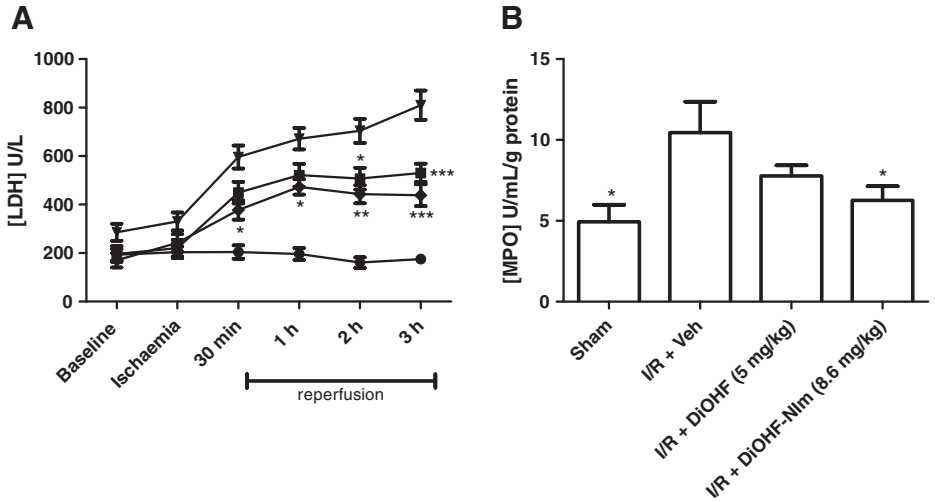


Figure 5. (A) Effect of flavonols on LDH release following hindquarters I/R. LDH levels in serum of blood samples taken from the anaesthetized sham-operated rats (●; $n = 9$); rats subjected to hindquarters I/R (▼; $n = 18$); I/R rats treated with DiOHF, 5 mg/kg (■; $n = 9$), and I/R rats treated with DiOHF–NIm **2**, 8.6 mg/kg (♦; $n = 9$). Hindquarters I/R caused a steady increase in LDH levels over time. All treatments were administered in a bolus dose 5 min before reperfusion. DiOHF treatment was able to prevent the increase in LDH levels, significant at 2 h ($p < 0.05$) and 3 h ($p < 0.001$) after reperfusion. Similarly, **2** was also able to prevent this increase, significant at all time points of reperfusion. Areas under curve were analysed using two way ANOVA with Bonferroni's multiple comparison post test. $* = p < 0.05$; $** = p < 0.01$; $*** = p < 0.001$. (B) Effect of DiOHF and **2** on muscle MPO level following hindquarters I/R. MPO activity in muscle samples obtained from anaesthetized sham-operated rats ($n = 5$); rats subjected to hindquarters I/R ($n = 8$); I/R rats treated with DiOHF, 5 mg/kg ($n = 6$) and I/R rats treated with **2**, 8.6 mg/kg ($n = 5$). Hindquarters I/R induced a significant increase in MPO levels in the muscle. DiOHF–NIm **2** was able to significantly reduce MPO levels in the muscle ($p < 0.05$, one way ANOVA, Dunnett's multiple comparison post test).

In summary, DiOHF–NIm **2** lacks vascular activity compared to the parent DiOHF. Both DiOHF and **2** possess functional antioxidant activity and can significantly reduce NADPH-activated superoxide ($O_2^{-\bullet}$) levels in vitro to similar extents. Studies in normoxic and hypoxic cells indicate selective consumption of **2** only under hypoxic conditions, consistent with the proposed mechanism of hypoxia-targeting by the 2-nitroimidazole group. In rat hindquarters I/R, where tissue injury was quantified by assaying serum LDH and muscle MPO levels, it was found that while both DiOHF and **2** were able to significantly reduce serum LDH levels, only **2** provided a significant reduction at the early time points of 30 min and 1 h. Finally, muscle MPO levels were significantly increased after hindquarters I/R in rats. Only DiOHF–NIm **2**, and not DiOHF, was able to significantly reduce muscle MPO levels after I/R. Taken together these results indicates that while both of these flavonols are protective against I/R-induced injury, the protective effect is enhanced in the hypoxia-targeted derivative **2**. A possible explanatory mechanism is that the antioxidant activity of **2** reduces the amount of ROS produced upon reperfusion, limiting the damage produced and the resultant inflammatory response, thereby protecting against neutrophil-mediated reperfusion injury.

Several aspects of this study deserve further comment. Firstly, the fact that DiOHF–NIm **2** lacks vasorelaxant activity yet exhibits improved efficacy relative to DiOHF in limitation of I/R injury suggests that the antioxidant activity of flavonols is the most important contributor to the pharmacological mechanism of action for protection against I/R injury. In some circumstances the co-occurrence of antioxidant and vasorelaxant activities in a cardioprotective agent may be a liability for clinical usage, such as in patients suffering a myocardial infarction where impaired cardiac output can threaten adequate organ perfusion and any further hypotensive effect in response to an administered therapy may exacerbate that situation. Secondly, these studies investigated the administration of flavonols with clinically relevant timing as a bolus dose five minutes before reperfusion. As this is late in the period of ischaemia, and as perfusion is incomplete due to the no reflow phenomenon, the potential for accumulation of **2** may well be limited. Alternative timings for the administration of flavonols in the course of the I/R program may be useful for other therapeutic applications such as organ preservation. Thirdly, I/R injury arises from a range of phenomena in addition to ROS stress. At the single dose at which these flavonols were administered, the antioxidant effects may already be maximized and so there may be no possibility for further benefit to be provided. The ability of DiOHF–NIm **2** to reduce I/R injury indicates that it deserves further investigation as an adjunctive therapy for the prevention and treatment of I/R injury, for example in acute myocardial infarction.

Acknowledgments

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Supplementary data

Supplementary data (complete experimental details for the synthesis of all new compounds and biological analyses) associated with this article can be found, in the online version, at doi:10.1016/j.bmcl.2011.03.040.

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