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REVIEW

# Extending the translational potential of targeting NO/cGMP-regulated pathways in the CVS

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The discovery of NO as both an endogenous signalling molecule and as a mediator of the cardiovascular effects of organic nitrates was acknowledged in 1998 by the Nobel Prize in Physiology/Medicine. The characterization of its downstream signalling, mediated through stimulation of soluble GC (sGC) and cGMP generation, initiated significant translational interest, but until recently this was almost exclusively embodied by the use of PDE5 inhibitors in erectile dysfunction. Since then, research progress in two areas has contributed to an impressive expansion of the therapeutic targeting of the NO-sGC-cGMP axis: first, an increased understanding of the molecular events operating within this complex pathway and second, a better insight into its dys-regulation and uncoupling in human disease. Already-approved PDE5 inhibitors and novel, first-in-class molecules, which up-regulate the activity of sGC independently of NO and/or of the enzyme's haem prosthetic group, are undergoing clinical evaluation to treat pulmonary hypertension and myocardial failure. These molecules, as well as combinations or second-generation compounds, are also being assessed in additional experimental disease models and in patients in a wide spectrum of novel indications, such as endotoxic shock, diabetic cardiomyopathy and Becker's muscular dystrophy. There is well-founded optimism that the modulation of the NO-sGC-cGMP pathway will sustain the development of an increasing number of successful clinical candidates for years to come.

## LINKED ARTICLES

This article is part of a themed section on Pharmacology of the Gasotransmitters. To view the other articles in this section visit http://dx.doi.org/10.1111/bph.2015.172.issue-6

## Abbreviations

sGC, soluble GC

## Tables of Links

| TARGETS | |
|---|---|
| **GPCRs**[a] | **Enzymes**[d] |
| $\beta_2$-adrenoceptor | Arginase |
| Endothelin receptors | COX |
| **Ligand-gated ion channels**[b] | Endothelial NOS (NOS3) |
| NMDA receptor | Inducible NOS (NOS2) |
| **Nuclear hormone receptors**[c] | Neuronal NOS (NOS1) |
| Glucocorticoid receptor | PDE family |
| PPAR-$\alpha$ | PDE2 |
| PPAR-$\gamma$ | PDE5 |
| | Soluble GC (sGC) |

| LIGANDS | |
|---|---|
| Angiotensin II | L-arginine |
| Aspirin | LPS |
| Ataciguat (HMR1766) | Methacholine |
| BAY41-2272 | NADPH |
| BH4 | Naproxen |
| cGMP | Nitric oxide (NO) |
| Cinaciguat (BAY58-2667) | Prednisolone |
| Flunisolide | Prostacyclin |
| Glyceryl trinitrate | Riociguat (BAY63-251) |
| GTP | Sildenafil |
| Hsp90 | Tadalafil |
| Isoprenaline | TNF-$\alpha$ |
| Isosorbide mononitrate | YC-1 |

These Tables list key protein targets and ligands in this article which are hyperlinked to corresponding entries in http://www.guidetopharmacology.org, the common portal for data from the IUPHAR/BPS Guide to PHARMACOLOGY (Pawson *et al.*, 2014) and are permanently archived in the Concise Guide to PHARMACOLOGY 2013/14 ([a,b,c,d]Alexander *et al.*, 2013a,b,c,d).

## Introduction

The recent progress in the generation of additional, therapeutic molecules that target the NO transduction pathway is in large part due to a more detailed understanding of the biochemical and mechanistic complexities of the downstream pathways this molecule triggers. That is, the soluble GC (sGC)–cGMP axis. cGMP is a ubiquitous intracellular signalling molecule that affects a wide spectrum of cellular, and thus physiological, processes from cell growth and apoptosis to ion channel gating. Especially in the CVS in which it has been best studied, cGMP regulates many vital homeostatic mechanisms, including endothelial cell permeability, vascular smooth muscle contractility and cardiomyocyte hypertrophy (Francis *et al.*, 2010). Of the two distinct GC systems that generate cGMP, this review exclusively focuses on the contribution of the NO-responsive arm to the detriment of the cGMP pool generated by natriuretic peptide hormones acting on membrane-bound, particulate forms of GC. Whereas there is considerable functional convergence of the two systems downstream, there is overwhelming evidence of spatial compartmentalization that results from the specific cellular co-localization of both the cGMP-generating systems as well as the cGMP-degrading PDEs, exemplified by the ability of PDE2 to selectively interfere with the natriuretic-stimulated cGMP pool, whereas PDE5 targets mainly the cytosolic cGMP pool, in cardiomyocytes (Castro *et al.*, 2006; Piggott *et al.*, 2006; Nausch *et al.*, 2008; Tsai and Kass, 2009; Zhang and Kass, 2011).

This review will highlight the molecules and mechanisms within this pathway whose further study has recently generated successful entries in the medical arsenal, including use in some novel medical indications, thus showing great future promise in contributing to the treatment and elimination of human disease, especially disorders of the CVS.

## Basic biology of the NO-sGC-cGMP pathway

### *Enzymatic generation of NO*

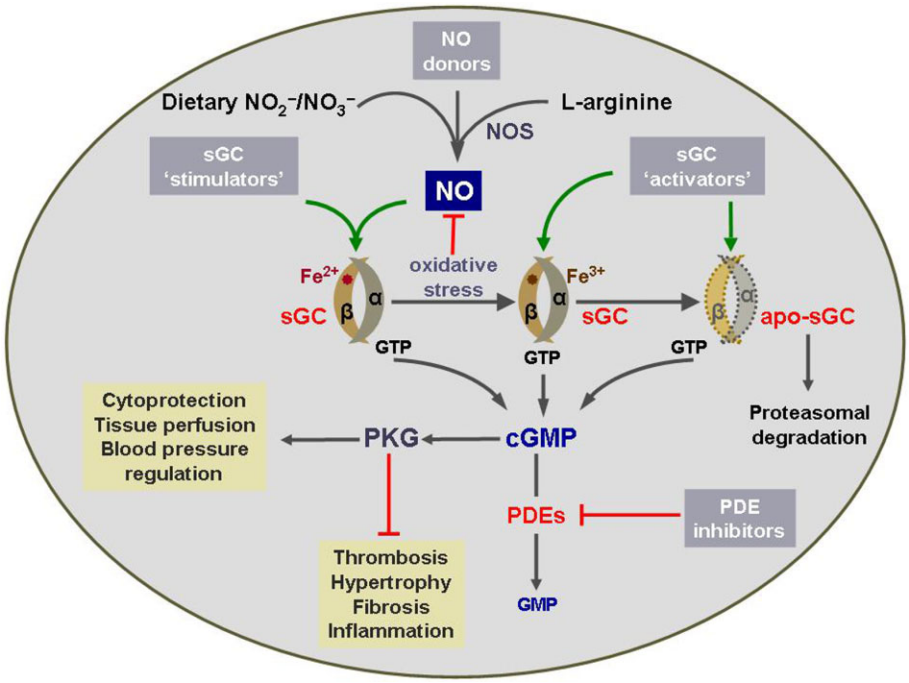
Three isoforms of NOS exist, each one with a different pattern of expression (Alderton *et al.*, 2001): neuronal NOS (nNOS or NOS-1), inducible NOS (iNOS or NOS-2) and endothelial NOS (eNOS or NOS-3). nNOS and eNOS are expressed constitutively whereas iNOS is not found in healthy cells but protein expression is induced following tissue injury or infection (Nathan, 1997). NOSs are capable of associating with the cell membrane, with cytosolic proteins or with the cytoskeleton, thus exhibiting dynamic subcellular localization (Oess *et al.*, 2006). NOSs facilitate the five-electron oxidation of the terminal guanidino moiety of the semi-essential amino acid L-arginine, utilizing NADPH and BH4 as electron sources, to generate NO and L-citrulline in the presence of molecular oxygen (Alderton *et al.*, 2001).

The regulation of NO bioavailability is complex and controlled by numerous mechanisms impacting *directly* NO levels, including NOS expression, substrate provision and chemical inactivation. For example, production of reactive oxygen species can inactivate NO (Münzel *et al.*, 2005), and endogenous asymmetric methylarginines appear to act as NOS inhibitors (Leiper and Nandi, 2011; Caplin and Leiper, 2012). Arginase activity decreases the availability of the NOS substrate, L-arginine (Morris, 2009), uncouples NOS (resulting in generation of cytotoxic superoxide) and is thought to underlie nitrate tolerance (Khong *et al.*, 2012). Modulation of

eNOS–caveolin interactions (Garcia-Cardena *et al.*, 1996) acts as an on/off switch for enzyme turnover and, more recently, interactions of NO with somatic haemoglobin (Straub *et al.*, 2012) can reduce NO bioavailability. Furthermore, pharmacological enhancement of NO signalling can also be achieved *indirectly*. For example, stimulation of the $\beta_3$-adrenoceptor in the heart has been shown to be coupled to the NO–cGMP pathway, to increase NO bioactivity and to prevent experimental maladaptive myocardial remodelling caused by isoprenaline or angiotensin II, an effect that deserves to be explored further clinically (Belge *et al.*, 2014). Several molecules targeting the above mechanisms have been developed and evaluated preclinically (e.g. a NOS–caveolin disruptive peptide; Bucci *et al.*, 2000); fewer have advanced in clinical trials. The latter include the arginase inhibitor N-hydroxy-nor-arginine, investigated in a phase I trial in coronary disease (Shemyakin *et al.*, 2012; NCT02009527). However, no clinical approval of molecules targeting these mechanisms has yet validated these approaches.

## *cGMP biosynthesis in response to NO*

The major biosensor of the generated NO is the enzyme sGC, which is found as an obligate heterodimer of α ($\alpha_1$ and $\alpha_2$) and $\beta_1$ subunits; the $\alpha_1\beta_1$ dimer seems to be the prevalent active form in most tissues with the exception of the nervous systems where equal amounts of $\alpha_1/\beta_1$ and $\alpha_2/\beta_1$ are detected. Each sGC subunit consists of (i) an N-terminal regulatory, haem-NO/oxygen (H-NOX) domain; (ii) a central Per-Arnt-Sim domain; (iii) a coiled-coil domain; and (iv) a C-terminal catalytic domain (Derbyshire and Marletta, 2012). There is one haem prosthetic group per heterodimer (Figure 1) that serves as the NO sensor and that is stimulated by nM concentrations of NO leading to an increase in enzymatic activity up to 400-fold (Kamisaki *et al.*, 1986; Tsai and Kass, 2009). The α and β subunits have been proposed to be organized in a parallel fashion and the low basal activity of sGC is thought to result from the inhibitory action exerted by the binding of the catalytic domain to the regulatory domain; this inhibition is relieved upon NO binding. The presence of a reduced ($Fe^{2+}$, ferrous) haem group is critical in NO sensing by sGC. For example, environmental cues, that increased the presence of reactive species such as superoxide ($\cdot O_2^-$) and peroxynitrite ($ONOO^-$) are translated into changes in the redox status of the haem group and therefore in the ability of sGC to respond to low concentrations of NO (Weber *et al.*, 2001; Stasch and Hobbs, 2009; Figure 1). The implications of this in disease are




## Figure 1

Schematic representation of the major targetable components of the NO pathway. Disease-modifying NO can be generated from three main, well-studied sources: (i) cellular conversion from L-arginine; (ii) bacterial-based, enterosalivary bioconversion of food nitrates; and (iii) nitrate drugs such as glyceryl trinitrate, either spontaneously or through cellular conversion. The bioavailability of NO is regulated by its generation by the synthetic NOS enzymes and by the tissue complexation and conversion of NO, for example, to nitrosyl-free radicals. Initially, NO bioactivity is in major part determined by its best-described cellular 'biosensor': sGC coupled to reduced haem. sGC 'stimulators' such as riociguat, which was recently approved for treatment of two forms of PH, can by themselves activate sGC or synergize with NO. Chemical modification of sGC or oxidation of the haem prosthetic group and dissociation from sGC can occur in pathophysiological situations such as PH and heart ischaemia. Apo-sGC has an impaired ability to respond to NO, thus 'uncoupling' the NO pathway. This form of sGC can be 'resuscitated' by sGC 'activators' such as cinaciguat and ataciguat. PDEs are themselves regulated by and participate in the catabolism of cGMP. PDE5 inhibitors such as sildenafil and tadalafil are approved for erectile dysfunction and treatment of PH. NO pathway modifying drugs are increasingly evaluated in clinical trials in indications as varied as heart failure, traumatic cerebral oedema and forms of skeletal muscle dystrophies.

crucial: it is thought that oxidative stress, a typical trigger for cardiovascular disease, can produce an NO-unresponsive ($Fe^{3+}$, Ferric) sGC that is rapidly ubiquitinylated and degraded (Evgenov *et al.*, 2006; Stasch and Hobbs, 2009). Furthermore, this sGC 'uncoupling' may result from S-nitrozation of vicinal thiols in the $\beta_1$ subunit in addition to oxidation of the haem group (Stasch *et al.*, 2006; Sayed *et al.*, 2008). Such impairment of sGC activity in cardiovascular disease, coupled to concomitant decreases in NO bioavailability, has been the bedrock on which novel NO and/or haem-independent sGC stimulators and activators have been developed and which will be examined below (Evgenov *et al.*, 2006; Follmann *et al.*, 2013; Gheorghiade *et al.*, 2013).

In addition to its upstream, direct effects on NO availability and sGC function, cellular oxidative stress may also interfere with the NO/cGMP pathway by inducing post-translational activation of the downstream cGMP effector PKG-Iα and thus affect adversely the progress of disease, something that has been experimentally shown to occur in sepsis (Rudyk *et al.*, 2013). Due to this complex, and in some cases antithetical, regulation of NO bioactivity, in such pathological settings a dual-pronged therapeutic approach, that combines upstream restoration of physiological cGMP generation and pharmacological intervention (e.g. anti-oxidants) could be optimal to preserve the physiological function of downstream effectors.

It is important to note that the downstream biochemical pathway of NO is far from limited to cGMP-mediated effects: cGMP-independent changes are undeniably part of the NO signalling repertoire, including NO-triggered protein S-nitrozation (Lima *et al.*, 2010) and effects on mitochondrial respiration and oxygen utilization (Erusalimsky and Moncada, 2007). One should keep in mind, however, that genetic inactivation of sGCβ1 (Friebe *et al.*, 2007) and cGMP-dependent kinase I (PKG1) abolishes the hallmark physiological effect of NO, that is, vasorelaxation (Pfeifer *et al.*, 1998), emphasizing the crucial involvement of cGMP in the effects of NO. It is also clear that the 'canonical' (cGMP dependent) NO pathway has provided the major impetus for translational progress and thus constitutes the central focus of the review.

### *Direct downstream signalling of cGMP*

Two main enzyme families are directly regulated and respond to cGMP, to impact the pathophysiology of the CVS: cGMP-dependent PKs (PKGs) and PDEs (Figure 1). In addition, ion channel function is also directly or indirectly (e.g. via PKG-dependent pathways) regulated by cGMP levels, although this phenomenon is largely restricted to sensory transduction (Biel and Michalakis, 2007; Francis *et al.*, 2010). To date, successful translational efforts have, however, focused primarily on the two upstream enzymatic targets: sGC and PDE. The ability of sGC to associate with the plasma membrane (Linder *et al.*, 2005) and the possible compartmentalization of cGMP degrading PDEs (Castro *et al.*, 2006; Nausch *et al.*, 2008; Zhang and Kass, 2011) may further complicate the downstream functions of spatially regulated cGMP levels and the therapeutic targeting of enzymes that regulate its levels in distinct diseases.

The dominant PKG in the CVS is PKG type 1, which consists of two isoforms: α and β (Hofmann *et al.*, 2006; Burley *et al.*, 2007). The binding of cGMP to a regulatory region of the kinase results in a conformational change that 'unrepresses' the catalytic activity of the kinase and permits phosphorylation on Ser/Thr residues of client proteins. Pharmacological targeting of PKG is attractive but has not been successful up to now, because selective PKG activators and inhibitors are lacking. In addition, PKG inhibition may result in smooth muscle dysfunction, based on experimental evidence provided by mice with genetic deletion of cGMP kinase I (Pfeifer *et al.*, 1998). Conversely, use of PKG activators to mimic the effects of sGC and pGC turnover is theoretically desirable in cardiovascular disease, but chronic use may be ultimately undesirable, given that gain-of-function genetic mutations in PKG found in humans are causally associated with aortic aneurysms and dissections (Guo *et al.*, 2013).

The second cGMP-responsive system that has been well-studied comprises the PDE family of cyclic nucleotide-hydrolyzing enzymes, which have arguably been the most successful 'cGMP-based' therapeutic targets. Of the 11 PDE families (PDE1-11, each consisting of one to four isozymes and their multiple isoforms), PDEs-2, -3, -5, -6 and -11 are regulated by cGMP, of which PDE2, 3 and 5 are expressed in the constituent cells of the CVS, with PDE11 being found in the heart. PDEs exist as dimers, each monomer comprises a characteristic for the isotype N-terminal regulatory domain and a relatively high homology C-terminal catalytic domain that can undergo post-translational prenylation or phosphorylation (Conti and Beavo, 2007; Keravis and Lugnier, 2012). Whereas PDE2 and PDE5 are activated by cGMP binding to their GAF regulatory domain, PDE3 is inhibited by competitive binding of cGMP to its catalytic site. Of these three PDEs, PDE2 and PDE3 can hydrolyse both cGMP and cAMP, while PDE5 is selective for cGMP (Bender and Beavo, 2006; Conti and Beavo, 2007). PDE5, which is highly expressed in the corpus cavernosum and in the lung, is the target of small-molecule inhibitors that have been approved to treat erectile dysfunction and pulmonary arterial hypertension [PAH; World Health Organization (WHO) group I] (Rosen and Kostis, 2003; Croom *et al.*, 2008). Additional preclinical data support a role for PDEs 1, 2, 3 and 10 in pulmonary hypertension, with proof-of-concept studies in cells and tissues from patients with the disease, implying that pharmacological blockade of other PDE isoforms might be beneficial (Phillips *et al.*, 2005; Schermuly *et al.*, 2007; Tian *et al.*, 2011; Bubb *et al.*, 2014). Further consideration of the therapeutic potential of PDE inhibitors, particularly PDE5, is discussed next.

## New lead molecules targeting the NO-sGC-cGMP pathway

Innovation in targeting the NO-sGC-cGMP pathway derives from either (i) development of new molecular entities; or (ii) extended clinical applications of already-approved therapeutic molecules. Research that has been conducted in the past 10–15 years has produced novel lead therapeutic molecules that have entered clinical evaluation and, on occasion, are now approved medicines.

Two main categories of novel chemical entities in the early or late clinical arena that target the NO-sGC-cGMP axis

are briefly explored below. First, there are established drugs that have been coupled to an NO-donating group to alleviate undesirable side effects of the 'parent' molecule. However, far more innovative is the second category, which includes sGC 'stimulators' and 'activators' and therefore this review will draw attention to their preclinical pharmacology and mode of action.

## NO-donating anti-inflammatory drugs

The most clinically advanced, major drug group that has been used as NO-donating, 'carrier' scaffold has been the steroidal and non-steroidal anti-inflammatory drugs (NSAIDs), including aspirin. These hybrid molecules are being tested in a wide array of indications, from colon cancer prophylaxis to reduction of vascular complications due to hypercholesterolaemia, not all of which can be thoroughly covered by this review.

The molecules that are perhaps closest to approval are NSAID conjugates whose therapeutic benefit relies (i) on the presumed gastroprotection that released NO would provide to the NSAID moiety, given the increased possibility of ulcer development (del Soldato *et al.*, 1999; Wolfe *et al.*, 1999; Bandarage and Janero, 2001); and (ii) on the counterbalancing of the modest, but significant, effect on blood pressure that certain NSAIDs can cause in some patient populations and that can limit the health benefit of the anti-inflammatory drug (White *et al.*, 2011). NSAIDs are among the most prescribed drugs in the world; however, it is now well established that their use carries the risk of upper gastrointestinal damage, including life-threatening bleeding complications, as side effects of their mode of action. The risk varies with the NSAID used and is especially frequent in certain populations prone to bleeding (Chan *et al.*, 2007). There are approved pharmacological strategies to prophylactically reduce the risk of gastrointestinal events due to NSAID intake, including, for example, co-administration of proton pump inhibitors (Chan *et al.*, 2007; Graham and Chan, 2008).

There is now ample experimental evidence from preclinical models that NO-releasing forms of approved steroidal and NSAIDs, including COX inhibitors such as aspirin and glucocorticoids such as prednisolone and flunisolide, exhibit similar or increased efficacy and a more favourable side effect profile than the parent molecules in several preclinical disease settings (Fiorucci *et al.*, 2002; Paul-Clark *et al.*, 2003; Turesin *et al.*, 2003; Wallace *et al.*, 2004). Such anti-inflammatory drug NO conjugates have been experimentally shown to modulate ovarian (Bratasz *et al.*, 2008) skin (Chaudhary *et al.*, 2013) or intestinal (Williams *et al.*, 2004) solid tumour growth, exert anti-inflammatory activity with reduced symptoms of gastric damage properties (Wallace *et al.*, 2004; Fiorucci *et al.*, 2007) and protect against or accelerate improvement of experimental colitis (Fiorucci *et al.*, 2002; Zwolinska-Wcislo *et al.*, 2011). The increased anti-inflammatory efficacy of at least one of them, the prednisolone derivative NCX-1015, may in part be attributed to glucocorticoid receptor nitration resulting in more robust signalling (Paul-Clark *et al.*, 2003).

A number of NO-conjugated COX inhibitors have also been evaluated in clinical trials. For example, NCX4016 (an aspirin-NO conjugate) has completed clinical testing in preventing colorectal cancer in patients at high risk for developing this disease (ClinicalTrials.gov identifier: NCT00331786) and in improving walking distance in patients with peripheral arterial occlusive disease (NCT01256775); however, no published report of trial outcomes is available at the writing of this review. Another 13 week clinical trial involves a naproxen–NO conjugate (naproxcinod) that is intended to treat 'hypertensive' patients (mean arterial pressure >125 mmHg) with osteoarthritis. In these individuals, naproxen induces a small rise (3–8 mmHg) in systolic BP, which increases significantly the risk of cardiac complications in this population. Naproxcinod exhibits a much lower tendency to increase systolic BP than naproxen, sparing the need for anti-hypertensive drugs taken concomitantly by this population (White *et al.*, 2011). However, the FDA has withheld approval until longer term effects of the drug are presented. In sum, none of these molecules has yet progressed to large-scale clinical evaluation, while, for the moment, the clinical use of NO-donating NSAIDs awaits convincing clinical data that for approval (Fiorucci and Distrutti, 2011).

## sCG stimulators

*Pharmacology and mode of action.* Given that reduced NO production is a defining feature of many cardiovascular diseases, including PH, the use of PDE inhibitors is likely to be limited as the efficacy of such molecules is dependent on endogenous cGMP generation. Thus, compounds that activate sGC directly, or that synergize with NO in activating the enzyme, appear a perfect fit as drug candidates in such indications. The initial discovery, by Taiwanese researchers in the mid-1990s, of the first 'sGC stimulator', YC-1 (Wu *et al.*, 1995), was paralleled by a wide search performed by a variety of pharmaceutical companies for molecules that could act in dual fashion: they synergize with NO in stimulating sGC and directly stimulate the enzyme in the absence of NO. Both activities are, however, dependent on the presence of a reduced, sGC-bound haem moiety (Hoenicka *et al.*, 1999).

The mechanistic basis of sGC stimulation by these molecules has been extensively studied, but not conclusively elucidated (Follmann *et al.*, 2013), mainly because there are no X-ray data of the full-length crystallized enzyme. Raman spectroscopic studies with sGC stimulators and structural modelling studies (based on the somewhat tenuous similarity to the AC catalytic domain) suggest that molecules such as YC-1 and BAY 41-2272 (i) induce a (indirect) change in the prosthetic haem group geometry that has bound NO, making the enzyme more active and stabilizing the nitrosyl–haem complex; and (ii) photoaffinity labelling of BAY-41-2272 and YC-1 analogues results in labelling of the α-subunit, following binding of the compound to a domain distinct from the catalytic site. However, it is not absolutely clear that the binding itself occurs on the α-subunit. It is possible that the site of binding is in the interface between the sGC subunits and thus elicits an allosteric interaction that results in a more active conformational shift of the enzyme and in the labelling of the α-subunit (reviewed in Derbyshire and Marletta, 2012; Follmann *et al.*, 2013). Alternatively, sGC stimulators have been suggested to relieve an autoinhibitory interaction between the H-NOX domain in the N-terminus, which harbours the haem moiety and the C-terminus catalytic domain (Winger and Marletta, 2005). In a recently published study, Purohit *et al.* (2014) demonstrated that YC-1 binding to the $\beta_1$

sGC subunit overcomes the allosteric inhibition by the $\alpha_1$ subunit. In all, the exact binding site of the sGC stimulators has not been assigned with certainty yet, and more structural studies have to be performed to finally understand how sGC stimulators bind to the protein.

Of the many molecules of the sGC stimulator class that have been developed, riociguat (BAY 63-2521) is the one that finished first in the translational race that led to its approval in the past year in the United States, Canada and in the European Union for the treatment of two forms of PH (Conole and Scott, 2013). Many sGC stimulator molecules, including YC-1, were abandoned because of lack of selectivity (YC-1 also inhibited PDEs) and poor pharmacokinetic characteristics (Stasch and Hobbs, 2009). One instructive reason for riociguat's success may be that very early, before full preclinical evaluation, all fellow candidate molecules were evaluated and discarded if they possessed a poor pharmacokinetic profile (Follmann *et al.*, 2013), allowing research to concentrate on candidates that were potent, selective and possessed a favourable bioavailability/pharmacokinetic profile. At the outset, riociguat showed good bioavailability and lack of interaction with the CYP metabolizing system, thus presenting the considerable advantage of future co-administration with other drugs (Follmann *et al.*, 2013). *In vitro* characterization of the drug showed strong synergy in combination with NO, ability to induce sGC activity in the absence of NO and dependence on a reduced haem prosthetic group. The preclinical evaluation of riociguat in key experimental animal models *in vivo* displayed, crucially, a long-preserved (several weeks) hypotensive effect in rats made tolerant to organic nitrates, effective inhibition or reversal of pulmonary vasoconstriction and remodelling (muscularization of small pulmonary arteries, hypertrophy of the right ventricle) in the monocrotaline model of PH (Schermuly *et al.*, 2008; Stasch *et al.*, 2011; Lang *et al.*, 2012), and reduction of heart and kidney fibrosis in the Dahl hypertensive rat, resulting in increased survival rates over time (Geschka *et al.*, 2011).

*Clinical success of the sGC stimulator, riociguat.* There are two clinical areas where considerable progress has been made in the last few years with the sGC stimulators: PH and heart failure, with pulmonary hypertension being the most successfully targeted clinical indication, based on riociguat's approval.

PH is a progressive, debilitating, multifactorial disease and exacts a high socio-economic toll. Most of the approved current treatments target one subgroup: PAH, a life-threatening form of the disease that is characterized by increased pulmonary vascular resistance, excessive remodelling of small vessels and of the pulmonary artery that lead, over time, to right heart failure and death (Baliga *et al.*, 2011; Galiè *et al.*, 2011; Schermuly *et al.*, 2011). Available treatments for PAH include endothelin receptor antagonists, PDE inhibitors, prostacyclin analogues and $Ca^{2+}$ channel blockers (Baliga *et al.*, 2011; Galiè *et al.*, 2011). The necessity of additional supportive drug therapy to treat concurrent pathophysiologies, which includes oral anticoagulants, digoxin for arrhythmias and diuretics to regulate fluid accumulation and blood pressure (reviewed by Galiè *et al.*, 2011) increases the risk of undesirable drug–drug interactions, especially with the anticoagulants. Approval of any new pharmacological options that are well-tolerated and display minimal drug–drug interactions would be a welcome addition to this therapeutic arsenal.

Among other PH forms, persistent PH of the neonate can be effectively treated with administration of inhaled NO (Roberts *et al.*, 1992; Vosatka *et al.*, 1994), but NO donors are not clinically useful for chronic treatment of PH because of partial patient response, development of severe tolerance over time, short-lived duration of the pulmonary vasodilation and the danger of methaemoglobinaemia with high NO doses (Ichinose *et al.*, 2004; Galiè *et al.*, 2011).

The exact molecular 'defect' in the NO-sGC-cGMP axis that may contribute to the development of the various forms of pulmonary hypertension in adults remains debatable and experimental and clinical data seem often contradictory (Giaid and Saleh, 1995; le Cras *et al.*, 1996; Xu *et al.*, 2004). Pharmacological potentiation of the NO pathway (Rossaint *et al.*, 1993; Klinger, 2007; Vermeersch *et al.*, 2007; Geschka *et al.*, 2011) has been the basis for the development of small-molecule inhibitors of PDE5A such as sildenafil and tadalafil, which were introduced in this clinical area in the past decade (Galiè *et al.*, 2009; 2011; Stasch and Hobbs, 2009). The issue, particularly in PAH, is reduced NO bioavailability: PAH is considered an NO-deficient state (Stasch and Evgenov, 2013). Because sGC expression is maintained or even up-regulated in PH, targeting it with a sGC stimulator (which can synergize with NO) seems a particularly beneficial approach.

Clinical trials with the sGC stimulator, riociguat, in two forms of PH were successfully concluded in 2013: the treatment met primary end points in patients diagnosed with PAH and with chronic thromboembolic pulmonary hypertension (CTEPH or WHO group IV). In the phase III trial (PATENT 1 ClinicalTrials.gov) in PAH patients who received riociguat alone or in combination with approved endothelin receptor antagonists or prostanoids for 12 weeks, the 6 min walk distance (6-MWD) increased by 36 m compared with placebo. In addition, there was significant improvement in pulmonary vascular resistance, cardiac output, N-terminal pro-B-type natriuretic peptide (NT-proBNP) plasma levels, time to clinical worsening, WHO functional class, Borg dyspnoea score and quality-of-life assessment. In addition, the benefit was also manifest at 24 weeks (Ghofrani *et al.*, 2013b). The second, 16 week phase III trial (CHEST-1) included patients diagnosed with CTEPH who were either inoperable or showed persistent or recurrent PH despite having undergone pulmonary endarterectomy, a standard surgical option for this group for which no pharmacological options exist. Riociguat increased the 6-MWD by 46 m compared with placebo and produced significant improvement in pulmonary vascular resistance, cardiac output, N-terminal pro-B-type natriuretic peptide level and WHO functional class (Ghofrani *et al.*, 2013a). In both trials, the safety profile of the sGC stimulator was reassuring, a major plus that warrants further evaluation of the molecule in additional indications.

In addition to the above indication, riociguat is also being tested clinically, and has shown beneficial effects, in proof of concept, pilot or phase II studies in patients with PH secondary to interstitial lung disease and chronic obstructive pulmonary disease (Bonderman *et al.*, 2013; Hoeper *et al.*, 2013; Stasch and Evgenov, 2013). The first report of a phase IIb trial

in patients with pulmonary hypertension caused by systolic left ventricular dysfunction, an indication with no approved medication, shows that treatment with riociguat did not meet the primary end point, which was the decrease in mean pulmonary artery pressure at 16 weeks (Bonderman *et al.*, 2013); however, it improved the secondary outcomes cardiac index and systemic and pulmonary resistance. Despite an attempt to decipher possible effects in patient populations after stratification, the study was not powered or designed to answer some critical questions, for example, whether riociguat elicited pulmonary vasodilation (inferred by the calculated drop in pulmonary vascular resistance) or whether variation of the drug dose and duration of treatment in specific patient subpopulations would successfully reach the primary end point. The mitigated results may leave the door open for a more prolonged trial, where long-term ventricular function is monitored and where, given riociguat's safety profile, higher doses are tested. Riociguat is also in early clinical stage evaluation for improvement of flow to the digits in Raynaud's syndrome patients (NCT01926847).

## sGC activators

*Preclinical pharmacology of sGC activators.* Additional screening of a compound library following the discovery of sGC stimulators at Bayer and further examination of hits revealed that a second series of dicarboxylic acids could up-regulate sGC activity in an NO-independent and haem-independent manner, thus inaugurating a quite different molecular class, termed sGC activators. More companies also arrived at similar-acting molecules (Schindler *et al.*, 2006; Costell *et al.*, 2012; Follmann *et al.*, 2013). Most of the second-generation molecules contain only one monocarboxylic acid moiety. An example of an activator that lacks carboxylic acid moieties also exists (HMR176). The mechanistic basis for the mode of action of sGC activators is arguably better understood than that of sGC stimulators. Data from functional, mutational and spectroscopic studies indicate that sGC activators bind in the haem cavity within the H-NOX domain of the $\beta_1$ subunit, competing with the native ligand (Pellicena *et al.*, 2004; Martin *et al.*, 2010; Follmann *et al.*, 2013). The $His^{105}$ in the $\beta_1$ H-NOX domain, which serves as a fifth coordination for the haem iron and is crucial for sGC activation, is displaced from the 'inactive' form, causing the rotation of the helix that harbours $His^{105}$ to a degree that depends on the sGC activator used (Follmann *et al.*, 2013). In this way, this class of compounds activate sGC in the absence of a haem moiety (Pellicena *et al.*, 2004; Follmann *et al.*, 2013). Of the sGC activators, the molecular mechanism of action of BAY 58-2667 (cinaciguat) has been characterized in most detail (Martin *et al.*, 2010). The carboxylic groups of BAY 58-2667 displace the haem propionic acids and interact with $Tyr^{135}$ and $Arg^{139}$ of the $\beta_1$ subunit and sGC activation results from a signal transmission triad composed of $His^{105}$, $Tyr^{135}$ and $Arg^{139}$ (Schmidt *et al.*, 2004).

Cinaciguat, and possibly other sGC activators, can prevent the degradation of sGC subunits that occurs following haem oxidation, apo-sGC formation and subunit ubiquitination in disease conditions. The ability of cinaciguat to closely mimic haem binding rescues sGC from proteasomal degradation by stabilizing the apo-sGC structure and thus possesses a dual mechanism of action (maintenance of sGC levels and sGC activation) in diseases associated with increased oxidative stress (Evgenov *et al.*, 2006; Martin *et al.*, 2010; Follmann *et al.*, 2013).

A more conclusive assessment of the sGC haem redox state in whole cells and in tissues would help improve decision making on which diseases might benefit from the administration of sGC activators (Ahrens *et al.*, 2011). There are two, recently described, methods that may allow this in different contexts in the future, provided that they are validated and confirmed by other laboratories. Fluorescence dequenching can be measured after the attachment of the biarsenical fluorophore FlAsH to the haem moiety (Hoffmann *et al.*, 2011) via energy transfer from this fluorophore to the haem. However, this technique for now is limited to live cells *in vitro* and has yet to be extended to *in vivo* applications. In addition, a biochemical determination can be performed by assessing the degree of sGC-Hsp90 complexation: the binding of Hsp90 is limited to the haem-lacking enzyme and Hsp90 is dissociated once sGC has incorporated a haem prosthetic group (Ghosh and Stuehr, 2012). Similar methods, once established, could be very useful in better directing the therapeutic applicability of sGC activators.

This class of NO- and haem-independent sGC activators, therefore, raised the possibility of therapeutic use in situations where sGC is present in its haem-free form. Increased levels of apo-sGC (leading to its ubiquitination and proteasomal degradation) occur during oxidative stress, exemplified by either full-blown, acute inflammatory responses or chronic, low-level inflammation (Stasch *et al.*, 2002; 2006). In these situations, the effect of PDE inhibitors or sGC stimulators is inherently limited (Evgenov *et al.*, 2006) due to a lack of intact NO–sGC signalling. Thus, sGC activators have been extensively characterized in preclinical models of disease to determine if they offer a greater therapeutic potential. For example, drugs modifying the haem-oxidized or haem-free enzyme would target diseased tissue. This proved to be the case with encouraging results observed in models of myocardial infarction, hypertension or congestive heart failure (reviewed by Follmann *et al.*, 2013). Cinaciguat, in a fast ventricular pacing model of congestive heart failure in dogs (Boerrigter *et al.*, 2007), reduced mean arterial, right atrial, pulmonary artery and pulmonary capillary wedge pressure; increased cardiac output and renal blood flow; and preserved glomerular filtration rate and sodium and water excretion, making it a prime therapeutic candidate for cardiovascular indications where sGC is impaired because of oxidative stress. In addition, cinaciguat was shown to antagonize crucial profibrotic mechanisms *in vitro* (Dunkern *et al.*, 2007), thought to operate in many pathological remodelling processes in chronic cardiovascular diseases. GSK2181236A a sGC activator developed by GlaxoSmithKline, was tested in spontaneously hypertensive stroke-prone rats on a high salt/fat diet, demonstrating organ-protective effects and reducing left ventricular hypertrophy (Costell *et al.*, 2012). Yet another sGC activator, HMR 1766 (ataciguat), was shown to improve *ex vivo* vascular function and reduce platelet activation (Schäfer *et al.*, 2010). Ataciguat also prevents and reverses pulmonary vascular remodelling and right ventricular hypertrophy in a mouse model of PH (Weissmann *et al.*, 2009). Collectively, these results warranted clinical evaluation in similar indications.

*Clinical testing of sGC activators*. HMR1766 (ataciguat) has been evaluated in two indications and trials have been completed: in the first, the primary end point was the reduction of pain in patients with neuropathic pain (NCT00799656) and in the second, the primary end point was improvement of intermittent claudication in patients with Fontaine stage II peripheral arterial disease (NCT00443287). The conclusions from these trials are still being awaited.

Cinaciguat has been tested in patients with acute decompensated heart failure, an indication where it seemed to be perfectly poised to succeed because of the strong evidence of NO pathway impairment in this disease and because of the experimentally based ability of the drug to limit fibrosis (reviewed by Tamargo and López-Sendón, 2011; Gheorghiade *et al*., 2013). Cinaciguat was delivered by i.v. administration at dose rates of 50–150 $\mu g \cdot h^{-1}$ and patients were monitored for up to 48 h. The trial, however, was terminated prematurely because of an increased occurrence of hypotension with all three doses (Gheorghiade *et al*., 2012; Erdmann *et al*., 2013a), which is an unfavourable occurrence in this patient population; in addition, there was no discernible effect of this treatment on either dyspnoea or on cardiac index and the small patient numbers did not allow stratification (Gheorghiade *et al*., 2012).

Although some of these clinical results have been disappointing, human genome-wide association studies have identified mutations in the genes encoding $\alpha_1$ (GUCY1A3) and $\beta_1$ (GUCY1B3) subunits of sGC, and in the sGC-stabilizing protein CCTη, which increase the risk of hypertension, thrombosis and myocardial infarction (Ehret *et al*., 2011; Erdmann *et al*., 2013b). Thus, there is strong evidence for a direct involvement of sGC impairment in thromboembolic human disease and in the regulation of blood pressure. Individuals carrying such mutations may be prime candidates for treatment with sGC stimulators or activators, as they are likely to be disease modifying. However, the ethnic divergence in phenotype which is associated with GUCY SNPs suggests that patient stratification to sGC modulating drugs may be necessary.

### *NOS cofactor supplementation*

One particular approach aiming to augment NO production is supplementation of the NOS cofactor tetrahydrobiopterin (BH4). Its bioavailability is reduced in a variety of cardiovascular pathologies, such as in atherosclerosis, at least in part as a result of overproduction of oxygen radicals, and correlates with NOS uncoupling (Förstermann and Li, 2011; Li and Förstermann, 2013). Pharmacological augmentation of BH4, therefore, aims to re-establish a healthy cofactor stoichiometry (Alkaitis and Crabtree, 2012; Starr *et al*., 2013) and direct eNOS catalytic activity towards producing NO rather than $O_2^-$. To achieve just that, several clinical trials have been conducted or are in progress in disease conditions that include systolic or systemic hypertension and peripheral artery disease; however, for the moment, results from these trials either do not reveal statistically significant changes or are still not reported (Alkaitis and Crabtree, 2012; Cunnington *et al*., 2012). Characteristically, supplementation of BH4 in patients with coronary artery disease, although it produced increased levels of BH4 in saphenous vein (but not in internal mammary artery), resulted in the presence of the oxidation product BH2, which lacks NOS cofactor properties, and failed to either reduce superoxide levels or improve vascular function (Cunnington *et al*., 2012). These results demonstrate that, while supplementation of NOS cofactor(s) is based on a sound therapeutic rationale, the establishment of a favourable target BH4 : BH2 ratio is hard to achieve. Therefore, a fundamentally different approach targeting BH4 may be more useful, such as indirectly increasing its recycling and preservation. Indeed, in atherosclerotic patients, supplementation with 5-methyl-tetrahydrofolate (which prevents peroxynitrite-driven oxidation of BH4) has been shown to reduce peroxynitrite-mediated BH4 oxidation, to ameliorate the BH4/total biopterin ratio and to increase NOS coupling, thus preserving *in vivo* and *ex vivo* vascular endothelial function (Antoniades *et al*., 2006).

## Repositioning of existing medicines and combination approaches

New molecular entities and modes of action have unquestionably boosted excitement in the NO field, and have advanced understanding of the physiology and pathology of sGC–cGMP signalling. However, significant translational progress has also been made with older, approved drugs. Quite a few of these have been, or are currently being, evaluated in indications that are either poorly served by available medications, or where an improvement of the currently obtainable therapeutic effect is desired.

One such example is the small (six patient), pilot clinical trial with a combination of the tried-and-tested organic nitrate, isosorbide mononitrate (ISMN), and the PDE5 inhibitor, sildenafil, in achieving better regulation of the blood pressure in patients afflicted with 'resistant' hypertension (Oliver *et al*., 2010). Monotherapy with either drug alone effectively reduced brachial systolic and diastolic blood pressure, and central systolic and diastolic arterial pressure. Combination of sildenafil and ISMN elicited significantly stronger reduction of brachial systolic blood pressure and central arterial systolic pressure, compared with either drug alone. Reduction of central arterial pressure with the combination reached a maximum of 26/18 mmHg (systolic blood pressure/diastolic blood pressure) compared with placebo (Oliver *et al*., 2010), thus opening the way for a study involving more patients and evaluation of longer administration of this combination in this challenging patient population.

Sildenafil also showed improvement in non-ischaemic, non-failing diabetic cardiomyopathy (i.e. at a relatively early stage) in a small, 3 month trial in 59 diabetic patients (NCT00692237), improving left ventricle contraction and preventing cardiac remodelling through, presumably, direct intramyocardial effects, independent of endothelial vasodilatation (Giannetta *et al*., 2012). Longer term results are expected in the next 48 months.

More impressively, in a 1 year prospective trial in 45 patients with stable, systolic heart failure, sildenafil, at 6 months and 1 year, improved left ventricle ejection fraction and elicited reverse remodelling of left atrial volume index and left ventricle mass index. These structural and functional ameliorations by sildenafil were coupled with improved exer-

cise performance, ventilation efficiency and quality of life, thus making sildenafil the first PDE5 inhibitor that demonstrably elicits structural and functional changes in the human heart (Guazzi *et al.*, 2011). A year later, the same group (Guazzi *et al.*, 2012) reported that sildenafil succeeded, in a group of patients with heart failure that presented oscillatory breathing during exercise (attributed to pulmonary vasoconstriction), to almost eliminate (in ~90% of the patients at 6 and 12 months) oscillatory breathing, a sign of poor prognosis for the progress of the disease, as well as to improve functional performance. These results were accompanied by reductions of pulmonary vascular resistance and pulmonary arterial pressure. Unfortunately, in the longer term follow-up RELAX study (Effectiveness of Sildenafil at Improving Health Outcomes and Exercise Ability in People With Diastolic Heart Failure), treatment of HFpEF patients with sildenafil failed to produce a significant change in exercise capacity, its primary outcome measure (Redfield *et al.*, 2013), despite the positive outcome achieved in systolic heart failure patients (Guazzi *et al.*, 2011).

Sildenafil was also tested in a 12 week clinical trial (NCT00517933) in patients with idiopathic pulmonary fibrosis (Zisman *et al.*, 2010). Although the primary end point (increase in the 6 min walk distance by more than 20%) was not met, secondary symptomatic end points such as oxygenation, dyspnoea and quality of life score were improved by sildenafil (Zisman *et al.*, 2010), raising the possibility of an expanded clinical investigation in the future.

Yet another approved PDE5 inhibitor, tadalafil, was the second molecule of its class to be approved for PAH in 2009 (Rosenzweig, 2010). Furthermore, in 2012, in a small pilot study, tadalafil proved effective in normalizing blood flow to the muscles of patients with Becker's muscular dystrophy (BMD). This genetic disease is linked to mutations in the gene encoding the skeletal muscle protein dystrophin, which induces defective sarcolemmal targeting of proteins, among which nNOSμ, and progressive muscle damage and wasting (Bushby *et al.*, 2010a,b). There is no pharmacological treatment directed to this disease, which is associated with cardiomyopathy and results in loss of ambulation. The investigators tested a small patient group (and a matched cohort control, $n$ = 10 each) for restoration of the exercise-induced attenuation of reflex sympathetic vasoconstriction. This is a physiological reflex that optimizes perfusion to the exercising muscles. This reflex was absent in 9/10 men carrying the disease and tellingly correlated with missing sarcolemmal nNOSμ. Tadalafil, given once, normalized this adaptive blood flow in response to sympathetic vasoconstriction in all participant patients (Martin *et al.*, 2012) and can therefore benefit people with BMD by preventing muscle damage due to pathological vasoconstriction during exercise. In addition, in a promising preclinical study in a related indication sildenafil reversed cardiac dysfunction in the mdx mouse model of Duchenne muscular dystrophy (Adamo *et al.*, 2010).

It can safely be said that the expectation, broadly shared by the research community, that PDE5 inhibitors would be clinically useful in treating heart failure (Zhang and Kass, 2011) or other diseases with a critical cardiac and/or vascular dysfunction (Kukreja *et al.*, 2011) is slowly but steadily being fulfilled, despite the occasional hiccup. The clinical success and failures of PDE5 inhibitors reveal both the potential and the limitations of their therapeutic utility. More diversified trials may be expected to near completion in the next 2–3 years, firmly positioning PDE5 inhibitors in the therapeutic arena for years to come.

## Thinking 'outside the box': re-examination of existing work and targeting novel therapeutic areas

Innovative rethinking of the role of the NO pathway in disease can open new opportunities, described briefly in the sections below. This is particularly true of the role of NO in sepsis, which points towards 'a window of opportunity' for sGC activators. In addition, dietary supplementation with inorganic nitrates offers an elegant example of how one can clinically improve cardiovascular disease by administering a simple, cheap and effective molecule. The therapeutic advantage of *inhibition* of the NO pathway has received relatively little attention, compared to efforts to *increase* NO activity; however, there are situations where this could provide therapeutic benefit. Lastly, the involvement of NO in energy expenditure is a topic with immense translational potential in atherometabolic diseases.

### *Time-sensitive apo-sGC stabilization in sepsis?*

After the recent withdrawal of recombinant activated protein C from the market, there are no other specifically approved medications for sepsis, a largely (>50%) lethal indication (Ranieri *et al.*, 2012). The hypothesis that boosting NO signalling may be of therapeutic interest in this life-threatening disorder is a novel concept, and directly opposite to the initial notion that iNOS inhibitors, which reduce the excessive NO production associated with systemic expression of this NOS isozyme in sepsis, would be the better approach (a thesis that failed to be substantiated in clinical evaluation; López *et al.*, 2004). The anti-inflammatory properties of NO are well documented and augmenting NO signalling shows positive preliminary results in animal models of endotoxaemia (Da *et al.*, 2007) and alleviates some symptoms in humans presented with adult respiratory distress syndrome (Taylor *et al.*, 2004). Furthermore, nitrite generates NO selectively in hypoxic conditions (Lundberg *et al.*, 2008) and can rescue mice subjected to LPS- or TNF-α-elicited shock (Cauwels *et al.*, 2009), an effect mediated by cGMP produced by $sGC\alpha_1/\beta_1$ (Buys *et al.*, 2009). However, initial experimental tests in endotoxin-exposed subjects that received inhaled NO have not yielded positive results (Hållström *et al.*, 2008). A recent study in mice (Vandendriessche *et al.*, 2013), though, has re-addressed this issue and has generated some very interesting observations, namely that a beneficial effect may critically depend on a combination of optimal timing and of apo-sGC stabilization. Mice that received an LPS injection were treated with sildenafil, the sGC stimulator BAY 41-2272 or the sGC activator cinaciguat, 3 or 8 h post-LPS challenge. Mortality was prevented only by cinaciguat, and only when it was given at the late, 8 h, time point after LPS. The effect

of late treatment with cinaciguat correlated with stabilized body temperature and reduced cardiomyocyte apoptosis (Vandendriessche *et al.*, 2013). This preclinical work demonstrates that 'reactivation/preservation' of apo-sGC is crucial in endotoxaemic shock and that the response critically depends on the time of treatment, when 'rescued' function of haem-free sGC is optimally amenable to impact the course of the disease. It is therefore of particular importance in future clinical trials in sepsis and systemic inflammatory response syndrome to correctly estimate this target window of apo-sGC responsiveness. It should be stressed that in sepsis, distinguishing between the effects of NO in the macrocirculation and in the microcirculation is important, and generation of NO selectively in the microcirculation may provide critical cytoprotective and tissue-protective effects. Indeed, treatment with nitrite, which is converted to NO selectively in hypoxic/acidic conditions, characteristic of the septic microvasculature, provides therapeutic benefit in preclinical murine models based on challenge by LPS or TNF-α, alleviating telltale symptoms of sepsis, such as organ damage and progressive hypothermia (Cauwels and Brouckaert, 2011).

## *Inorganic nitrite and nitrate*

Although organic nitrates have been used for the treatment of angina and heart failure for more than 150 years, the physiological importance and pharmacodynamic properties of inorganic nitrite ($NO_2^-$) and nitrate ($NO_3^-$) have only recently been established (Lundberg *et al.*, 2008; 2009). Initially considered to be simply inactive oxidation products of NO, it is now clear that these molecules can be reduced, preferentially under conditions of hypoxia and acidosis, to bioactive NO. This 'non-canonical' route of NO generation (Figure 1) is dependent on reduction of nitrate to nitrite by anaerobic bacteria that colonize the tongue, concentration of nitrite in the saliva, followed by absorption through the gut wall and entry into the systemic circulation (Lundberg *et al.*, 2008; Kapil *et al.*, 2010b). Production of NO from nitrite is then catalysed by 'nitrite reductase' enzymes, including xanthine oxidoreductase (Millar *et al.*, 1998; Zhang *et al.*, 1998; Webb *et al.*, 2008a) and globins (Doyle *et al.*, 1981; Basu *et al.*, 2007; Tiso *et al.*, 2011). In preclinical models, augmentation of this 'nitrate-nitrite-NO' pathway lowers systemic blood pressure, protects against ischaemia–reperfusion (I/R) injury and ameliorates pulmonary hypertension (Hunter *et al.*, 2004; Webb *et al.*, 2004; 2008b; Hendgen-Cotta *et al.*, 2008; Casey *et al.*, 2009; Zuckerbraun *et al.*, 2010; Baliga *et al.*, 2012). Such positive observations and the comparative ease of pharmacological and/or dietary manipulation of nitrite/nitrate levels has led to rapid translation of this phenomenon to healthy volunteers and patients with cardiovascular disease. Inorganic nitrite and nitrate have both been shown to lower systemic blood pressure in healthy volunteers (Cosby *et al.*, 2003; Larsen *et al.*, 2006; Webb *et al.*, 2008b; Kapil *et al.*, 2010a) and dietary nitrate supplementation reduces blood pressure in hypertensive patients (Ghosh *et al.*, 2013) with a significantly increased potency, suggesting the beneficial effects of modulating nitrate-nitrite-NO signalling for therapeutic benefit are enhanced in disease. Further clinical evaluation has been conducted in patients presenting with acute myocardial infarction undergoing percutaneous coronary intervention. In a randomized, placebo-controlled, double-blind phase II evaluation, a 5 min i.v. administration of sodium nitrite prior to angioplasty did not reduce infarct size (the primary end point), although a subgroup of patients with diabetes did show some improvement (Siddiqi *et al.*, 2013; NCT01388504 and ISRCTN57596739). This lack of efficacy is disappointing, given the preclinical observations, but may be dose related as the 70 μmol $NaNO_2$ administrated was insufficient to significantly increase circulating $NO_2^-$ concentrations, at least to levels shown to be required for blood pressure effects in healthy volunteers and hypertensive patients. Thus, further studies with higher doses of nitrite (and/or nitrate) and using different routes of administration (e.g. intracoronary) are warranted. Several further studies, primarily to assess the pharmacokinetic and safety profile of inorganic nitrite or nitrate, are also underway or nearing completion in patients with cardiovascular disease (e.g. cerebral vasospasm, sickle cell, peripheral arterial disease). Nitrite, at least in part via bioconversion to NO, can also provide tissue and organ protection following ischaemia (Rassaf *et al.*, 2014), whether the experimental ischaemic insult is established in heart, kidney, brain or liver. In addition, nitrite also offers protection from experimental hypoxia-induced pulmonary hypertension (Rassaf *et al.*, 2014). Based on these data, the beneficial effects of inhaled nitrite are currently being investigated in a phase I clinical trial, determining the changes in pulmonary vascular resistance in patients with pulmonary hypertension that undergo right heart catheterization (NCT01431313). In sum, raising plasma nitrite levels by pharmacological or dietary means represents a novel and inexpensive strategy to augment sGC–cGMP signalling for therapeutic gain.

## *Therapeutic potential of NOS inhibitors*

High NO concentrations can compromise the blood–brain barrier and lead to brain oedema. The expression of iNOS and the levels of NO peak about 24–48 h after traumatic brain injury in humans (Clark *et al.*, 1996). The improved pathology in mice subjected to cryogenic cerebral trauma that have been subjected to genetic (Jones *et al.*, 2004) or pharmacological (Rinecker *et al.*, 2003) ablation of iNOS indicates a deleterious role for NO in the recovery in this disease setting. VAS203 (6R,S)-4-amino-5,6,7,8-tetrahydro-L-biopterin) is an allosteric NOS inhibitor which, in a preclinical mouse model of intracranial oedema formation subsequent to cerebral trauma, showed improvements in short-term (24 h) oedema formation and in long-term functional preservation (Terpolilli *et al.*, 2009). VAS203 is being tested in the clinic (NOSTRA: NO-Synthase inhibition in TRAumatic brain injury), in a European multicentre trial that is ongoing. Preliminary phase IIa results (according to a communiqué of the company) seem promising; however, the end of the trial has to be awaited to conclude on the efficacy of this molecule. Nonetheless, these findings are welcome because to date, iNOS inhibitors have failed to make the positive clinical impact predicted by animal models, particularly in the setting of sepsis.

NO production by NOS isoforms is regulated through protein–protein interactions. In particular, nNOS has been found to exist in a ternary complex with the synaptic scaffolding protein PSD95 and the NMDA receptor. Activation of this complex by glutamate following stroke and excessive NO

production contributes to neuronal excitotoxicity and brain damage, making nNOS–PSD95 uncoupling a therapeutic approach to limit neurotoxicity (Cao *et al.*, 2005). Tat-NR2B9c is a chimeric peptide that consists of the HIV-1 Tat protein transduction domain to facilitate cell penetration fused to a sequence that binds to the PDZ domains of PSD95 disrupting downstream neurotoxic signalling pathways, without blocking NMDA receptor activity. It was demonstrated that i.v. administration of Tat-NR2B9c 1 h after middle cerebral artery occlusion in non-human primates led to a reduction in infarct volume by 70% after 30 days. An improved, dimeric version of this peptide (NA-1) was generated and first tested in mice with favourable results (Bach *et al.*, 2012). NA-1 was subsequently tested for its ability to improve the outcome of iatrogenic strokes occurring during aneurysm repair and assessed in a phase II trial (ENACT, NCT00728182). Although no differences in infarct volumes were observed between the saline and NA-1 groups, patients who received NA-1 exhibited significantly fewer new brain lesions than those receiving saline (Hill *et al.*, 2012). This landmark study provides a proof of concept that neuroprotection is feasible in humans; however, the efficacy of NA-1 in community-onset stroke needs to be further established in more extended studies.

NO is also involved in nociceptive processing in the brain and contributes to cerebral artery vasodilatation, which is a symptomatic epiphenomenon of migraine (Hoffmann and Goadsby, 2012). iNOS seems to play a role in the pathogenesis of the disease, and for this reason iNOS inhibitors have also been in various stages of preclinical and clinical development to treat migraine, of which GW274150 is the most advanced. This molecule has been clinically tested both as a prophylactic treatment and as a treatment in acute migraine (NCT00242866 and NCT00319137). Results from both trials show that at doses that are predicted to inhibit iNOS by 80–90%, GW724150 was ineffective in reducing pain (Høye *et al.*, 2009; Palmer *et al.*, 2009; Høivik *et al.*, 2010). Taken together, therefore, these data suggest that iNOS inhibition is unlikely to provide therapeutic relief in this indication.

iNOS inhibitors have also been, or are being, tested in additional indications. Elevated NO biosynthesis has been linked with increased angiogenesis, bone resorption and destruction of connective tissue in rheumatoid arthritis (Farrell *et al.*, 1992; Stefanovic-Racic *et al.*, 1993; Sakurai *et al.*, 1995), manifestations that correlate with the pathogenesis and progress of the disease. GW274150 has been under clinical evaluation for use in this indication (NCT00370435 and NCT00379990). Final evaluation of 28 day treatment with GW274150 in reducing synovial thickness and vascularity in a rheumatoid arthritis in an early phase trial showed only a non-statistically significant trend (Seymour *et al.*, 2012). The results of additional concurrent trials are being awaited. Last, GW274150 has also been tested in the treatment of mild asthma (NCT00273013). The conclusions of the study were negative, as GW274150 did not inhibit early or late asthmatic challenges to allergen or to methacholine-induced responses (Singh *et al.*, 2007).

These mixed clinical results suggest that it may be important in the future to focus testing selective NOS inhibitors in a subset of carefully chosen clinical indications.

### *Regulation of fat phenotype and energy expenditure by NO*

Our understanding of the molecular mechanisms that determine adipose tissue phenotype and of the respective pathophysiological roles of white and brown fat has made impressive progress lately (Bartelt and Heeren, 2014). Hence, ways to pharmacologically control and modulate fat phenotype can have a potentially enormous impact on various pathologies, including atherometabolic diseases. Pharmacological inhibition of NO activity *in vitro* or eNOS genetic inactivation *in vivo* results in decreased mitochondrial biogenesis, which is ascribed to altered cGMP generation; these interventions also interfere with non-shivering thermogenesis by brown fat and with energy expenditure (Nisoli *et al.*, 2003). Conversely, eNOS transgenic mice (overexpressing eNOS under the pre-proendothelin promoter) on high fat diet display increased systemic metabolic rate (not attributed to hyperthyroidism) and adipose cell hypertrophy, while their adipose tissue shows signs of 'browning', with higher mitochondrial activity and elevated PPAR-α and PPAR-γ expression (Sansbury *et al.*, 2012). In addition to NO-dependent pathways, natriuretic peptide signalling can also trigger a brown fat thermogenic programme in white adipocytes (Bordicchia *et al.*, 2012). Collectively, these data clearly show anti-obesity effects of cGMP-mediated signalling and raise the possibility that increased NO bioactivity may help control some crucial features of the metabolic syndrome. Importantly, in the study by Sansbury *et al.*, eNOS overexpression did not affect blood glucose handling. These exciting results point to a novel biochemical pathway that can be effectively targeted, even with currently available medications, to control clinical features of metabolic disorder associated with obesity.

## Summary

### *A promising future for molecules targeting the NO-sGC-cGMP pathway in cardiovascular diseases*

The collective research effort to better understand the biochemical and mechanistic complexity of the NO-sGC-cGMP pathway, combined with the progress in elucidating its regulation and involvement in pathophysiology (Figure 1), have successfully guided the translational development of medicines to address important human therapeutic needs. The extraordinary robustness of the field is mainly due to three factors: (i) the existence of already-approved molecules with good safety profile that continue to be pillars of therapy; (ii) the successful, steady repositioning of approved molecules in new therapeutic niches; and (iii) the continuous development of a significant number of lead molecules that employ a novel mechanism of action or target molecular components of the system (Table 1) that had received poor attention before (e.g. riociguat and NA-1 respectively). It can be predicted that an increasing number of new therapeutic candidates that target the NO-sGC-cGMP pathway will be seeking clinical assessment and approval in the next few years to benefit the treatment of therapeutically challenging, or even intractable, human pathologies (Figure 1).

## Table 1

Summary of the therapeutically amenable molecular targets within the NO-cGMP-sGC axis discussed in this review

| Target | Molecules | Function | References |
|---|---|---|---|
| Arginase | N-hydroxy-nor-arginine | Arginase inhibition; L-Arg preservation | Shemyakin *et al.*, 2012; NCT02009527 |
| NOS | Caveolin-derived peptide | Disruption of NOS–caveolin interaction | Bucci *et al.*, 2000 |
| | BH4 | Cofactor supplementation; enhancement of NOS coupling | Alkaitis and Crabtree, 2012; Cunnington *et al.*, 2012; Antoniades *et al.*, 2006 |
| | VAS203 (6R,S)-4-amino-5,6,7,8-tetrahydro-L-biopterin | NOS inhibition | Terpolilli *et al.*, 2009 |
| | NA-1 peptide | NO-PSD95-(NMDAR) complex uncoupling | Bach *et al.*, 2012; Hill *et al.*, 2012 |
| | GW274150 | iNOS inhibition | Høye *et al.*, 2009; Singh *et al.*, 2007 |
| sGC | NO anti-inflammatory drug conjugates | NO release–anti-inflammatory action | NCT00331786; NCT01256775; White *et al.*, 2011 |
| | sGC 'stimulators' | NO-independent, haem-dependent sGC activation | Follmann *et al.*, 2013; Ghofrani *et al.*, 2013a,b |
| | sGC 'activators' | NO- and haem-independent sGC activation | Follmann *et al.*, 2013; Vandendriessche *et al.*, 2013 |
| | Nitrate–nitrite | Bioconversion to NO | Lundberg *et al.*, 2008; Cosby *et al.*, 2003; Kapil *et al.*, 2010a |
| PDE5 | PDE inhibitors | Enhancement of cGMP signalling | Keravis & Lugnier, 2012 |

Targets and molecules that have been validated preclinically or clinically are shown.

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## Conflict of interest

A. J. H. has acted as a consultant/advisory board member for Bayer AG, Novartis, Merck and Palatin Technologies.

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