

## REVIEW

# Recent developments in nitric oxide donor drugs

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During the 1980s, the free radical, nitric oxide (NO), was discovered to be a crucial signalling molecule, with wide-ranging functions in the cardiovascular, nervous and immune systems. Aside from providing a credible explanation for the actions of organic nitrates and sodium nitroprusside that have long been used in the treatment of angina and hypertensive crises respectively, the discovery generated great hopes for new NO-based treatments for a wide variety of ailments. Decades later, however, we are still awaiting novel licensed agents in this arena, despite an enormous research effort to this end. This review explores some of the most promising recent advances in NO donor drug development and addresses the challenges associated with NO as a therapeutic agent.

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**Keywords:** nitric oxide; nitric oxide donor drugs; S-nitrosothiol; diazeniumdiolate; NONOate; furoxan; nitroaspirin; organic nitrate

**Abbreviations:** ACE, angiotensin-converting enzyme; DEA/NO, diethylamine NONOate; EDHF, endothelium-dependent hyperpolarizing factor; GSNO, S-nitroso-glutathione; GTN, glyceryl trinitrate; ISMN, isosorbide mononitrate; mtADH, mitochondrial aldehyde dehydrogenase; NCI, National Cancer Institute; NO, nitric oxide; eNOS, nitric oxide synthase; iNOS, inducible nitric oxide synthase; NSAID, nonsteroidal anti-inflammatory drug; PETN, pentaerythryl tetranitrate; PPI, protein pump inhibitor; PSA, prostate-specific antigen; sGC, soluble guanylate cyclase; SNAP, S-nitroso-N-acetylpenicillamine; SNO-Cap, S-nitroso-captopril; SNP, sodium nitroprusside; SNVP, S-nitroso-N-valerylpenicillamine; SPER/NO, spermine NONOate; TNF- $\alpha$ , tumour necrosis factor- $\alpha$ ; t-PA, tissue-type plasminogen activator; VSMC, vascular smooth muscle cell; vWF, von Willebrand factor

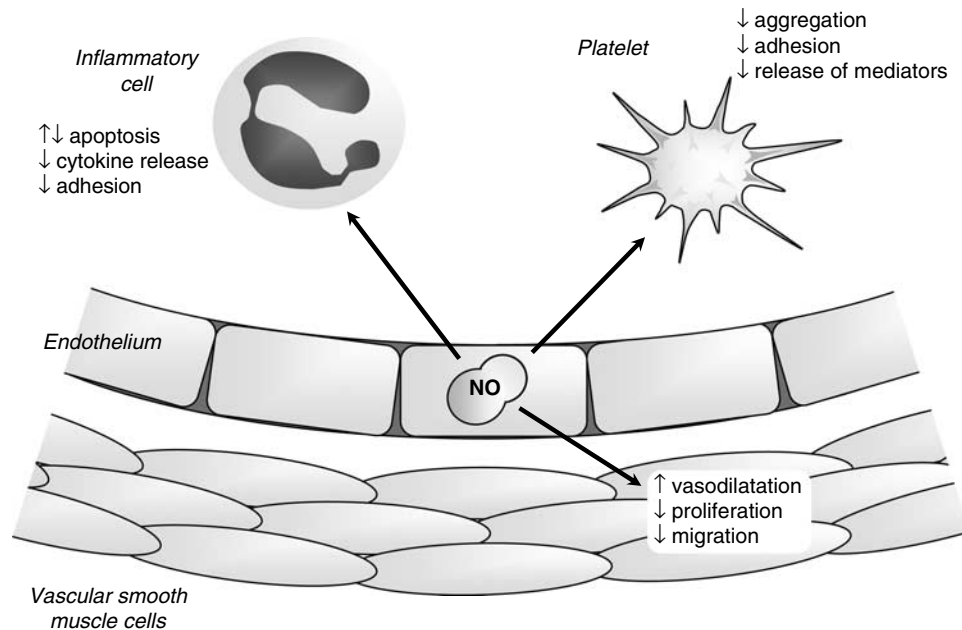
## Introduction

In 1992, the free radical, nitric oxide (formula  $\bullet\text{N}=\text{O}$ , abbreviated to NO), was awarded the curious accolade of 'molecule of the year' (Culotta and Koshland, 1992) and the extensive body of research already built around this small molecule continues to increase unabated, with ~13 000 papers focused on the topic in the past 5 years alone. Despite its structural simplicity, NO has a complex chemistry, endowing the free radical with wide and varied biological actions. NO is synthesized by a number of cell types, where it acts as a highly regulated autocrine and paracrine signalling molecule. Importantly, its sphere of influence is likely to only extend to ~100  $\mu\text{m}$  of its origin on account of its reactive nature. This property of NO has important consequences: first, it means that NO must be rapidly synthesized on demand in response to stimuli and second, it is truly a local mediator that does not require complex metabolism for clearance; it is simply diluted and oxidized to nitrite and

nitrate as it diffuses away from its source. This property is both the greatest weakness and strength of NO in relation to its use as a therapeutic agent, as will be discussed later. It is worth noting, however, that nitrite (Gladwin *et al.*, 2006) and/or some of the possible sinks for NO (haemoglobin; Singel and Stamler, 2005, albumin; Crane *et al.*, 2002) are not necessarily inactive by-products but might themselves be complex NO 'donors'. This aspect of NO biology is highly complex and contentious (Hobbs *et al.*, 2002) and is beyond the remit of this review.

The most studied actions of NO are in the cardiovascular system (Figure 1), where it is continuously produced by the endothelial cells that line the lumen of blood vessels. Here, L-arginine is converted into NO by the endothelial isoform of the enzyme, NO synthase (eNOS), in response to mechanical and chemical stimuli that act by mobilizing intracellular calcium. NO diffuses in three dimensions away from the cell of origin, passing easily through membranes of neighbouring cells, where it can bring about a range of physiological effects. In vascular smooth muscle cells (VSMCs), it acts almost exclusively (Miller *et al.*, 2004) via the enzyme soluble guanylate cyclase (sGC) to stimulate the generation of cyclic guanosine-3',5'-monophosphate (cGMP) and elicit

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**Figure 1** The multifaceted action of NO in the cardiovascular system.

vasodilatation via cGMP-dependent protein kinases. NO is also recognized to inhibit VSMC proliferation by a cGMP-dependent mechanism (Jeremy *et al.*, 1999). Elsewhere, NO also has an impact on circulating platelets, where both cGMP-dependent and -independent (Crane *et al.*, 2005) mechanisms in response to endothelium and platelet-derived NO play a part in a powerful inhibitory effect on aggregation and adhesion. Similarly, endothelium-derived NO is a powerful inhibitor of inflammatory cell activation and, most notably, is recognized to be an important inhibitor of monocyte activity (Bath, 1993). Interestingly, the relative role of NO compared to other endothelium-derived vasodilators (prostacyclin, endothelium-derived hyperpolarizing factor; EDHF) is not uniform across the vascular system: NO generally predominates in large conduits, whereas EDHF is apparently dominant in resistance vessels. This localization of NO to conduits that have little impact on blood pressure determination might suggest that the primary role of NO lies in its antiatherothrombotic properties rather than its vasodilator effects, a suggestion that is supported by the evident impact of endothelial dysfunction in conduit arteries on atherogenesis.

It is important to recognize that the concentrations of NO required to mediate the primarily protective effects described above are extremely low (picomolar to nanomolar). An important feature of NO is that its properties and cellular targets at higher concentrations are profoundly different, particularly under conditions of oxidative stress, where it rapidly reacts with superoxide to form peroxynitrite ( $\text{ONOO}^-$ ). Under these circumstances, NO is highly cytotoxic, a feature that is exploited by inflammatory cells in response to invading pathogens by expressing an inducible form of NOS (iNOS) in concert with activation of NAD(P)H oxidase to cogenerate NO and superoxide, forming highly cytotoxic and cytostatic  $\text{ONOO}^-$ . At high concentrations, additional chemical reactions become relevant, especially those with

molecular oxygen, generating nitrosating species capable of regulating protein and cell function (Gow *et al.*, 2004). Of particular note is the inhibitory impact of NO, together with endogenous S-nitrosothiols and  $\text{ONOO}^-$  on cellular respiration through interaction with complexes in the respiratory chain (Beltran *et al.*, 2000; Brown and Borutaite, 2004). High concentrations of NO and related species also mediate apoptosis in inflammatory cells (Taylor *et al.*, 2003).

#### Endothelial dysfunction

Endothelial dysfunction is implicated in the development of a wide range of cardiovascular risk factors and conditions including atherosclerosis (Ludmer *et al.*, 1986), heart failure (Katz *et al.*, 1992), diabetes (Calver *et al.*, 1992b), hypertension (Calver *et al.*, 1992a), cigarette smoking (Newby *et al.*, 1999), hypercholesterolaemia (Drexler and Zeiher, 1991). Depression of the NO:sGC pathway is a key feature of endothelial dysfunction, occurring at a number of levels, including downregulation of eNOS expression and activity, uncoupling of NOS, scavenging of NO by oxygen-centred free radicals and decreased sensitivity of VSMCs to vasodilators, together with NO-independent alterations (Feletou and Vanhoutte, 2006). Loss of endogenous NO activity has a number of detrimental actions, most notably, vasoconstriction, increased smooth muscle cell proliferation, as well as increased activity and adherence of platelets and inflammatory cells at sites of endothelial damage. As the endothelium deteriorates, blood flow is disturbed and vessels become occluded by atheromatous plaque, or their associated thrombosis or emboli, ultimately leading to infarction of downstream organs, resulting in myocardial infarction, stroke and peripheral ischaemia.

At first sight, delivery of exogenous NO is an attractive therapeutic option, particularly with a view to slowing progression of atherosclerosis and reducing the risk of

thrombosis, on account of its multifaceted action. NO from existing organic nitrates effectively alleviates symptoms of angina through improved blood flow to the ischaemic region of the heart via dilatation of diseased vessels and collateral coronary arteries. Systemic dilatation of veins and resistance arteries also reduces both pre- and afterload, reducing the cardiac workload and oxygen consumption and limiting the pain associated with hypoxia. In addition, however, NO would be expected to inhibit VSMC proliferation and migration, limiting the development of the complex plaque, and inhibit platelet activation, aggregation and adhesion to areas of damage, reducing the extent of thrombosis. Furthermore, NO would be expected to inhibit inflammatory cell activation, preventing infiltration into the plaque and also the propagation of proinflammatory signals. Unfortunately, however, the latter benefits do not appear to be realized with organic nitrates and these drugs remain effective only as symptomatic treatments, rather than agents that might slow disease progression and improve outcome (see below). Whilst the multifaceted impact of NO should, at least in theory, constitute a benefit in this setting, it might equally be argued that the enormous variety of effects of NO in different tissues and systems might be a considerable limitation to systemic NO delivery, with unwanted side effects outside the target tissue.

#### NO donor drugs

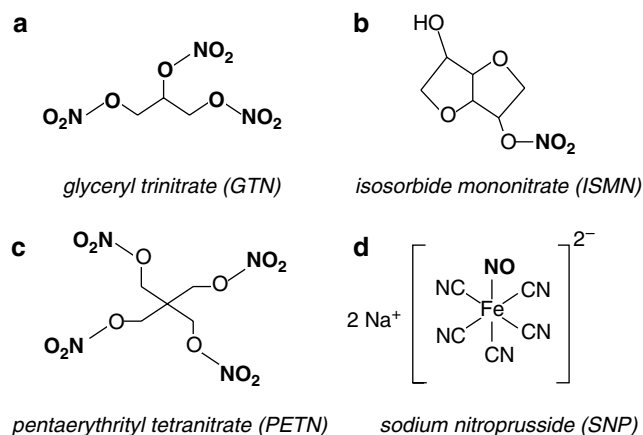
NO gas is notoriously difficult to handle on account of the problems associated with complete exclusion of oxygen to prevent oxidation to nitrogen dioxide. Nevertheless, the gas itself can be used therapeutically, particularly in pulmonary hypertension (Griffiths and Evans, 2005) and in neonates (Greenough, 2000), where it is delivered to the lungs via inhalation. This specific use apart, however, we are reliant on molecular carriers of NO (NO donor drugs) to stabilize the radical until such time as its release is required. Given the enormous potential of NO in cardiovascular medicine, it is somewhat astonishing that only two types of NO donor drug are currently used clinically and no new NO donors have reached the market since the discovery of NO as a crucial cardiovascular mediator in the 1980s. Recently, there have been a plethora of reviews on NO donor drugs, providing detailed descriptions of the different classes and neatly summarizing decades of research (Megson, 2000; Yamamoto and Bing, 2000; Burgaud *et al.*, 2002; Ignarro *et al.*, 2002; Megson and Webb, 2002; Napoli and Ignarro, 2003). This review will therefore focus on recent advances in NO donor drug development within the past 5–10 years, with special attention to diazeniumdiolates, *S*-nitrosothiols, NO donor hybrid drugs and NO-generating materials that show particular promise. Although the primary focus of the review is the prevention or treatment of cardiovascular conditions, it also highlights novel drug design with other goals in mind.

#### NO donors in current clinical use

Before considering novel NO donor drugs, it is necessary to briefly comment on existing drugs because there are several

new studies that have expanded our understanding of the compounds and might alter clinical practice.

The organic nitrates are the most commonly used NO donor drugs. Glyceryl trinitrate (GTN; also known as nitroglycerin; Figure 2a) is the best-studied nitrate, used mainly in acute relief of pain associated with angina, whereas other slower release preparations, such as isosorbide mononitrate (ISMN; Figure 2b), are used for the treatment of chronic angina. GTN ointments are also routinely used for the treatment of anal fissure (Fenton *et al.*, 2006), transdermal patches in heart failure and chronic angina, whereas nebulized GTN may have benefits in certain subgroups with pulmonary hypertension (Yurtseven *et al.*, 2003; Goyal *et al.*, 2006). GTN contains three nitro-oxy ester (from now on referred to as nitrate) groups, but releases only one molar equivalent of NO from the terminal position after bioactivation (Bennett *et al.*, 1989). However, the metabolism of GTN to NO has strict requirements beyond that of simple chemical reduction and it is now almost universally agreed that specific enzymes mediate this process *in vivo* (Thatcher *et al.*, 2004), although the requirement for NO release at all was recently challenged in an interesting paper (Kleschyov *et al.*, 2003). The identity of the nitrate-activating enzyme(s) has been intensely studied for over 30 years, generating a host of candidates and a mountain of confusing and often contradictory data. Recently, the work of Chen *et al.* (2002) has sparked a flurry of interest by convincingly showing that the enzyme, mitochondrial aldehyde dehydrogenase (mtADH), is a suitable candidate. Subsequent work has supported this proposal (Daiber *et al.*, 2004; Sydow *et al.*, 2004; Zhang *et al.*, 2004; Kollau *et al.*, 2005) and it has now been shown that mtADH meets most criteria of the physiological GTN-activating enzyme: it is an intracellular enzyme that is activated by low concentrations (nanomolar) of GTN; it contains active sulphhydryl groups that are redox regulated; it generates the correct metabolites (1,2-glycerol-dinitrate and NO) via an *S*-nitrosothiol or nitrite intermediate; it releases NO from physiologically active concentrations of GTN, inhibitors of mtADH inhibit 1,2-glycerol-dinitrate production and vasodilatation and its activity is reduced



**Figure 2** Chemical structure of NO donor drugs used clinically: the organic nitrates (a–c) and sodium nitroprusside (d). The NO-containing moiety is shown in bold.

with continuous use of GTN (summarized by Ignarro, 2002). In addition, transgenic mice lacking the mtADH genes do not have the high affinity pathway of GTN vasodilatation seen in wild-type mice (Chen *et al.*, 2005). The latter two points are crucial, because the main limitation of the organic nitrates is the well-documented development of tolerance with prolonged continuous use (succinctly reviewed in Gori and Parker, 2002a, b). The only reliable means to avoid tolerance is to incorporate a nitrate-free interval in the therapeutic regimen, which can be problematic for some forms of angina and is a clear impediment to the use of nitrates for management of chronic conditions. Additionally, *in vivo* studies in animals (Munzel *et al.*, 1995, 1999, 2000) and humans (Sage *et al.*, 2000) have shown that tolerance may be associated with endothelial dysfunction and enhanced oxidative stress. This may be a contributory factor in reports that long-term nitrate use causes a paradoxical increase in risk of cardiac events (Ishikawa *et al.*, 1996; Nakamura *et al.*, 1999). The underlying cause of tolerance and better methods of bypassing this problem might emerge once the mechanism of action of nitrates has been fully elucidated. Although the role of mtADH is compelling, contradictory findings have already been published (DiFabio *et al.*, 2003; de la Lande *et al.*, 2004) and a number of other important mechanistic questions remain, most notably: why is the activity of mtADH only altered by specific thiols and not others, for example glutathione (Ignarro 2002)? What chemical reactions allow the nitrate moiety from GTN to react with thiols and/or lead to NO production (Ignarro, 2002; Daiber *et al.*, 2004)? Why does the nitrate pentaerythrityl tetranitrate (PETN; Figure 2c) cause less desensitization of the esterase activity of mtADH and is less susceptible to tolerance (Daiber *et al.*, 2004)? Why do inhibitors of mtADH not inhibit all nitrates, for example ISDN (Daiber *et al.*, 2004)? Does the cellular distribution of mtADH explain why nitrates are venoselective (Ignarro, 2002), if indeed they are (Sogo *et al.*, 2000a; Miller *et al.*, unpublished results). In the face of a wide range of NO donors that do not develop tolerance (Brilli *et al.*, 1997; Miller *et al.*, 2000) and are better antiplatelet agents (Sogo *et al.*, 2000b), the prevalence of these drugs in the clinical setting may diminish in favour of other agents.

The only other notable advance with this class of drugs is the launch of BiDil (isosorbide dinitrate with hydralazine; Sica, 2006) in 2005 for use in heart failure in African Americans (Pukrein and Yancy, 2005). The development has revived interest in the arena, more for the controversy stirred up by its targeting at a specific racial group (Haga and Ginsburg, 2006) than for any particular novelty in the therapeutic approach.

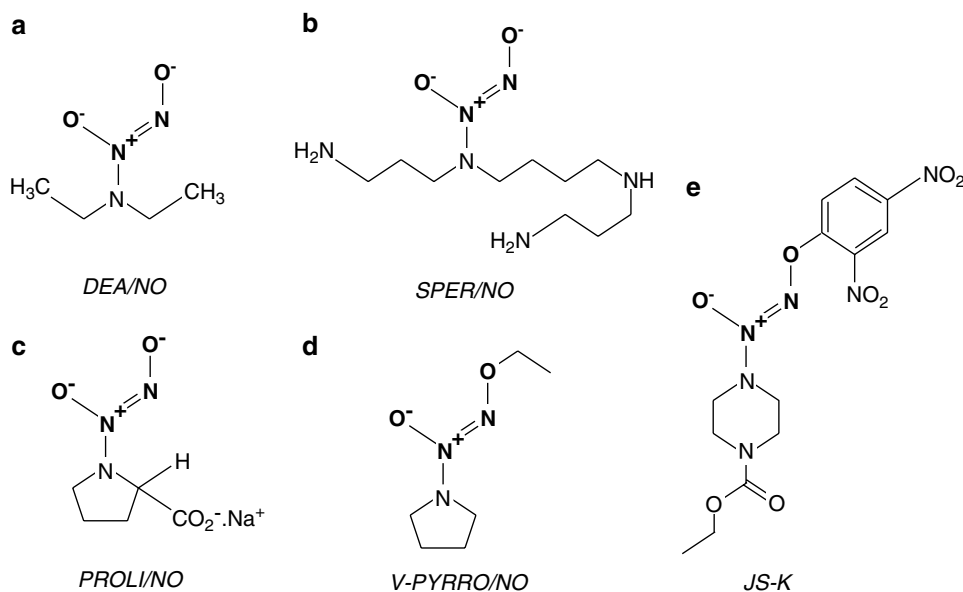
The other clinically relevant NO donor in current use is sodium nitroprusside (SNP; Figure 2d). SNP is used on-site in hospitals to provide rapid lowering of blood pressure in hypertensive crises. SNP is also the drug of choice in clinical studies, where it is recognized as the gold standard NO-dependent, but endothelium-independent vasodilator, although the complex mechanism involved in NO release might indicate that some of the novel NO donors that are emerging might be better suited for this purpose.

The mechanism of NO release from SNP in biological tissue is more complex than is often assumed and has not yet been fully elucidated. Contrary to popular belief, SNP is relatively stable and does not release NO spontaneously in the physiological environment; instead, NO generation requires either light or a tissue-specific mode of release (Butler and Megson, 2002; Grossi and D'Angelo, 2005). Of particular concern with this NO donor is the potential for release of any of the five cyanide groups incorporated in the structure (Bates *et al.*, 1991). Indeed, there have been isolated reports that long-term use of this agent can be associated with cases of cyanidosis, albeit rarely (see Butler and Glidewell, 1987). Further limitations for the use of SNP are its requirement for intravenous administration, sensitivity to photolysis once in solution and its remarkable potency, which can make dose titration difficult (Megson, 2000). Given these limitations, it is difficult to envisage a wider application of this NO donor drug.

### Diazeniumdiolates (NONOates)

Diazeniumdiolates (also known as 'NONOates') have been recognized for many years. The first of this class to be described was an adduct of diethylamine and NO (diethylamine NONOate; DEA/NO), first synthesized in 1960 (Drago and Paulik, 1960). However, diazeniumdiolates only became the focus of attention in the NO world in the 1990s, when their NO donor properties were considered in biological settings (Maragos *et al.*, 1991). These compounds consist of a diolate group  $[N(O-)N=O]$  bound to a nucleophile adduct (a primary or secondary amine or polyamine) via a nitrogen atom (Maragos *et al.*, 1991). NONOates decompose spontaneously in solution at physiological pH and temperature, to generate up to 2 molar equivalents of NO. The rate of decomposition is dependent on the structure of the nucleophile (Hrabie *et al.*, 1993). A range of NONOates have now been described with half-lives varying from seconds to hours (Morley and Keefer, 1993). An attractive feature of this class of compounds is that their decomposition is not catalysed by thiols or biological tissue, unless specifically designed to (see below) and, because NO release follows simple first-order kinetics (Morley *et al.*, 1993; Mooradian *et al.*, 1995; Kavdia and Lewis, 2003), the rate of NO release can be accurately predicted. Subsequently, biological activity such as vasodilatation (Maragos *et al.*, 1991; Morley *et al.*, 1993), inhibition of platelet aggregation (Diodati *et al.*, 1993; Sogo *et al.*, 2000b), inhibition of blood coagulation (Nielsen, 2001) and inhibition of VSMC proliferation (Mooradian *et al.*, 1995) closely correlate with the amount of NO generated *in vitro*. Additionally, the lack of tissue requirement for NO release is most likely responsible for the apparent lack of tolerance experienced with these compounds (Brilli *et al.*, 1997).

At present, NONOates are not used clinically, although they have been tested frequently in experimental models of cardiovascular disease. For example, DEA/NO (Figure 3a) prevents and reverses vasospasm in a primate model of subarachnoid haemorrhage, without affecting systemic blood pressure (Pluta *et al.*, 1997). Several different NONOates



**Figure 3** Chemical structure of five examples of the diazeniumdiolate (NONOate) class of NO donor drug. The diolate group (shown in bold) releases NO in solution, although prior cleavage of the molecule to release the terminal oxygen may be required (i.e. for V-PYRRO/NO and JS-K).

have been shown to lower pulmonary vascular resistance, with (Vanderford *et al.*, 1994) or without (Brilli *et al.*, 1997) decreasing systemic vascular resistance, depending on the design of the nucleophile adduct. Actions can be further improved by using formulations to aerosolize NONOates in combination with surfactants (Jacobs *et al.*, 2000), making these drugs useful alternatives in the treatment of pulmonary hypertension or acute lung injury. NONOates may also have a use in the treatment of erectile dysfunction by enhancing blood flow to the penis (Talukdar and Wang, 2005), although it remains to be determined whether these drugs can be applied in a way that would have advantages over the sildenafil-type drugs.

The primary cardiovascular focus for NONOates has been in the prevention of thrombosis and neointimal formation following vascular injury, an inevitable result of interventional cardiology techniques, such as balloon angioplasty, bypass grafting or placement of stents. Spermine NONOate (SPER/NO; Figure 3b), applied perivascularly, reduces neointimal formation induced by peri-arterial collars (Yin and Dusting, 1997), balloon angioplasty (Kaul *et al.*, 2000) and also in bypass veins (Chaux *et al.*, 1998). MAHMA/NO-eluting stents also reduce platelet adhesion to artificial grafts (Hanson *et al.*, 1995). Infusion of PROLI/NO (Figure 3c) decreases platelet deposition to downstream polyester vascular grafts in the baboon without affecting mean arterial pressure (Saavedra *et al.*, 1996). Local infusion devices releasing PROLI/NO also reduce cell proliferation following endarterectomy-induced injury (Chen *et al.*, 1997). PROLI/NO, and other NONOates have been incorporated into an insoluble solid matrix that can be used to coat surfaces such as glass (Saavedra *et al.*, 1996) and polymer films (Mowery *et al.*, 2000) to minimize the thrombogenicity of localized delivery devices, extracorporeal circuits and blood pressure monitors. The same group have also synthesized a NONOate

adduct of heparin that is still able to inhibit thrombin-induced platelet aggregation, but has the advantage over conventional heparin in that it can also inhibit ADP-induced platelet aggregation (Saavedra *et al.*, 2000a). However, this compound has only had limited success when incorporated into a polymer film (Mowery *et al.*, 2000). NONOates have also been used to coat the external surface of oxygen-sensing electrodes to prevent thrombus formation on the sensor, which can interfere with measurements of blood gases and electrolytes (Schoenfisch *et al.*, 2000).

Modification of the structure of the nucleophile adduct to protect the terminal oxygen of the diolate moiety can stabilize the drug in solution and potentially engender a selective NO release in different organs, vascular beds or specific cell types. For example, Saavedra *et al.* (1997) have made O-alkenyl/O-alkyl derivatives of existing NONOates (e.g. V-PYRRO/NO; Figure 3d) to target NO release in liver cells. Of five cell types tested, V-PYRRO/NO blocked tumour necrosis factor- $\alpha$  (TNF- $\alpha$ )-induced apoptosis only in hepatocytes and protected against experimentally induced liver toxicity in rats *in vivo*. Similarly, another NONOate prodrug was synthesized that was stable in solution, but released NO following esterase activity within cells, causing apoptosis of the human leukaemia cell lines studied (Saavedra *et al.*, 2000b). The group have also used piperazine as a linker molecule to produce a fluorescent NONOate (GLO/NO), and a method whereby the NONOate group can be linked to nonsteroidal anti-inflammatory drugs (NSAIDs), vitamin B<sub>3</sub> and polyethylene glycol (Saavedra *et al.*, 1999). The use of these compounds in biological systems largely remains at a theoretical level at present.

The use of NONOate adducts has received interest from the viewpoint of targeting delivery of high concentrations of NO specifically to tumour cells. Wang's group (Tang *et al.*, 2001) coupled PYRRO/NO to a chain of amino acids

recognized to be a substrate for prostate-specific antigen (PSA). PSA is inactive in blood plasma, but upregulated within prostate cancer metastases. This conjugated NONOate did not release NO in solution in the absence of PSA, offering the possibility that NO release will occur exclusively within cancer cells rather than in the blood. The same group have also synthesized sugar-conjugated NONOates, which they envisage will be preferentially taken up by cancer cells that overexpress the GLUT-1 transporter (Wu *et al.*, 2001). A NONOate group has also been attached to the frequently used antitumour agent, 5-fluorouracil, engendering greater cytotoxicity on the prostate and cancer cells tested (Cai *et al.*, 2003). More recently, the group has described the antitumour activity of a  $\beta$ -galactosyl-linked NONOate (Chen *et al.*, 2006a), although the authors note that for clinical purposes, gene therapy to transfect tumour cells with LacZ would be required before drug administration. The same compound was also a highly efficient antibacterial agent (Chen *et al.*, 2006b). JS-K (Figure 3e), a terminal oxygen-protected NONOate, has been developed by the US National Cancer Institute (NCI). The glutathione *S*-transferase enzymes are responsible for cleaving the compound to expose the diolate group and release NO, but despite the ubiquitous nature of these enzymes, JS-K slows the growth of cancer cells without harming healthy cells (Shami *et al.*, 2003). Since being accepted as part of the NCI's 'Rapid Access to Interventional Development (RAID) programme, JS-K has been shown to be active against a wide range of cancer cells (Cai *et al.*, 2005).

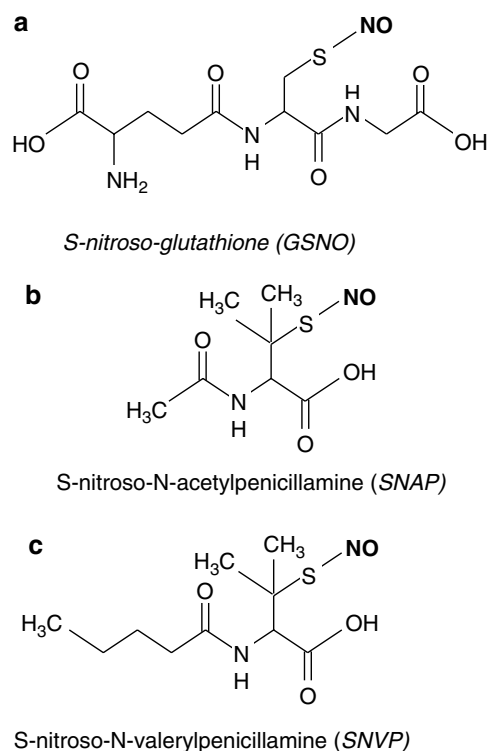
We are currently aware of only a single study using NONOates in humans (in patients with respiratory distress syndrome; Lam *et al.*, 2002). However, the predictable nature of NO release from NONOates will undoubtedly lead to further clinical investigations, once long-term safety has been established. The toxicity of by-products needs to be more fully confirmed (Lam *et al.*, 2003), especially as subsequent reactions between decomposition products could lead to the formation of carcinogenic nitrosamines (Maragos *et al.*, 1991). Incorporation of NONOates into polymers may represent a means of preventing the leaching of by-products (Mowery *et al.*, 2000). At present, conjugated NONOates hold a great deal of promise, especially for the treatment of certain cancers, although further characterization of these drugs is essential before they reach larger clinical trials. The potential for oral preparations of NONOates has yet to be fully clarified, although transdermal preparations have already been developed (Shabani *et al.*, 2001). However, experimentally, the NONOates remain an invaluable scientific tool for researching NO physiology.

## S-Nitrosothiols

The *S*-nitrosothiol class of NO donors covers a vast array of different compounds which contain a single chemical bond between a thiol (sulphydryl) group (R-SH) and the NO moiety. Biological activity of *S*-nitrosothiols is highly influenced by the molecular environment of the parent thiol. That said, the complex chemistry of NO release from even the most basic *S*-nitrosothiol gives these compounds several means by which they can confer NO bioactivity. For

instance, *S*-nitrosothiols are considered to be NO<sup>+</sup> donors (see below) and transfer of NO<sup>+</sup> across the plasma membrane via protein disulphide isomerases (Zai *et al.*, 1999) may allow even large molecule weight *S*-nitrosothiols to transfer oxides of nitrogen across cell membranes to subcellular targets. The complex chemistry of NO release from *S*-nitrosothiols is beyond the scope of this review and has been reviewed elsewhere (Williams, 1999; Al-Sa'doni and Ferro, 2000; Megson and Webb, 2002). However, it is important to acknowledge that a vast number of factors are capable of releasing NO from *S*-nitrosothiols, including light, heat, transition metals, thiols, superoxide and enzymes such as xanthine oxidase (Trujillo *et al.*, 1998), superoxide dismutase (Jour'dheuil *et al.*, 1999), protein disulphide isomerase (Ramachandran *et al.*, 2001) and various dehydrogenases (Liu *et al.*, 2001). Subsequently, *S*-nitrosothiols have advantages over other classes of NO donors, such as the nitrates, as they have far less stringent metabolic requirements and this may be the reason that they do not induce tolerance with long-term use (Hanspal *et al.*, 2002; Shaffer *et al.*, 1992; Miller *et al.*, 2000).

*S*-Nitrosothiols have a number of potential advantages over other classes of NO donor. Firstly, some examples show tissue selectivity: *S*-nitroso-glutathione (GSNO; Figure 4a) is selective for arteries over veins, giving them a different haemodynamic profile of action than those of classical organic nitrates. Additionally, *S*-nitrosothiols are potent antiplatelet agents, inhibiting aggregation at doses that do not influence vascular tone (de Belder *et al.*, 1994; Ramsay *et al.*, 1995). Furthermore, the ability of *S*-nitrosothiols to directly transfer NO<sup>+</sup> species allows biological activity to be



**Figure 4** Chemical structure of *S*-nitrosothiols. The NO moiety is shown in bold.

passed on through a chain of other thiols without the release of free NO. This mechanism of bioactivation may make *S*-nitrosothiols less susceptible to conditions of oxidative stress by effectively protecting the NO moiety from attack by oxygen-centred free radicals. It may be somewhat ambitious to suggest that *S*-nitrosothiols could also act as antioxidants conferred by the parent thiol, although GSNO has at least the potential to concurrently boost intracellular levels of the endogenous antioxidant, GSH. What is certain is that the endogenous nature of *S*-nitrosothiols such as GSNO (found at concentrations as high as 250 nM in some tissues; Bryan *et al.*, 2004), coupled with *in vitro* evidence from high concentration, long-term incubations (20 h; Miller *et al.*, 2000) and *in vivo* data (Shaffer *et al.*, 1992), suggests that such *S*-nitrosothiols are unlikely to carry significant cytotoxicity and pharmacologically relevant concentrations.

*S*-Nitrosothiols are not used clinically at present, but there are a large number of animal and clinical studies demonstrating their advantageous features, especially in the cardiovascular system. GSNO has been shown to decrease the occurrence of cerebral embolism after carotid endarterectomy in patients already receiving aspirin and heparin (Molloy *et al.*, 1998; Kaposzta *et al.*, 2001). Additionally, GSNO reduces platelet adhesion in bypass grafts (Salas *et al.*, 1998), thrombosis following coronary angioplasty (Langford *et al.*, 1994) and emboli that dissociate from carotid plaques (Kaposzta *et al.*, 2002). GSNO also shows beneficial haemodynamic effects in pre-eclampsia, without influencing fetal Doppler indices (Lees *et al.*, 1996). A 2-week administration of *S*-nitroso-*N*-acetylcysteine, at a dose without vasomotor activity has been shown to more than halve lesion size in a transgenic mouse model of early atherosclerosis (Krieger *et al.*, 2006). A 3-min intracoronary infusion of *S*-nitroso-*N*-acetyl-penicillamine (SNAP; Figure 4b) before the ischaemic period has been shown to decrease infarct size and improve coronary endothelial function (Gourine *et al.*, 2002). *S*-nitrosoalbumin, a naturally occurring *S*-nitrosothiol, which can be formed from other NO donors (Crane *et al.*, 2002), reduces platelet adhesion and neointimal thickening in angioplasty-damaged blood vessels, when given as an infusion (Marks *et al.*, 1995) or as a stent-coating (Maalej *et al.*, 1999).

The advantageous effects of *S*-nitrosoalbumin may be partly owing to its adhesive properties. We have carried out extensive experimentation on a group of novel *S*-nitrosothiols with alterations to their chemical structure to increase lipophilicity. We demonstrated that bolus injection of these lipophilic *S*-nitrosothiols produced a sustained vasodilatation (>4 h) in endothelium denuded arteries that was not seen with conventional *S*-nitrosothiols in isolated rat (Megson *et al.*, 1997; Megson *et al.*, 1999) and human (Sogo *et al.*, 2000a) blood vessels, as well as human hand veins *in vivo* (Sogo *et al.*, 2000c). We hypothesized that these novel compounds are retained in the lipophilic areas of the subendothelium where they slowly release NO over a prolonged period (Megson, 2002). Because the sustained effects were not seen in endothelium-intact arteries, these compounds may be a way of targeting NO to areas of endothelial damage and, therefore, minimize side effects such as large sustained falls in blood pressure. Subsequently,

we showed that one such compound, *S*-nitroso-*N*-valerylpenicillamine (SNVP; Figure 4c) inhibited platelet adhesion to rabbit carotid arteries damaged by balloon angioplasty *in vivo* suggesting a wider application of these drugs in vascular disease (Miller *et al.*, 2003). Importantly, these novel compounds do not produce tolerance with long-term continuous use and remain fully active in nitrate-tolerant blood vessels (Miller *et al.*, 2000).

Outside the cardiovascular system, GSNO has been shown to have neuroprotective properties via regulation of antioxidant and apoptotic enzymes and, subsequently, may have a role in delaying progression of neurodegenerative disorders or even encouraging neuroregeneration (Rauhala *et al.*, 2005). GSNO has also been shown to minimize liver deterioration when added to University of Wisconsin (UW) solution during cold storage during liver transplantation (Quintana *et al.*, 2001). Supplementation of endogenous *S*-nitrosothiols may also encourage wound healing: Achuth *et al.* (2005) demonstrated in rats that systemically administered GSNO at concentrations that did not cause hypotension increased collagen deposition at sites of cutaneous incisions, without affecting matrix metalloproteinase/collagenolytic pathways. These results are encouraging for the development of dressings for local delivery of NO to wounds. A cell-permeable form of *S*-nitroso-cysteine has been synthesized which can inhibit intracellular superoxide formation in neutrophils (Clancy *et al.*, 2001). *S*-Nitrosoalbumin has also been shown to have several beneficial actions that reduce inflammation and pulmonary vascular remodelling following hypoxia and reoxygenation in a mouse model of sickle cell disease (de Franceschi *et al.*, 2006).

Several new *S*-nitrosothiol drugs have been described in the past few years. A more stable analogue of GSNO, LA810 (Lacer, Barcelona, Spain) has been shown to have a marginally greater antithrombotic action than GSNO, in whole blood using a Badimon chamber as a model for *ex vivo* thrombus formation (Vilahur *et al.*, 2004). A polyethylene glycol-conjugated form of *S*-nitrosoalbumin has been described with improved distribution and prolonged NO release in the circulation (Katsumi *et al.*, 2005). Finally, 'fillers' have been designed to blend NO donors into polymers, allowing the release of NO but not other by-products (Frost and Meyerhoff, 2005). This publication also presents encouraging data showing that *S*-nitrosothiols such as SNAP can be sandwiched into the centre of a tri-layer of polymers for use as a film on medical devices. NO release could be 'switched on and off' using light irradiation (or provision of Cu(I) ions) and levels controlled and maintained over 12 h by varying the design of the films. Importantly, NO is not liberated in plasma, suggesting the pool of NO would not be released until a trigger was provided.

In light of the preclinical data discussed above, *S*-nitrosothiols should have a promising future. The foundations for targeted delivery have already been provided and will likely be expanded upon, by incorporation of these drugs to devices for cardiological intervention. Despite cost implications, much research has been garnered into drug-eluting stents (Eisenberg, 2006; Ryan and Cohen, 2006) and the flexibility of *S*-nitrosothiols makes them ideal candidates

for preventing in-stent thrombosis, at least in the short term. Manipulating chemical properties such as lipophilicity may provide targeted delivery of NO and different routes of administration, including transdermal preparations. Indeed, preliminary work has already been carried out using topical application of synthetic *S*-nitrosothiols to skin microvessels (Khan *et al.*, 1997; Seabra *et al.*, 2004).

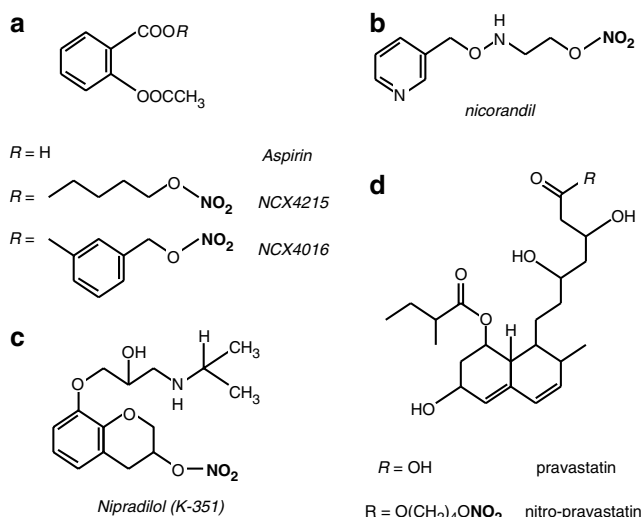
## NO hybrid drugs

Hybrid NO donor drugs represent a novel approach to the design of NO-releasing compounds. This is a broad grouping that covers a range of established drugs that have been structurally modified to incorporate NO-containing molecules. The aim of this strategy is to synthesize drugs that retain the pharmacological activity of the parent compound, but also have the biological actions of NO. Importantly, the release of NO must be balanced to provide sufficient activity within the concentration range of the parent compound (Bandarage *et al.*, 2000).

### NO-NSAIDs

The high efficacy and low cost of NSAIDs in the treatment of both mild and severe inflammatory conditions, have led to drugs such as aspirin becoming invaluable in a number of clinical fields. Subsequently, use of NSAIDs is commonplace for the treatment of persistent inflammation, such as rheumatism and arthritis. Low-dose aspirin is also routinely used prophylactically to reduce the risk of thrombotic events associated with a wide range of cardiovascular conditions. However, prolonged use of aspirin leads to serious side effects in the gastrointestinal tract that have been reported to cause ~16 000 deaths each year in the USA (Keeble and Moore, 2002). Furthermore, a recent report highlights a further increase in the risk of upper gastrointestinal bleeding attributed to the co-administration of multiple antithrombotic therapies regularly prescribed for cardiovascular conditions (Hallas *et al.*, 2006). NO has a number of effects in the gastrointestinal tract that could counteract the loss of protective prostanoids caused by aspirin. NO increases secretion of protective gastric mucus (Brown *et al.*, 1993), increases blood flow to the gastric mucosa, promoting repair and removal of toxins (Hallas *et al.*, 2006), decreases interaction of neutrophils with the gastric microcirculation (Wallace, 1997) and may also promote the healing of gastric ulcers (Ma and Wallace, 2000). Therefore, incorporating NO-releasing properties into a NSAID may minimize the gastric side effects of drugs such as aspirin.

The first NO-NSAID compounds designed and released commercially were the NicOx compounds, NCX4016 and NCX4215 (Figure 5a). Both are derivatives of aspirin (often referred to as 'nitroaspirins') adapted to contain a nitrate group. These compounds have been shown to retain the ability of aspirin to inhibit inflammation and nociception without causing gastric ulcers seen with equivalent concentrations of aspirin (del Soldato *et al.*, 1999; Fiorucci *et al.*, 2003; Turnbull *et al.*, 2006b). Also, several studies have shown that these compounds have comparable or greater



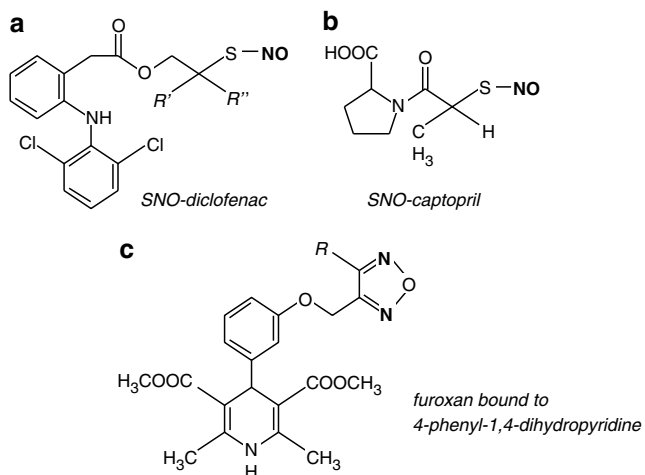
**Figure 5** Chemical structure of NO donor hybrid drugs containing a nitro-oxy moiety. The nitro-oxy group is shown in bold.

antiplatelet effects than the parent NSAID, without causing excessive vasodilatation or hypotension (Lechi *et al.*, 1996; del Soldato *et al.*, 1999; Wallace *et al.*, 1999a; Momi *et al.*, 2000). The biological actions of nitroaspirins have received much attention and have been reviewed extensively elsewhere (Keeble and Moore, 2002; Wallace *et al.*, 2002; Chiroli *et al.*, 2003; Wallace and Del Soldato, 2003; Turnbull *et al.*, 2006b). Here, we will concentrate on a few new developments using nitroaspirins and then focus on emerging novel NO-NSAID compounds.

In the past 5 years, there has been considerable investigation into the mechanism that underpins the anti-inflammatory properties of NO generated from nitroaspirins (Keeble and Moore, 2002). Caspases are a family of proteases involved in cytokine release and apoptosis. NO from NCX4016 inhibits the action of caspase-1, and, subsequently, the propagation of other cytokines such as IL-1 $\beta$  and IL-8 (Fiorucci *et al.*, 2000). NCX4016 induced inhibition of caspase-1 is mediated through *S*-nitrosylation of a sulphhydryl group (Dimmeler *et al.*, 1997), therefore, other NO-NSAIDs, such as *S*-nitroso-diclofenac (see Figure 6a) may be more effective inhibitors of caspase-1 through transnitrosation reactions. Nitroaspirins also inhibit the release of TNF- $\alpha$  from lipopolysaccharide-stimulated macrophages (Minuz *et al.*, 2001), although it is difficult to determine whether this is a direct effect of NO or through inhibition of other cytokines. Regardless, this property would likely provide benefits in the treatments of a range of inflammatory diseases, above that of conventional aspirin (Fiorucci and Del Soldato, 2003).

NO-NSAIDs have attractive properties in a number of cardiovascular conditions. On top of the antiplatelet actions of NO-NSAIDs, the NO-mediated anti-inflammatory properties would be useful in vascular injury and atherosclerosis, given the central role of inflammatory cells in the process (Ross, 1999). NCX4016, but not aspirin, has been shown to reduce experimental restenosis in hypercholesterolemic (Napoli *et al.*, 2001) and aged (Napoli *et al.*, 2002) mice. NCX4016 also reduces infarct size in several different models





**Figure 6** Chemical structure of NO donor hybrid groups containing a (a, b) *S*-nitrosothiol or a (c) furoxan moiety. The NO containing moiety is shown in bold.

of myocardial ischaemia-reperfusion injury (Rossoni *et al.*, 2000, 2001; Wainwright *et al.*, 2002; Burke *et al.*, 2006) and has been shown to reduce platelet-monocyte interaction in humans to a greater extent than aspirin alone (Fiorucci *et al.*, 2004).

Nitroaspirins also show potential in cancer therapy. Upregulation of cyclooxygenase-2 leading to enhanced prostaglandin output is a feature of a number of cancers (Baron, 1995). Both the gastro-sparing properties of nitroaspirins and the direct effect of NO on cell proliferation could be beneficial, although obtaining suitable balance between the different facets of a nitroaspirin might prove difficult. That said, NCX4016 have been shown to be 250–6000-fold more effective at inhibiting the growth of a number of different cancer cell lines (Kashfi and Rigas, 2005). Additionally, the compound also reduced tumour growth in an *in vivo* rat model of colonic adenocarcinoma to a greater extent than aspirin itself (Bak *et al.*, 1998). Aside from cancer, NO-NSAIDs may have applications in other areas of the body (Keeble and Moore, 2002). A NO-releasing derivative of flurbiprofen has been shown to have beneficial actions in models of renal ablation (Fujihara *et al.*, 1998), bone degeneration (Armour *et al.*, 2001) and Alzheimer's disease (Jantzen *et al.*, 2002).

Given the tolerance issues associated with organic nitrates, it is surprising that the majority of nitroaspirins investigated so far exploit the same NO donor moiety, especially as it has been shown for at least one example that the mechanism of NO release is the same in hybrid drugs (Turnbull *et al.*, 2006a). This is a pressing concern considering the rapid emergence of many new NSAIDs/anti-inflammatory/analgesic agents with nitrates groups, for example paracetamol (Marshall *et al.*, 2006), flurbiprofen (Fujihara *et al.*, 1998), naproxen (Young *et al.*, 2005), mesalamine (Wallace *et al.*, 1999b), gabapentin (Wu *et al.*, 2004), prednisolone and other steroids (Tallet *et al.*, 2002). However, the nitrate ester of nitroaspirin has been replaced with a furoxan moiety (Cena *et al.*, 2003; Turnbull *et al.*, 2006a) and *S*-nitroso- (Bandarage *et al.*, 2000) and diazeniumdiolate (Velazquez *et al.*, 2005)

forms of other NSAID drugs (e.g. aspirin, diclofenac, indomethacin and ibuprofen) have also been described. These alternative forms of NO-NSAID has advantages over nitrate-linked NSAIDs in that they are likely to have less strict requirements for the release of NO and, therefore, may exert greater antiplatelet activity and would be unlikely to produce tolerance (Turnbull *et al.*, 2006b).

#### Other nitrate-hybrid drugs

There are several other drugs of note that have been successfully adapted to contain a nitrate to provide NO bioactivity. The first, nicorandil (Figure 5b), a nicotinamide derivative with a nitrate group that has both NO donor and mitochondrial  $K_{ATP}^+$  channel-opening properties. Although existing organic nitrates are extremely effective at relieving the acute symptoms of angina, clinical trials have not found that nitrates improve outcome (Yusuf *et al.*, 1988; GISSI-3 study group, 1994; ISIS-), indeed, they may even worsen long-term prognosis (Ishikawa *et al.*, 1996; Nakamura *et al.*, 1999). Nicorandil, however, has been shown to decrease coronary events in over 5000 patients with stable angina during a mean follow-up period of 1.6 years (IONA study group, 2002), and subsequently, this drug is beginning to overshadow classical nitrates in the minds of many physicians. The additional beneficial properties of nicorandil have been largely attributed its actions on  $K^+$  channels, causing dilatation of peripheral arteries and coronary resistance vessels, reducing the afterload of the heart (Simpson and Wellington, 2004; Herman and Moncada, 2005). This proposal is somewhat puzzling, as it is regularly argued that the reason behind organic nitrate effectiveness in the relief of angina, is their ability to selectively dilate peripheral veins over arteries (Kojda, 2000), and large coronary arteries over small (Winbury *et al.*, 1969; Kurz *et al.*, 1991). Should the dual action of nicorandil on preload and afterload be the reason for its success, it would suggest that other NO donors with more balanced arteriovenous selectivity may also be useful in the treatment of angina and ischaemic heart disease. Alternatively, actions other than those on blood vessels may be important; for example, encouragement of fibrinolysis by decreasing the activity of type-1 plasminogen activator inhibitor (PAI-1) (Sakamoto *et al.*, 2004) or by preconditioning the heart against ischaemic episodes (Matsuo *et al.*, 2003). Currently, it is unclear whether long-term use of nicorandil is restricted by the development of tolerance, like other nitrates, with some studies showing no cross-tolerance to GTN with respect to vasodilatation (Simpson and Wellington, 2004), whereas others suggest that, while peripheral vasodilatation is unaffected, tolerance develops to the anti-ischaemic preconditioning (Rajaratnam *et al.*, 1999; Loubani and Galinanes, 2002). Replacement of the nitrate group of nicorandil with a furoxan group (see below) has been described, (Cai *et al.*, 2005), that may circumvent the problems of tolerance.

Nipradilol (K-351; Figure 5c) is an orally active compound that was first described in the early 1980s as a nonspecific  $\beta$ -receptor antagonist containing a nitrate group (Uchida *et al.*, 1983). It was hypothesized that combining a NO donor with an adrenoceptor antagonist would provide additional

beneficial cardiovascular actions by counteracting the undesirable side effects of the individual drugs. Addition of the nitrate group instills the drug with vasodilator actions similar to those of GTN, as well as enhancing the  $\beta$ -antagonistic potency and, surprisingly, providing a degree of antagonism for  $\alpha$ -receptors (Uchida *et al.*, 1983). There is conflicting evidence as to whether nipradilol shows selectivity for conduit (Uchida *et al.*, 1983) or resistance (Lamping and Bloom, 1995) arteries. More recently, Thakur *et al.*, (2002) demonstrated that nipradilol inhibits the development of atherosclerotic lesions in a rabbit model of hypercholesterolemia and eNOS inhibition. Interestingly, the beneficial actions of nipradilol appear to be in part owing to increased eNOS expression (Jayachandran *et al.*, 2001; Thakur *et al.*, 2002), although a mechanism for this has yet to be established. As with the conventional organic nitrates, the development of tolerance with continuous use may limit nipradilol's effectiveness.

Lastly, it is worth briefly mentioning another type of nitrate hybrid drug that, while only preliminary experimental data have been published so far, will undoubtedly come under close scrutiny. Statins are highly effective inhibitors of 3-hydroxy-methylglutaryl CoA reductase that are used to lower cholesterol. They have had a dramatic impact on clinical outcome in a number of cardiovascular conditions. It is now clear that statins have a number of additional molecular mechanisms beyond lipid-lowering, that ultimately modulate inflammation and smooth muscle cell proliferation. Ongini *et al.*, (2004) have synthesized nitrate-linked forms of pravastatin (Figure 5d) and fluvastatin, which they believe could complement the existing statin effects in conditions where there is endothelial dysfunction, such as diabetes and atherosclerosis. *In vivo* effects have yet to be tested, but experiments in cell lines showed that the NO-statins had a far greater ability to inhibit cell proliferation compared to their parent compound, and were also shown to decrease iNOS expression and activity (Ongini *et al.*, 2004). A very recent contribution to work in this area has been made by Dever *et al.* (2007) who have clearly shown a nitrate-derivative of pravastatin (NCX6550) to have significant inhibitory effects on ROS generation and adhesiveness in splenocytes from the Apo-E<sup>-/-</sup> murine model of atherosclerosis, as well as wild-type controls. NCX6550 was also shown to enhance endothelium-dependent vasodilatation in aortic rings from Apo-E<sup>-/-</sup> mice; of the benefits observed with NCX6550, only inhibition of ROS was shared by the parent compound, pravastatin.

#### Other S-nitroso-hybrid drugs

A hybrid approach has also been applied to inhibitors of angiotensin-converting enzyme (ACE). Captopril is an example of an ACE inhibitor that contains a SH group, which can be nitrosated, forming S-nitrosocaptopril (SNO-Cap; Figure 6b) (Loscalzo *et al.*, 1989). SNO-Cap has sGC-mediated vasodilator and antiplatelet actions, yet retains the ability to inhibit ACE (Cooke *et al.*, 1989; Loscalzo *et al.*, 1989). Additionally, SNO-Cap appears to preferentially dilate coronary arteries over peripheral vessels (Cooke *et al.*, 1989). Intravenous SNO-Cap produces a long-lasting hypotensive

effect *in vivo* (Shaffer *et al.*, 1991) and, similarly to other S-nitrosothiols, is less susceptible to tolerance (Shaffer *et al.*, 1991; Zhang *et al.*, 1994; Matsumoto *et al.*, 1995). ACE inhibition may also contribute to the NO-mediated actions of SNO-Cap, as the enzyme also inactivates bradykinin, an endogenous endothelium-dependent vasodilator. Despite the obvious appeal of such a drug, there have been very few reports of SNO-Cap within the last 10 years, the most recent being a detailed study of NO release from SNO-Cap in the absence of tissue (Aquat and Dasgupta, 2004). This may be owing to the poor stability of SNO-Cap, making it difficult to purify and crystallize (Jia *et al.*, 2000). In addition, several groups have been unable to replicate the synthesis of SNO-Cap and confirmation of the synthesis is required. That said, others have managed to synthesize red flake crystals of SNO-Cap and showed that these crystals have potent antiangiogenic effects in embryonic tissue (Jia *et al.*, 2000), although it is not clear if conventional S-nitrosothiols would have a similar effect.

Tissue-type plasminogen activator (t-PA) is an endogenous enzyme synthesized by the endothelium. Fibrin, a constituent of thrombus, binds to t-PA stimulating the conversion of plasminogen into plasmin: a powerful fibrinolytic agent. t-PA contains a single SH group which can be S-nitrosated allowing t-PA to directly inhibit platelets as well as exerting a slightly greater fibrinolytic activity than native t-PA (Stamler *et al.*, 1992). The combined antithrombotic and anti-inflammatory action of SNO-t-PA has been shown to reduce cardiac necrosis following ischaemia-reperfusion injury *in vivo* (Delyani *et al.*, 1996). Von Willebrand factor (vWF) is synthesized and released by damaged blood vessels. The binding of vWF to platelet glycoprotein receptors induces platelet activation, causing adhesion to damaged regions of blood vessels. Recombinant fragments of vWF, such as AR545C, bind to platelet receptors, competing with the binding of endogenous vWF to platelet receptors and therefore preventing the initiation of thrombosis. Inbal and co-workers have shown that S-nitrosated AR545C causes greater inhibition of platelet adhesion and aggregation than recombinant vWF that is not S-nitrosated (Inbal, 1999). As far as we are aware, the application of SNO-t-PA and SNO-vWF has not been taken further, although the authors remain convinced of their therapeutic potential and aim to readdress these compounds within the near future (J Loscalzo; personal communication).

#### Other NO-hybrid drugs

Furoxans are a group of compounds that has been of considerable interest to chemists for decades, yet have received relatively little attention from biologists, despite their NO-releasing properties. The complicated chemistry of NO release from the many different compounds in this class are reviewed elsewhere (Gasco and Schoenafinger, 2005), here we focus on existing pharmacological agents that can be modified to contain the pentavalent furoxan group.

The dihydropyridine class of calcium antagonists can be linked a furoxan group (Figure 6c) and these compounds cause vasodilatation through sGC stimulation as well as inhibition of voltage-dependent Ca<sup>2+</sup> ion channels on

VSMCs (Di Stilo *et al.*, 1998). The same group have also linked furoxans to  $\alpha_1$ - (Fruttero *et al.*, 1995) and  $\beta_1$ -antagonists (Boschi *et al.*, 1997). Both produce vasodilatation of isolated aortic strips through a combination of adrenoceptor antagonism and NO release. A range of furoxan (and nitrated) phenols have also been synthesized, to provide a balanced NO-mediated vasodilatory response with antioxidant defence, with the underlying goal of treating atherosclerosis (Boschi *et al.*, 2006).

Outside the cardiovascular system, furoxans have also been linked to a histamine H<sub>2</sub> receptor antagonist, and provide a greater protection against gastric ulcers than antihistamine drugs alone (Coruzzi *et al.*, 2000). Furoxan-linked H<sub>3</sub>-antagonists have also been described (Bertinaria *et al.*, 2003b). Protein pump inhibitors (PPIs) have been a major success in a range of gastric pathologies. A furoxan form of the PPI, rabeprazole, has been shown to reduce histamine secretion and indomethacin-induced gastric lesion development in rats *in vivo* (Sorba *et al.*, 2003). Remaining in the gastrointestinal system, furoxan derivatives linked to the antimicrobial drug metronidazole have been shown to have potent activity against *Helicobacter pylori* in metronidazole-resistant strains, although it is unclear if this property is owing to NO release *per se* (Sorba *et al.*, 2003; Bertinaria *et al.*, 2003a). Lastly, a uroselective furoxan linked  $\alpha_1$ -antagonist, REC15/2739, has a dual vasodilator action on noradrenaline-contracted rat vas deferens, that may be of benefit in the treatment of conditions such as benign prostatic hyperplasia (Boschi *et al.*, 2003).

## Zeolites

A novel approach to storage and delivery of NO has recently been adopted using ion-exchanged zeolites (Wheatley *et al.*, 2006). These microporous insoluble materials form a framework containing metal ions that can bind NO. Exposure of the solid to NO gas results in NO binding to the metal ions within the pores, facilitating highly efficient packing of NO within the solid. NO-zeolites of this type are very stable in the anhydrous state, but NO is displaced by water on immersion in an aqueous environment. The beauty of these materials is that they constitute very high capacity stores for NO and the rate of release can be modulated by altering the porosity of zeolite, the metal ion in the framework and the composition and nature of the binder (Frost *et al.*, 2005; Wheatley *et al.*, 2006). This infinite flexibility with respect to NO release allows for development of a range of different NO donor materials for different purposes ranging from fast-acting antimicrobial coatings for urinary catheters and wound dressings to durable, slow acting antithrombotic coatings for stents, bypass tubing, cannulae and catheters. Although at an early stage, this approach represents a novel means of site-selective delivery of NO that optimizes the benefits of NO as a local mediator.

## Summary and conclusions

Depression of the NO:sGC pathway is a feature of many cardiovascular conditions and delivery of low concentrations

of exogenous NO is an attractive therapeutic option. In contrast, delivery of high concentrations might have completely different therapeutic targets and act via the cytotoxic actions of NO. The chemical versatility of NO has led to the synthesis of a wide range of NO donors, each with different modes and rates of NO release. Subsequently, NO donor drugs can be chosen or tailor-made to suit the disease target. Long-term use of current NO donors is limited by development of tolerance and toxicity issues, ensuring that there is a clinical need for novel alternatives.

NONOates have proved extremely popular in the experimental setting due to the predictable nature of NO release. However, use of these compounds should not be restricted to the bench; NONOates have been successfully applied in a number of cardiovascular conditions, especially where a slow prolonged release rate is desirable. In pulmonary hypertension, for example, NONOates are less likely to cause rebound hypertension on cessation than inhaled NO (Butler and Russell, 2005). There has been an explosion of interest in the possibility of delivering cytotoxic levels of NO to cancer cells to promote tumour regression. Subsequently, a series of NONOates have been designed with protected diolate groups that release NO upon metabolism by factors specific to tumour cells. However, the potential toxicity of by-products of NONOate metabolism still remains to be fully investigated before the clinical potential of these compounds can be fully assessed.

S-nitrosothiols are another group of NO donors that can be structurally modified to release NO at varying rates in specific conditions. Alterations of the structure of S-nitrosothiols to modify lipophilicity may allow us to target these compounds to areas of endothelial damage (Megson *et al.*, 1997; Miller *et al.*, 2003) and, therefore, minimize side effects. Both S-nitrosothiols and NONOates hold a great deal of promise as coatings for stents, extracorporeal circuits and catheters used in interventional cardiological procedures, surgery and renal replacement therapy. Polymeric materials are used to coat many such devices and all currently induce biological response (Frost *et al.*, 2005). NO donor containing polymers will likely reduce a number of unwanted complications such as thrombosis and restenosis without the need for systemic administration of heparin or potent antiplatelet agents (Annich *et al.*, 2000). Further research is now required to design agents that can release active concentrations of NO over a significant period of time.

Finally, the development of hybrid NO donor drugs has increased exponentially in recent years. The concept is best described by NO-NSAIDs that retain the actions of their parent compound (analgesia, inhibition of inflammation, decreased thrombosis), yet have additional beneficial actions attributed to NO, most notably in protecting against gastric damage caused by aspirin. Results from the use of NO-NSAIDs in animal models are promising but it remains to be seen whether the progress of these drugs is hindered or enhanced by the recent high-profile withdrawal of cyclooxygenase-2 specific inhibitors (Fitzgerald, 2004; Garcia Rodriguez *et al.*, 2004). Regardless, the concept has been extended to a vast array of pharmacological agents such as anticoagulants, antitumour agents, ion channel inhibitors, ACE inhibitors and histamine receptor inhibitors. It remains to be

seen whether use of these agents in clinical trials is more practical or have greater effects than co-administering individual drugs or using combinations tablets (Boschi *et al.*, 2006; Turnbull *et al.*, 2006b).

It has transpired that the development of a superior NO donor drug to rival conventional organic nitrates has proved highly elusive. Despite the obvious potential, there have been very few notable advances in terms of licenced medicines that have emerged from the euphoria surrounding its identification as an important biological messenger. Indeed, far more progress has been made in targeting downstream sGC and cGMP, most notably with sildenafil (Sawatzky *et al.*, 2005), which is likely to have many cardiovascular benefits beyond its well-documented application in erectile dysfunction. Indeed, it is fair to say that these downstream mechanisms might represent a more user-friendly means of systemic modulation of the NO pathway, whereas the ability to release NO itself may be either be too simple and/or rapid, leading to instability of the parent compound and lack of specificity, or too complex, resulting in tolerance associated with enzyme-mediated processes.

In assessing where developments might realistically emerge in the future, it is perhaps worth reconsidering the endogenous roles of NO, where it acts primarily as a local mediator. *In vivo*, the short biological half-life is exploited to facilitate local effects in response to specific stimuli; NO then simply dissipates through diffusion and oxidation to nitrite and nitrate, without the need for complex metabolism. Technologies that utilize this inherent special quality of NO have a great chance of success and a number of developments in this area have already begun to emerge, as described above. Wound healing is a particularly interesting target, given the multiple benefits that NO might have, from antimicrobial agent, through vasodilator and pro- or anti-inflammatory agent, depending on the concentration of NO generated and experimental materials (Masters *et al.*, 2002) have shown some benefit in this setting. However, central to the success or otherwise of this (Schulz and Stechmiller, 2006) or any other NO donor therapy is generation of the correct amount of NO in the right place for the right length of time; cracking these problems will undoubtedly lead to a new range of NO donors that successfully exploit specific elements of the wide range of physiological roles played by this critical endogenous mediator.

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## Conflict of interest

ILM held a consultancy at Strakan Pharmaceuticals (2003–2005).

MRM has no conflict of interest.

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