Nitric Oxide Synthase Inhibitors into the Clinic at Last

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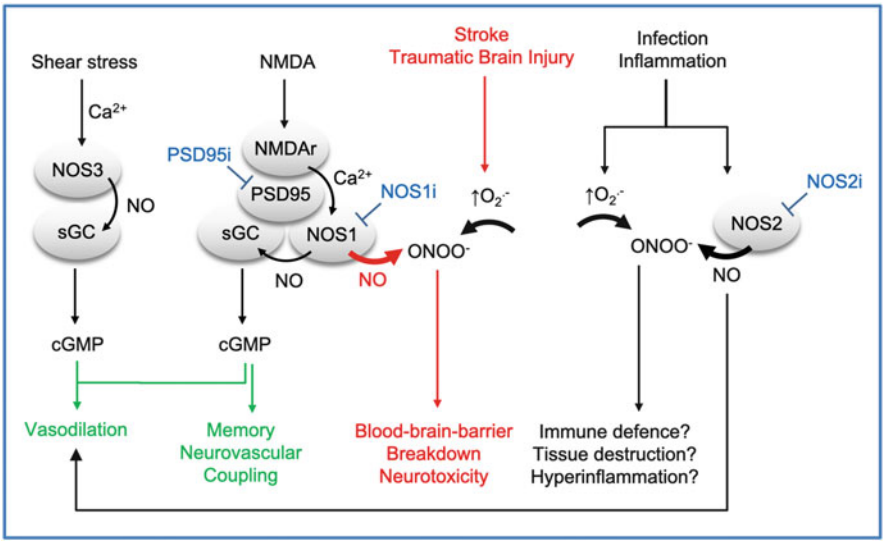
Abstract

The 1998 Nobel Prize in Medicine and Physiology for the discovery of nitric oxide, a nitrogen containing reactive oxygen species (also termed reactive nitrogen or reactive nitrogen/oxygen species) stirred great hopes. Clinical applications, however, have so far pertained exclusively to the downstream signaling of cGMP enhancing drugs such as phosphodiesterase inhibitors and soluble guanylate cyclase stimulators. All clinical attempts, so far, to inhibit NOS have failed even though preclinical models were strikingly positive and clinical biomarkers correlated perfectly. This rather casts doubt on our current way of target identification in drug discovery in general and our way of patient stratification based on correlating but not causal biomarkers or symptoms. The opposite, NO donors, nitrite and enhancing NO synthesis by *e*NOS/NOS3 recoupling in situations of NO deficiency, are rapidly declining in clinical relevance or hold promise but need yet to enter formal therapeutic guidelines, respectively. Nevertheless, NOS inhibition in situations of NO overproduction often jointly with enhanced superoxide (or hydrogen peroxide production) still holds promise, but most likely only in acute conditions such as neurotrauma (Stover et al., J Neurotrauma 31(19):1599–1606, 2014) and stroke (Kleinschnitz et al., J Cereb Blood Flow Metab 1508–1512, 2016; Casas et al., Proc Natl Acad Sci U S A 116 (14):7129–7136, 2019). Conversely, in chronic conditions, long-term inhibition of NOS might be too risky because of off-target effects on *e*NOS/NOS3 in particular for patients with cardiovascular risks or metabolic and renal diseases.

Graphical Abstract



Nitric oxide synthases (NOS) and their role in health (green) and disease (red). Only neuronal/type 1 NOS (NOS1) has a high degree of clinical validation and is in late stage development for traumatic brain injury, followed by a phase II safety/efficacy trial in ischemic stroke. The pathophysiology of NOS1 (Kleinschnitz et al., J Cereb Blood Flow Metab 1508–1512, 2016) is likely to be related to parallel superoxide or hydrogen peroxide formation (Kleinschnitz et al., J Cereb Blood Flow Metab 1508–1512, 2016; Casas et al., Proc Natl Acad Sci U S A 114(46):12315–12320, 2017; Casas et al., Proc Natl Acad Sci U S A 116(14):7129–7136, 2019) leading to peroxynitrite and protein nitration, etc. Endothelial/type 3 NOS (NOS3) is considered protective only and its inhibition should be avoided. The preclinical evidence for a role of high-output inducible/type 2 NOS (NOS2) isoform in sepsis, asthma, rheumatic arthritis, etc. was high, but all clinical development trials in these indications were neutral despite target engagement being validated. This casts doubt on the role of NOS2 in humans in health and disease (hence the neutral, black coloring).

Keywords

Nitric oxide · Nitric oxide synthase · NOS · NOS inhibitor · NOS isoforms

Abbreviations

ADMA	Asymmetric dimethyl arginine
ADME	Absorption, distribution, metabolism, and excretion
CaM	Calmodulin
cHL	Classical Hodgkin lymphoma
CLL	Chronic lymphocytic leukemia
DAMP	Damage-associated molecular pattern
*e*NOS/NOS3	Endothelial nitric oxide synthase
GLP-2	Glucagon-like peptide-2
H4Bip	Tetrahydrobiopterin
HNSCC	Neck squamous cell carcinoma
Hsp	Heat shock protein
IDH	Intradialytic hypotension
*i*NOS/NOS2	Inducible nitric oxide synthase
JSN	Joint space narrowing
L-NIL	L-*N* iminoethyl lysine
L-NMMA	N^G-monomethyl-L-arginine
NF-κB	Nuclear factor kappa B
NMDA	*N*-methyl-D-aspartate
*n*NOS/NOS1	Neuronal nitric oxide synthase
PAMP	Pathogen-associated molecular pattern
PD-1	Programmed death-1
PSD95	Post synaptic domain
sGC	Soluble guanylate cyclase
SMTC	*S*-methyl-L-thiocitrulline

1 NOS Isoforms, Regulation and Dysregulation

Nitric oxide (NO) synthases (NOS) are homodimeric NADPH binding flavo-heme proteins additionally regulated by the redox-sensitive cofactor tetrahydrobiopterin (H4Bip), calmodulin and several other modulatory interactions (Nedvetsky et al. 2002) to convert L-arginine (Schmidt et al. 1988; Nedvetsky et al. 2002) to NO. Three isoforms exist, originally named according to their first observed cellular/tissue localization or expressional regulation, i.e., neuronal, inducible, and endothelial (i.e., NOS1, NOS2, and NOS3) (Schmidt et al. 1991; Förstermann et al. 1992; Chakrabarti et al. 2012; Liu et al. 2012; Caviedes et al. 2017).

All three NOS isoforms generate NO by conversion of L-arginine to L-citrulline by a stoichiometric five electron oxidation utilizing molecular oxygen (O_2) and reduced nicotinamide adenine dinucleotide phosphate (NADPH) as co-substrates and several cofactors including 6R-tetrahydrobiopetrin (BH4), flavin adenine dinucleotide (FAD), and flavin mononucleotide (FMN) (Bredt et al. 1991; Knowles and Moncada 1994).

The NOS monomer structure has two domains, the NOS heme containing amino-terminal and the carboxy-terminal domain harboring binding sites for FAD, FMN, and NADPH. Both domains are connected by linking to an calcium-binding regulatory protein, i.e., calmodulin (CaM) (Smith et al. 2013) enhancing electron flux within the reductase domain and thus is essential for O_2 to bind to NOS heme to start the NO synthesis. Ca^{2+}-dependent CaM binding occurs in NOS1 and NOS3 following an increase in intracellular Ca^{2+}concentrations, while NOS2 binds CaM at low intracellular Ca^{2+}level thus independent of elevation of intracellular Ca^{2+}level (Cho et al. 1992).

A key step of NOS maturation which has been shown for NOS1 and 2 involves the insertion of heme by associating to different heat shock proteins (Hsp) such as Hsp 90 and Hsp 70 (Ghosh et al. 2011; Peng et al. 2012). Then, in the presence of NOS heme, the monomers can form dimers by coupling their ferric hemes and thus become fully active to generate NO. This takes place in the N-terminal oxygenase site that catalyzes first hydroxylation then oxidation of L-arginine to L-citrulline and NO, initiated by O2 binding to reduced ferric heme that utilized electrons provided by the reductase C-terminal domain of the opposite monomer via NADPH to FAD to FMN (Stuehr et al. 2001; Forstermann and Sessa 2012; Ramasamy et al. 2014). Intermediate formed heme-dioxy species are then reduced by NOS coupled BH4 resulting in reactive heme-oxy species that react either with L-arginine or *N*-hydroxy-L-arginine (Masters et al. 1996; Stuehr and Haque 2019). Hence, oxidation of BH4 results in BH3 radical or radical cation that can be reversed by NOS itself transferring an electron (Crabtree and Channon 2011) or by ascorbic acid (Kuzkaya et al. 2003). In addition, all NOS enzymes contain a zinc-tetrathiolate binding two cysteine residues provided by each monomer Masters et al. 1996; Raman et al. 1998; Li et al. 1999; Hemmens et al. 2000), which is catalytically inactive at the dimer interface (Forstermann and Sessa 2012) but stabilizing the active homodimer and appear to promote BH4 binding (Chreifi et al. 2014). In addition, the zinc-tetrathiolate cluster can be targeted by selective *S*-nitrosation (Wynia-Smith and Smith 2017).

1.1 Neuronal NOS/Type 1 NOS (nNOS/NOS1)

NOS1 is abundantly expressed in neurons of the central and peripheral nervous systems (Dudzinski et al. 2006), e.g., in hypothalamic supraoptic nucleus and the paraventricular nucleus, in some parts of rat glial cells (Korzhevskiĭ et al. 2007), in rat astrocytes and the adventitia of rat brain blood vessels (Yuan et al. 2004), but is also found in skeletal muscle, pulmonary epithelium, the gastrointestinal system, and the genitourinary system (Zhou and Zhu 2009).

Amongst the three NOS isoforms only NOS1 encodes a unique regulatory protein–protein interaction domain, i.e., PSD/Disc-Large/ZO-1 (PDZ) at the N-terminus, relevant for changes of subcellular localization as it can interact directly with other proteins containing PDZ domains or adapter proteins (Courtney et al. 2014; Candemir et al. 2016). Further, regulatory aspects leading to activation of

constitutively expressed NOS1 are besides CaM-binding, the AKT phosphorylation site at the C-terminus, that helps CaM to bind (Gantner et al. 2020). Another allosteric activator that is shown to activate NOS producing NO is Hsp90 which associates to *n*NOS, thus enhancing calmodulin binding to *n*NOS (Bender et al. 1999; Song et al. 2001). In addition, there is an autoinhibition site close to the CaM binding domain controlling NOS activity (Salerno et al. 1997).

NOS1-derived NO and its signaling has been implicated in not only antegrade but also retrograde signaling from the postsynaptic neuron, thus contributing to long-term potentiation (Böhme et al. 1993; O'Dell et al. 1994), fear conditioning (Ota et al. 2010), and neurogenesis (Chong et al. 2018). Furthermore, NO signaling is involved in mediating excitotoxicity in neurons driven by excessive glutamate-dependent overstimulation of NMDA receptors following Ca^{2+}overload in the cell, and consequently activation of Ca^{2+}-sensitive enzymes such as NOS1 (Sattler and Tymianski 2000; Chong et al. 2018) leading to cell death.

Mechanistically, neuronal NOS1 signaling requires adapter proteins harboring a PDZ motif such as syntrophin (Aquilano et al. 2014), PSD95 or PSD93 (Brenman et al. 1996) to anchor NOS1 and thereby target NOS1 to the proximity of the NMDA receptors at the postsynaptic membranes. Another adapter protein is NOS1AP (former Capon) that binds to the PDZ motif of NOS1 via its C-terminal domain (Jaffrey et al. 2002).

Besides the brain, NOS1 is involved in other physiological regulations such as skeletal muscle metabolism in response to exercise training (Percival 2011). This is regulated by different splice variants of the highly conserved NOS1 consisting of 29 exons and about 240 kb. These are an almost full length *n*NOSα, *n*NOSμ with 34 additional amino acid insertions, the PDZ lacking *n*NOSβ and *n*NOSγ and finally *n*NOS2 (Gantner et al. 2020).

The enzymatic activity of the splice variants may differ depending on their subcellular localizations but their activation also requires calcium and phosphorylation by PI3K/Akt (Gantner et al. 2020). Of those *n*NOSμ in skeletal muscle is bound to the dystrophin glycoprotein complex, at the sarcolemma and has been shown to contribute to better muscle blood flow, resisting fatigue by endurance training (Percival 2011). Furthermore, NOS1 has been implicated in inflammatory response (Baig et al. 2015) cardiac and smooth muscle physiology involving to cardiac protection and vascular tone (Seddon et al. 2008, 2009; Shabeeh et al. 2017).

1.2 Inducible NOS/Type 2 NOS (iNOS/NOS2)

Mammalian inducible NOS2 is a 131 kDa protein composed of 1,153 amino acids that lacks the PDZ domain and in contrast to NOS1&3 also the autoinhibition site and is not constantly expressed but only by induction of the cell. Depending on cell type and species strong stimulants of transcription of NOS2 expression are tumor necrosis factor (TNF), interleukin (IL-1β), interferon (IFN-γ), and lipopolysaccharide (LPS) exerting synergistic effects when combined (Cinelli et al. 2020). However, constitutively expressed NOS2 has been found tissue specific

in the human colonic and lung epithelium as well as in primate lungs and could be a response to the local microbiota (Mattila and Thomas 2014).

Accumulated NOS2-derived NO plays important roles in innate and adaptive immunity such as regulating T-cells, B-cells, and myeloid-derived suppressor cells (Bogdan 2015) and helping macrophages to defend against pathogens. The latter has been best established so far (Weinberg et al. 1995; MacMicking et al. 1997; Fang 2004; Nathan 2006).

Once NO is generated, it rapidly reacts with superoxide to form radical peroxynitrite ($ONOO-$) that can cause, damage to DNA (Pacher et al. 2007), modifications of proteins (Casas et al. 2015; Dao et al. 2015; Bartesaghi and Radi 2018) and reactions with unsaturated lipids (Jones 2012). Thus, also host tissue can be targeted. Therefore, regulation of NOS2 gene expression is strictly bound to transcriptional processes (Scheschowitsch et al. 2015).

Briefly, pathogen (PAMP) and damage (DAMP) associated molecular patterns bind to pattern recognition receptors (Amarante-Mendes et al. 2018), and proinflammatory cytokines such as TNF-α and IL-1 bind to the cell surface, e.g., one popular PAMP, LPS, binds to toll like receptor 4 in macrophages (Hume et al. 2001), starting the signaling cascade by activation of transcription factors, including nuclear factor κB (NF-κB) and (STAT-1a) while IFN-γ activates the JAK/STAT-1α pathway to induce NOS2 mRNA expression (Dell'Albani et al. 2001; Ganster et al. 2001).

Control of NO output is also regulated by autoregulation of its own expression in a feedback manner (Ganster et al. 2001). For example, posttranslational regulations involving *S*-nitrosylation of NF-κB binding partners lead to a stop of mRNA transcription (Kelleher et al. 2007). Further, *S*-nitrosation at the Zn^{2+} tetrathiolate cluster discards the Zn^{2+} leading to destabilization of the active homodimer resulting in dissociation to the inactive monomeric form (Wynia-Smith and Smith 2017). In contrast, inhibition of phosphorylation on phosphotyrosine residues leads to increased NOS2 activity (Pan et al. 1996).

1.3 Endothelial NOS/Type 3 NOS (eNOS/NOS3)

NOS3 is constitutively expressed mostly in endothelial cells but also in cardiac myocytes, platelets (Forstermann and Sessa 2012), and macrophages (Mattila and Thomas 2014). NOS3-derived NO exerts various physiological functions such as vasodilation through its receptor soluble guanylyl cyclase leading to increasing cyclic GMP in smooth muscle cells (Förstermann et al. 1986), inhibition of platelet aggregation (Förstermann et al. 1986; Alheid et al. 1987), platelet and leukocytes adhesion to the vascular wall (Kubes et al. 1991), vascular remodeling, anti-inflammatory effects (Ahluwalia et al. 2004), and angiogenesis (Wei et al. 2020).

Its structure has important features, including (1) an autoinhibitory loop within the FMN binding domain where CaM can be removed in the absence of Ca^{2+} to stop catalytic reaction, (2) the loss of PDZ domain, (3) an AKT phosphorylation site at the C-terminal, and (4) an additional acylation site (palmitoylation and myristoylation) in the oxygenase domain.

Another regulatory element that differs from the other NOS isoform is a shorter and less active hinge, which is responsible for binding FMN to the reductase domain. Notably, mammalian NOS3 has the weakest activity amongst the NOS family (Haque et al. 2007, 2012).

NOS3 can be found in sarcolemmal caveolae where it is bound by posttranslational myristoylation and palmitoylation to caveolin-1 which tonically inhibits NOS3 activity. A rise of intracellular Ca^{2+} level induces CaM binding and interaction of NOS3 with heat shock protein 90 results in disruption of the NOS3-caveolin-1 heterodimer complex leading to NOS3 activation (Averna et al. 2008).

However, NOS3 activation can also be regulated by mechanical changes, i.e., fluid shear stress, leading to NOS3 up-regulation in endothelial cell and rodents (Nishida et al. 1992; Sessa et al. 1994; Awolesi et al. 1995; Fukai et al. 2000; Dao et al. 2016). The mode of shear stress has different effects on NOS3 regulation. While acute changes affect immediate vascular tone, chronic shear stress induces gene expression and remodeling of blood vessels (Garcia and Sessa 2019). The mechanism underlying shear stress involves not only intracellular calcium rise but depends directly on phosphorylation by serine/threonine (Ser/Thr) protein kinase Akt/PKA (Dimmeler et al. 1999). In this regard, studies in mutant *AKT1* mice have shown that AKT1 is an important NOS3 kinase in vivo that phosphorylates Ser1176 (human Ser1177) (Schleicher et al. 2009).

Besides fluid shear stress, other stimulants can regulate NOS3 activation such as vascular endothelial growth factor induced phosphorylation by AKT1 at Ser/Thr site, bradykinin-induced phosphorylation at Ser1177 by Ca^{2+}/calmodulin dependent protein kinase II, insulin-mediated Akt1 and AMP-activated protein kinase activation (Forstermann and Sessa 2012) or hydrogen peroxide (Drummond et al. 2000; Thomas et al. 2002; Searles 2006; Dao et al. 2011).

Several phosphorylation sites including serine, threonine, and tyrosine residues of NOS3 have been discovered such as Y81, S615, S633, and S1177 (equivalent to Y83, S617, S635, and S1179 of bovine NOS3) responsible for stimulation (Fulton et al. 2005; Fulton 2016), while S114, T495, and Y657 leads to inhibition of NOS3 activity (Loot et al. 2009; Fulton 2016). Briefly, phosphorylation of the Ser1177 increases, while constitutively phosphorylated Thr495 in endothelial cells appears to interfere with CaM binding (Heiss and Dirsch 2014). This plays a role in *e*NOS uncoupling (Lin et al. 2003), and decreases enzyme activity.

Other posttranslational modifications are also described to change NOS3 regulation, including *S*-nitrosylation (at C94 and C98) (Erwin et al. 2005) leading to reduced activity, while acetylation (K609, S765, and S771) increases its activity (Jung et al. 2010). Glutathionylation in the C-terminal reductase domain (C689 and C908) uncouples NOS3 thus forming superoxide anion (Chen et al. 2010).

At transcriptional level regulation of NOS3 mRNA expression is decreased by DNA methylation of the promoter thus reducing Sp1, Sp3, and Ets1 transcription factor binding (Chan et al. 2004) and controlled by histone modification at NOS3 promoter (Fish et al. 2005, 2010). In addition, NOS3 mRNA expression can be up-regulated by long noncoding RNAs (lncRNAs) in endothelial cells induced via transcription factor KLF2 (Man et al. 2018).

2 Dysregulation of NOS Isoforms

Qualitatively, NOS can exist in three different functional states: (1) normal state that produces physiological levels of NO which signals mainly via its receptor, soluble guanylate cyclase (sGC) (Schmidt et al. 1994), (2) uncoupled state that produces superoxide rather than NO resulting in endothelial dysfunction and cardiovascular diseases (Li et al. 2015), and (3) hyperactive state that produces excessive NO leading to cellular and tissue injury (Kleinschnitz et al. 2016), e.g., in stroke and myocardial infarction (Fig. 1).

The uncoupled state is induced upon depletion of the NOS substrate, L-arginine, or oxidation of its cofactor, tetrahydrobiopterin (H4Bip) (Schmidt et al. 1992;

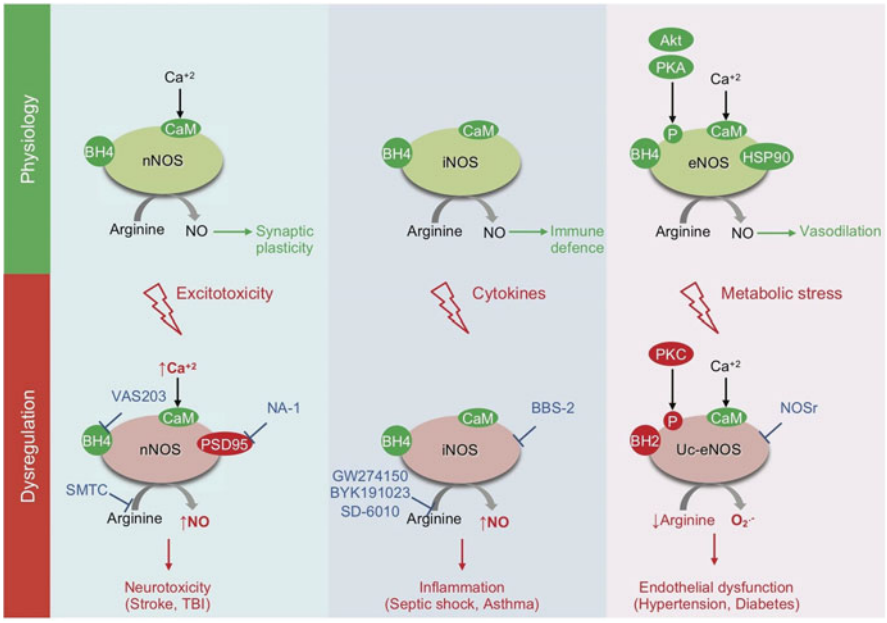


Fig. 1 NOS isoforms and their regulation and dysregulation. In physiology, the three NOS isoforms, *n*NOS, *i*NOS, and *e*NOS, bind to the substrate, arginine, the cofactor, BH4, and calmodulin (CaM) to produce NO. Ca^{+2} is required for CaM binding in *n*NOS and *e*NOS. The latter is phosphorylated by PKA/Akt. The NO produced by *n*NOS, *i*NOS, and *e*NOS performs several biological functions including synaptic plasticity, immune defense, and vasodilation, respectively. Under disease conditions, dysregulation of NOS enzymes takes place. In excitotoxicity, e.g., in stroke and traumatic brain injury (TBI), Ca^{+2} is increased leading to binding of *n*NOS to postsynaptic density protein 95 (PSD95) and increased NO production resulting in neurotoxicity. Under inflammatory conditions, e.g., in asthma and septic shock, cytokines activate *i*NOS to produce high levels of NO that further aggravates inflammation and induces cytotoxicity. Metabolic stress causes uncoupling of *e*NOS by reducing arginine, oxidation of BH4 to BH2 and PKC-induced phosphorylation resulting in formation of superoxide ($O_2^{\cdot-}$) rather than NO and endothelial dysfunction. *n*NOS and *i*NOS inhibitors and *e*NOS recoupling agents (NOSr) are written in blue

Bömmel et al. 1998), leading to NOS monomerization (Reif et al. 1999) and inhibition of the enzyme activity (Kotsonis et al. 1999; Reif et al. 1999). However, recently NOS monomerization has been shown to be irrelevant for uncoupling (Gebhart et al. 2019). NOS uncoupling can also be induced by the accumulation of methylated arginine analogs, in particular, asymmetric dimethyl arginine (ADMA), which is a competitive inhibitor of NOS (Antoniades et al. 2009).

The hyperactive state of NOS is induced in many conditions that involve inflammation and hypoxia leading to formation of high and non-physiological levels of NO that cause harmful effects, most likely in a cGMP-independent manner. Several mechanisms have been proposed that explain the high NO-induced cytotoxicity. These include protein *S*-nitrosylation (Shahani and Sawa 2012; Dao et al. 2020), formation of peroxynitrite via interaction with superoxide (Mendes et al. 2002; Pacher et al. 2007), activation of inflammatory signaling pathways, e.g., NF-κB (Mendes et al. 2002), decreased expression and activity of sGC, and formation of the NO-insensitive *apo*-sGC (Dao et al. 2020). Two NOS isoforms have been shown to be activated in disease conditions, *n*NOS/NOS1 and *i*NOS/NOS2. With respect to the former, it is activated to produce high levels of NO in response to excitotoxicity, which is an important event in the pathophysiology of stroke and traumatic brain injury (Ito et al. 2010; Luo et al. 2019). The activation *n*NOS/NOS1 in these conditions is dependent on its interaction with PSD-95 and results in neurotoxic effects (Zhou et al. 2010; Luo et al. 2019). With respect to *i*NOS/NOS2, it is activated mainly in inflammatory conditions in response to cytokines. The inappropriately high NO concentration produced by *i*NOS can result in cytotoxic effects and is associated with a variety of human diseases, including septic shock, asthma, and cancer (Zamora et al. 2000; Cinelli et al. 2020).

3 Discovery and Clinical Development of NOS Inhibitors

A large number of nitric oxide synthase inhibitors with various degrees of selectivity for NOS isoenzymes are available and claimed in patents Table 1. However, few have reached the clinical trial stage Table 2, and not all of these meet the criteria for pharmaceutical developability.

3.1 Which Site to Target?

NOS are proteins with a high number of binding and regulatory sites (Nedvetsky et al. 2002). The earliest attempts to develop NOS inhibitors were based on the physiological substrate, L-arginine (Alderton et al. 2001). Still to date, most compounds target that site. Later, cofactor and regulator sites were added, of which only those targeting BH4 (Bömmel et al. 1998; Fröhlich et al. 1999; Reif et al. 1999; Kotsonis et al. 2001; Pantke et al. 2001; Matter et al. 2002, 2005) and PSD-95 interaction (Cui et al. 2007) made it into clinical testing. Other sites such as calmodulin (ketoconazole) were never tested for NOS inhibition in humans.

Table 1 Overview of clinically applied and developed NOS inhibitors

Compound	Binding site	Reversibility
Unspecific		
L-NMMA	Arg	Reversible
L-NAME	Arg	Reversible
VAS203	H_4Bip	Reversible
2-Iminobiotin	Arg	Reversible
MTR 104	Arg	Reversible
nNOS/NOS1 specific		
S-methyl-L-thiocitrulline	Arg	Reversible
Tat-NR2B9c (NA-1)	PSD95	Reversible
ARL17477	Arg	Reversible
NXN-462 and NXN-188	Arg	Reversible
*i*NOS/NOS2 specific		
GW274150 and GW273629	Arg	NADPH dependent inactivation
Cindunistat (SD-6010)	Arg	Irreversible
BYK191023	Arg	Reversible/irreversible
L-NILTA (prodrug of L-NIL)	Arg	Reversible
BBS-2	Heme-containing *i*NOS monomer	Reversible

3.2 Nonspecific Inhibitors

L-NMMA (N^G-monomethyl-L-arginine) is approximately equipotent on all three isoforms of NOS (Alderton et al. 2001). The acetate salt is also known as tilarginine; the less hygroscopic HCl salt is also known as 546C88. L-NAME is an inactive prodrug of N^G-nitro-L-arginine, L-NOARG (Pfeiffer et al. 1996). This is modestly selective for *n*NOS and *e*NOS versus *i*NOS (Alderton et al. 2001). Other-nitroarginine esters include L-N^G-benzylarginine, L-N^G-aminoarginine, and iminoethylornithine. Moreover, specific inducible NOS inhibitors, i.e., acetamidine derivatives, have been synthesized and evaluated showing high isoform-specificity with submicromolar concentrations (Fantacuzzi et al. 2016). The currently most advanced NOS inhibitor under development is Vasopharm's ronopterin (VAS203; 4-amino-tetrahydrobiopterine) (Ott et al. 2019; Tegtmeier et al. 2020a, b). Ronopterin is a potent pterin-based inhibitor that competes with exogenous BH4 (Werner and Schmidt 2000; Schinzel and Tegtmeier 2017). This drug has shown promising results in patients with traumatic brain injury (see below). Vasopharm holds several patent applications on this compound and related pteridines (WO/2005/037286 and WO/2004/084906). Another nonspecific NOS1/NOS2 inhibitor, 2-iminobiotin, originally patented by the Amsterdam University Medical Center for the treatment and prevention of perinatal asphyxia (WO/2001/074351), is being developed by the Dutch company Neurophyxia B.V. The company has published an international patent application claiming the compound for cerebral

Table 2 Phase II and higher clinical trials involving NOS inhibitors

Drug name(s)	Originator company, *active companies	Therapy area, *active indications	Target-based actions	Highest status
L-NMMA.HCl (546C88)	Glaxo Group Ltd.	Infection; Neurology/ psychiatric *Stroke; septic shock	Nonspecific NOS inhibitor	Discontinued (phase 3)
L-NMMA	Toronto University Health Network	GLP-2 mediated intestinal lipoprotein release	Nonspecific NOS inhibitor	Ongoing (phase 3)
Gingivex (GED; Inotek; Guanidinoethyl disulfide)	Rocket Pharmaceuticals Inc.	Gastrointestinal; inflammatory; ocular; endocrine/ metabolic	*i*NOS/NOS2	Discontinued (phase 2)
GW274150	Glaxo Group Ltd.	Neurology/ psychiatric; inflammatory; immune; gastrointestinal; respiratory	*i*NOS/NOS2	Discontinued (phase 2)
ONO-1714	Ono Pharmaceutical Co Ltd.	Cardiovascular; infection *Sepsis; hypotension	*i*NOS *e*NOS	Discontinued (phase 2)
Cindunistat hydrochloride maleate (PHA-728669F; SD-6010)	Pfizer Inc.	Inflammatory	*i*NOS/NOS2	Discontinued (phase 3)
Pimagedine (aminoguanidine)	Rockefeller University	Dermatologic; endocrine/ metabolic; gastrointestinal; genitourinary/ sexual function	Nitric oxide synthesis inhibitor	Discontinued (phase 3)
S-Ethylisothiourea diethylphosphate (MTR 104)	Meditor Pharmaceuticals Ltd., *TrioxBio Inc.	Neurology/ psychiatric, *migraine	NOS inhibitor	Launched
		Cardiovascular, *hypotension	NOS inhibitor	Launched
		Neurology/ psychiatric, *cluster headache	NOS inhibitor	Phase 2
		Cardiovascular, *hypotension	NOS inhibitor	Phase 2

(continued)

Table 2 (continued)

Drug name(s)	Originator company, *active companies	Therapy area, *active indications	Target-based actions	Highest status
XQ-1H (Ginkgo biloba lactone B mesylate)	Jiangsu Carephar pharmaceutical Co Ltd.	Neurology/ psychiatric, *brain ischemia	*i*NOS/NOS2; platelet activating factor receptor agonist	Phase 1
OsteoDex (ODX; dextran-guanidine-bisphosphonate conjugate)	Dextech medical AB	Cancer; musculoskeletal, *bone metastases; hormone refractory prostate cancer	NOS inhibitor	Phase 2
NXN-462	NeurAxon Inc.	Neurology/ psychiatric, *movement disorder; Parkinson's disease	*n*NOS/NOS1	Phase 2
2-iminobiotin	Universiteit Utrecht	Other/ miscellaneous, *asphyxia	*i*NOS/NOS2; *n*NOS/NOS1	Phase 2
Ronopterin (VAS203)	Vasopharm GmbH	Neurology/ psychiatric, *traumatic brain injury	NOS inhibitor	Phase 3
Nerinetide (NA-1, tat-NR2B9c)	NoNO Inc.	Neurology/ psychiatric, *brain ischemia; stroke; traumatic brain injury	Discs large homolog 4 inhibitor; NMDA receptor antagonist; *n*NOS/NOS1	Phase 3

hypoxia-ischemia and reperfusion injury (WO/2017/105237), and another one claiming pharmaceutical formulations (WO/2011/149349). Finally, MTR 104, a low molecular weight isothiourea derivative (*S*-ethylisothiouronium diethylphosphate) and nonspecific NOS inhibitor (Garvey et al. 1994), has received orphan drug designation from the FDA. Meditor Pharmaceuticals has begun development through TrioxBio.

3.3 *n*NOS/NOS1 Inhibitors

Although much of the focus on creating selective NO synthase inhibitors has focused on *i*NOS (NOS2) and certainly steered well clear of NOS3 (*e*NOS), a few *n*NOS selective inhibitor programs have emerged and one of the first of these came from Fisons' research laboratories in Rochester, New York. The best known of these compounds is ARL17477 (alternatively known as AR-R17477 or initially FPL17477). This compound represents a series of heterocyclic substituted amidines, the full synthesis of which has only been covered in the patent literature (WO 95/05363). Of relevance in this respect are some notable differences in the amino acid sequence between NOS isoforms around the substrate or inhibitor binding site. Some of these are highlighted in a paper by Fedorov and colleagues (Fedorov et al. 2004) who present crystal structures of AR-R17477 in the different NOS isoforms to explain why the compound selectively inhibits *n*NOS. A potentially important observation in this respect is a key difference in sequence that is observed within the active site. The aspartate residue (rat *n*NOS; N597) becomes an asparagine in *e*NOS (bovine *e*NOS; D368). Li et al. demonstrated the importance of this residue in providing selectivity between isoforms for a series of L-nitroarginine based dipeptide inhibitors. However, these papers used bovine *n*NOS—human would be the same; but in mouse and rat (the species most commonly used in pharmacological studies), the aspartate residue is conserved (Li et al. 2005). Therefore, selectivity may be seriously compromised in these experiments. Site directed mutagenesis showed that switching these residues did indeed alter selectivity profiles (Li et al. 2005). There is a distinct possibility that failing to recognize this structural feature may have compromised some in vivo pharmacological or safety studies, blocking further progression of otherwise interesting, selective NOS inhibitors.

The AR-R series of compounds failed to produce clinical candidates for AstraZeneca, but the series was adopted elsewhere, most notable by Neuraxon in Canada. Although initially (Annedi et al. 2011, 2012) Neuraxon presented analogs of the thiophene amidines such as AR-R17477 and particularly AR-R17338 (Reif et al. 2000) which led to novel inhibitors with good selectivity toward *n*NOS, one of these, NXN-323, showed some efficacy in animal models of allodynia (Felice et al. 2010). A further compound, NXN-462, is reported to have entered trial for post-herpetic pain; to date, no results from the study have been released. Subsequently, the group moved on to produce dual functionality ligands including molecules that combine NOS inhibition with μ-opiate agonism or noradrenaline re-uptake inhibition, but notably NXN-188 a mixed *n*NOS and 5-$HT_{1B/D}$ agonist. Whenever one sees a mixed function drug it is fair to question whether one or other pharmacological activity dominates. NXN-188 is said to be a selective *n*NOS inhibitor with potency similar to L-NMMA and to have similar 5-HT potency to Sumatriptan. This potential drug prevented CGRP-release from preparations of several migraine-relevant brain areas (dura mater, trigeminal nucleus caudalis, and trigeminal ganglion (Bhatt et al. 2013). After Phase I trials of the drug showed suitable pharmacokinetics and that it was well tolerated in both single and multiple dose protocols (Vaughan et al. 2010), the compound entered a single center, double-blind,

randomized cross-over study in patients suffering migraine with aura (Hougaard et al. 2013). While the results of this study were seen as encouraging, with a reduction in patients reporting headache, the study's primary endpoints were not achieved as the trial suffered a high drop-out rate and only a small sample of patients completed the placebo-controlled cross-over study. Consequently, the data failed to achieve statistical significance. In 2015, this drug was licensed to Knight Therapeutics (Canada) and a further Phase II trial is reported to be ongoing (as of June 2015: source adisinsight.springer.com).

S-Methyl-L-thiocitrulline (SMTC) is an amino acid derivative that is selective for *n*NOS/NOS1. Lack of patentability has so far prevented further development of this compound. However, it has been employed in a number of investigational human studies that studied the effects of either local infusion in different vascular beds or systemic infusion in healthy volunteers. However, stability issues and (more importantly) also lack of patentability prevented its further development.

In excitotoxicity, the postsynaptic density protein PSD95 recruits the calcium-dependent *n*NOS to NMDA receptor channels leading to neurotoxic effects (Cui et al. 2007; Kleinschnitz et al. 2016). Therefore, inhibition of PSD-95/NMDA interactions has been suggested as a potential therapeutic approach for neurotoxicity, e.g., in stroke and traumatic brain injury (Cui et al. 2007). Tat-NR2B9c (NA-1 or nerinetide), a synthetic peptide and a PSD-95 inhibitor, has been tested clinically for stroke and preliminary results seem promising (Bruder 2012; Hill et al. 2012; Matsumoto 2013) (see below). ZL006 is also a small molecule drug that blocks the PSD95/*n*NOS interaction and has been tested only preclinically (Zhou et al. 2010). However, its binding to the PSD95/*n*NOS has been doubted (Bach et al. 2015).

3.4 iNOS/NOS2 Inhibitors

There is considerable evidence from animal models for a potential pathological role of excessive NO production in numerous chronic inflammatory diseases and for the beneficial effects of treatment with *i*NOS inhibitors (Cheshire 2001; Tinker and Wallace 2006). However, much of this data has been derived using compounds that are far from optimal with regard to potency and specificity. Considerable effort has been directed at discovering truly selective inhibitors of this isoform that will prevent over-production of NO while maintaining the basal formation of NO from constitutive NOS that is required for normal physiological function. The analogs of arginine have been widely used as inhibitors of NO synthases, with considerable success although they lack some of the "drug-like" properties sought by pharmaceutical research programs. The simplest analogs of arginine are highly hydrophilic, and so incapable of readily diffusing across biological membranes; hence, they may also rely upon cationic amino acid transporters to enter cells (Baydoun et al. 2006). Consequently, many companies have tried to design inhibitors that move away from these pharmacophores.

Compounds unrelated to arginine can also inhibit NO synthase. Often these are fairly simple, small compounds, such as 2-aminopyridines; featuring an aromatized amidine that can mimic the binding of the basic guanidino side chain in arginine to an active site glutamyl residue. Aminopyridines offer scope to design and synthesize novel inhibitors, with potential improvements in "drug-like" properties. Connolly et al. at AstraZeneca reported on a series of analogs of 2-amino-4-methyl pyridine leading to a potent (IC_{50} = 71 nM), selective *i*NOS inhibitor, AR-C133057 (Connolly et al. 2004). In crystal structures of the ligand bound to the active site of *i*NOS the compound was observed to have adopted a flip in binding of the pyridine ring in order to accommodate an *N*-(1-acyl-4-piperidinyl) group that could be further elaborated to derive the series of *i*NOS inhibitors. Unfortunately, these aminopyridine analogs often exhibited poor pharmacokinetics with low volumes of distribution and weak in vivo activity.

Probably the best known *i*NOS inhibitor to emerge from AstraZeneca was AR-C102222. This compound is a potent inhibitor of human *i*NOS (IC_{50} 35 nM) and also exquisitely selective (>1,000-fold against *e*NOS). The inhibitor arose through studies focusing on aminopyridine analogs intended to exhibit improved drug-like properties. Two studies (Beaton et al. 2001a, b) identified a pair of series of bicyclic amidines: 3,4-dihydro-1-isoquinolinamines and closely related thienopyridines. Some of these molecules exhibited reasonable potency against *i*NOS, with a range of selectivity. The breakthrough came with the introduction of a nitrogen atom to create quinazolinamine inhibitors followed by a limited parallel synthesis approach that identified spirocyclic dihydroquinazoline molecules as *i*NOS inhibitors. This modification removed a stereochemical center, making the molecules rather simpler to work with whilst simultaneously opening scope for further chemical elaboration. Further, parallel synthetic studies extended this substitution and identified some highly selective compounds, including AR-C102222 (Tinker et al. 2003). Surprisingly, it achieved much of its selectivity through a cascade of interactions involving conserved residues close to the active site. Crystal structures of the *i*NOS oxygenase domain with AR-C102222, or other similar ligands, bound in the active site show displacement of a glutamine residue (Gln257 in mouse *i*NOS). This conformation can only be achieved by further movements of residues beyond the immediate region of the active site (Garcin et al. 2008) and such movements are impaired in *n*NOS and largely blocked in *e*NOS by more bulky amino acid side chains in these more distant positions. The lead spirocyclic compound maintained reasonable potency against *i*NOS in cell-based assays and also offered good oral bioavailability.

There are very few NO synthase inhibitors that bind in the enzyme's active site, but do not include an isostere of the guanidinium present in arginine to bind to the crucial glutamic acid residue. However, a few examples of inhibitors that lack this functionality and therefore do not interact directly with this acidic side chain do exist. 7-nitroindazole and chlorzoxazone actually displace the glutamate carboxylic acid in order to bind, locating in the active site through a π–stacking interaction with the heme-porphyrin ring (Rosenfeld et al. 2002a). Indeed, the induced fit afforded by this movement was shown to compromise the binding affinity of these compounds.

Building upon this observation Cheshire et al. presented a new series of selective, non-amidine *i*NOS inhibitors that made a similar interaction with the heme, but could also access the region close to Gln257 that conferred selectivity to compounds such as AR-C102222 (Cheshire et al. 2011).

NOS inhibition by competition with L-arginine comes with potential difficulties. The L-arginine dependence of NO synthase activity has been reported in several studies. Typical values for K_m range between 1 and 10 μM (Bredt and Snyder 1990; Sherman et al. 1993) with similar potency reported in direct measurements of arginine binding (Berka et al. 1996). Inhibitors must compete with the relatively high concentrations of this substrate present in cells. Intracellular levels of arginine tend to be similar to plasma levels, around 100–200 μM (Armstrong and Stave 1973), some tenfold or more higher than K_m. This implies that an inhibitor acting by a purely substrate-competitive mechanism will need to have high affinity for the enzyme in order to show significant activity in cells and in vivo at a reasonable dose. Indeed, it is not uncommon to see potency losses of 30-fold to 100-fold when comparing *i*NOS inhibition in cells with potency in enzyme assays. Experimentally, this is further complicated by variations in the arginine content in different culture media. For example, RPMI-1640 is often used to culture human cells and typically contains around 1 mM arginine.

Circumventing this issue, one type of *i*NOS inhibitors has been described which have no effect on either the active NOS enzymes themselves or the stimulated production of NOS protein. Instead, these compounds appear to act by preventing the assembly of the initially synthesized monomeric NOS protein into the functional homodimer. The initial evidence for compounds acting by this mechanism came in a study of the antifungal imidazoles clotrimazole and miconazole from Stuehr's laboratory in Ohio (Sennequier et al. 1999). Although these compounds are fairly weak inhibitors, further work by from Berlex (later part of Schering AG) (Blasko et al. 2002) identified compounds with nanomolar activity toward *i*NOS. The Berlex group reported X-ray diffraction data for a complex between one of these compounds, BBS-1, and the *i*NOS monomer, which shows that the imidazole unit is acting as a ligand for the heme iron whilst the rest of the molecule binds to more remote parts of the NOS protein. This binding appears to cause conformational changes in the monomer which preclude dimer formation. Other companies followed suit with compounds from Fujisawa (FR-260330; see (Chida et al. 2005)) apparently using a pyridine to ligate the heme, SSP Co (PPA250; see (Ohtsuka et al. 2002)) and Adolor (Chu et al. 2009). Compounds from both the Berlex and Fujisawa series have shown beneficial effects in in vivo models of transplant rejection (Szabolcs et al. 2002; Ouyang et al. 2005) with the former also effective in a sepsis model in mice (Ichinose et al. 2003) and in lung injury caused by burns and smoke inhalation in sheep (Enkhbaatar et al. 2003). As these compounds prevent assembly and dimerization of the *i*NOS protein upon induction typical assays involve LPS treatment of mouse macrophage cell lines, or cytokine stimulation of human, DLD-1, cells followed by analysis of nitrite production or enzyme activity. Mallinder and colleagues reported technical information on using a proprietary cell-based, β-galactosidase enzyme complementation method to screen for

*i*NOS inhibitors acting via this mode-of-action in a more convenient HTS format (Mallinder et al. 2009). The assay system is known as InteraX™ and employs fusion proteins of *i*NOS oxygenase domains and β-galactosidase mutants as reporter enzymes. The individual mutants are inactive, but dimerization of the *i*NOS domains, fused in suitable orientation to the β-galactosidase reconstituted the galactosidase activity, which could then be used as a functional readout. The assay technique was shown to identify dimerization inhibitors, but more traditional *i*NOS inhibitors that bind in the active site were found to enhance the signal suggesting that they can promote dimerization as has been shown with the natural substrate, arginine. This methodology was reported to have been used in a high-throughput screen of around 800,000 compounds, but no data on the output from the screen has been published.

Wellcome/GlaxoWellcome/GlaxoSmithKline (GSK) ran a large *i*NOS/NOS2 program. Whilst 1400W, one of the first selective *i*NOS/NOS2 inhibitors (Garvey et al. 1997; Thomsen et al. 1997; Kankuri et al. 2001; Vuolteenaho et al. 2001; Pérez-Asensio et al. 2005; Järvinen et al. 2008), a non-amino acid compound, never made it into the clinic because of preclinical toxicity, GW274150 and GW273629, GSK's lead selective *i*NOS/NOS2 inhibitors, both amino acids (Alderton et al. 2005), were taken into clinical studies. These were both highly selective for *i*NOS inhibition, with slow or no reversal of inhibition and both orally bioavailable. GW274150 is transported by amino acid transporters such as y+-LAT (Baydoun et al. 2006). The imidazo[4,5-*b*]pyridine derivative BYK191023 has been identified by Altana Pharma/Nycomed as an *i*NOS/NOS2 selective inhibitor, which binds to the L-arginine site (Grädler et al. 2011). In the absence of NADPH, BYK191023 acts as a reversible L-arginine competitive inhibitor, whereas an NADPH and time-dependent irreversible inactivation mechanism with heme depletion is observed at low L-arginine levels and in intact cells (Tiso et al. 2008). AstraZeneca and Berlex also developed a line of NOS inhibitors (Rosenfeld et al. 2002b, c, d, e; Cheshire et al. 2011). A selective irreversible *i*NOS inhibitor developed by Pfizer/Pharmacia, cindunistat (SD-6010), is close to GSK's GW274150 in structure. Its entry into cells or tissue may be impaired by the (α)-methyl which is unlikely to be a substrate for amino acid transporters. This compound was investigated by Pfizer in osteoarthritis (50 or 200 mg/day) and failed to slow the disease progression (Hellio le Graverand et al. 2013). One of the well-known arginine analogs that have been tested in humans is L-NIL (L-*N* iminoethyl lysine), in the form of a pro-drug known as L-NILTA (and also as SC51 or SD3651). This was a product of the research teams at Pharmacia at a time when many acquisitions and mergers were occurring in the pharmaceutical industry, the work originated within G.D. Searle & Company and is now part of Pfizer. L-NIL is widely recognized as a selective *i*NOS inhibitor, but the compound is hygroscopic. While this can be managed in a research laboratory it can pose a problem in clinical trials if it becomes difficult to confidently and consistently prepare exactly the same concentration solutions for dosing. Unlike most pro-drugs this substance was not designed to circumvent issues with ADME or PK; rather L-NILTA, the tetrazolinium amide of L-NIL, is a stable, non-hygroscopic solid. Upon dosing the amide is rapidly removed, in effect dosing the parent drug L-NIL.

4 Clinical Applications of NOS Inhibitors

Clinical applications have focused mainly on the use of NOS inhibitors to vasoconstrict, inhibit inflammation, and neuroprotect. The role of the involved NOS isoform has not always been entirely clarified. In conditions such as inflammation and traumatic and ischemic damage additional interaction with reactive oxygen species is likely.

4.1 Vasoconstriction in Sepsis and for Blood Flow Disruption

The earliest clinical translational attempt for a NOS inhibitor has been in sepsis. Increased production of nitric oxide has been demonstrated in both experimental and clinical sepsis; the increased production of nitric oxide has subsequently been associated with hypotension, decreased responsiveness to vasoconstrictors, and development of multiple organ dysfunction (Petros et al. 1994; Grover et al. 1999; López et al. 2004). Reducing the overproduction of nitric oxide by partial inhibition of NOS could be postulated as a beneficial intervention in the treatment of septic shock. Previous experimental studies have produced conflicting results from the use of NOS inhibitors in models of septic shock provoked by either endotoxin or bacterial challenge. Clinical studies have shown that the administration of NOS inhibitors (L-NMMA; nitroarginine, L-NNA) to patients with septic shock can restore hemodynamics and the vascular responsiveness to vasoconstrictor therapy without significant acute adverse effects. However, a phase 3 study of infusion of L-NMMA (as 546C88) in septic shock showed that mortality was increased overall (López et al. 2004). Although post hoc analysis of the mortality by dose suggested that low doses (546C88, 5 $mg \cdot kg^{1} \cdot hr^{1}$ or below) provided an overall survival benefit, this was not regarded as strong enough to progress and the project was discontinued.

Preclinical shock models provide some support for the hypothesis that selective *i*NOS inhibition would be a better therapeutic approach to septic shock, but given the large phase 3 trial that would be needed to test this on the required mortality endpoint it doesn't seem likely to be tried.

Recently, non-specific L-arginine derived NOS inhibitors such as tilarginine (L-NMMA) have been employed as blood flow disruptors; in the gut to prevent GLP-2 from releasing gut lipid stores (ongoing study sponsored by the Toronto University Health Network, NCT03534661); in cancer, to overcome cancer-related immunosuppression. The nature of the involved isoform is unclear, although in cancer *i*NOS/NOS2 has been suggested to be involved.

The gut is able to retain some fat for many hours after a fatty meal. The gut hormone glucagon-like peptide-2 (GLP-2) is known to release these fat stores in the gut, but it is not known how GLP-2 achieves this. One possibility is that GLP-2 increases blood flow in the gut. NG-monomethyl-L-arginine (L-NMMA) is a substance that inhibits nitric oxide synthase (an enzyme that helps make nitric oxide which increases blood flow). This protocol examines whether blocking gut blood flow with L-NMMA is able to prevent GLP-2 from releasing gut lipid stores. Healthy

participants were treated with a combination of Teduglutide (a resistant form of GLP-2) and L-NMMA and their respective controls.

With a focus on elucidating blood pressure physiology and the role of NOS1 therein, the NOS1-specific inhibitor, *S*-methyl-thiocitrulline (SMTC), has been tested in experimental medicine investigational studies (Seddon et al. 2008, 2009; Melikian et al. 2009; Shabeeh et al. 2013; Khan et al. 2015). These studies demonstrated that *n*NOS and *e*NOS appear to have distinct roles in the regulation of vascular tone and blood flow, at least in healthy humans. Local intra-arterial infusion of SMTC in the forearm (Seddon et al. 2008) or intracoronary circulation (Seddon et al. 2008; Ammar et al. 2020), suggesting that *n*NOS contributes to the maintenance of basal blood flow in healthy humans. Locally infused SMTC also inhibits mental stress-induced increases in blood flow in the forearm and coronary circulations (Seddon et al. 2008; Khan et al. 2017). Systemic infusion of SMTC in healthy volunteers resulted in a significant increase in systemic vascular resistance and blood pressure without inhibiting *e*NOS-dependent flow-mediated dilatation (Shabeeh et al. 2017). These effects were of a similar magnitude to those previously observed with the infusion of non-selective L-NMMA, suggesting that the major NOS isoform involved in the regulation of blood pressure in healthy humans may be *n*NOS. This study does not establish the site of action on SMTC, i.e., central or peripheral, but ongoing work is examining the effects of SMTC in the human brain (unpublished data).

In persistent cardiogenic shock, systemic inflammation, including expression of inducible nitric oxide synthase (NOS) and generation of excess nitric oxide, is believed to contribute to pathogenesis and inappropriate vasodilatation. Preliminary, single-center studies had indeed suggested a beneficial effect of NOS inhibition on hemodynamics, renal function, and survival in these patients (TRIUMPH Investigators 2007). However, when tilarginine was tested in acute myocardial infarction complicated by refractory cardiogenic shock (the TRIUMPH trial, NCT00112281), it failed to reduce mortality (Bailey et al. 2007; Kielstein et al. 2007; Salem and Mebazaa 2007; Teerlink 2007; TRIUMPH Investigators 2007).

MTR 104 (Garvey et al. 1994) has received orphan drug designation from the FDA and addresses a variety of acute and chronic therapeutic indications associated with hypotension. Meditor Pharmaceuticals has begun through TrioxBio as developer a phase II clinical trial for MTR 104 in intradialytic hypotension (IDH). The double-blind clinical study will involve chronic renal failure patients who experience IDH.

4.2 Inflammatory Diseases and Conditions

A key target in NOS drugs discovery has been *i*NOS/NOS2 and its possible role in inflammation, although *n*NOS/NOS1 may play a role herein as well (Baig et al. 2015). Elevated exhaled NO is a characteristic feature of human atopic asthma and correlates with the degree of inflammation and can be further increased by exposure to allergens (Kharitonov et al. 1995). Exhaled NO is recognized as a suitable

biomarker to guide asthma treatment (Smith et al. 2005). The majority of this is believed to come from *i*NOS, and consistent with that, selective *i*NOS inhibitor GW274150 was shown to decrease exhaled NO in asthma patients in a dose-dependent manner to a maximum inhibition of >90% and persisting over 24 h when dosed once daily. Acute animal model studies with GW274150 showed inhibition of the late asthmatic response to allergen in guinea pigs similar to that of prednisolone, along with inhibition of exhaled NO, and inhibition of airway hyper-responsiveness in sensitized and challenged mice. Furthermore, GW274150 was active in some other models of lung inflammation in mice and rats. On the basis of these results, GW274150 was taken into a clinical trial in atopic asthma, looking at the responses to an allergen challenge. Although the expected inhibition by GW274150 of exhaled NO was observed, this was not accompanied by any benefit on the endpoints of early or late airway response, airway responsiveness to methacholine or AMP, or airway inflammation (Singh et al. 2007). This puts into question the acute animal models of asthma that were current at the time, and indeed studies in more chronic, complex allergen models showed a lack of beneficial effects consistent with the clinical findings (Evans et al. 2012; Mercer et al. 2015). Another compound, the prodrug L-NILTA, showed efficacy against allergen challenge in rats (Eynott et al. 2002) and moved on to a double-blind, placebo-controlled study monitoring exhaled NO in asthmatics. This compound reduced exhaled nitric oxide in asthma patients and also resulted in some depression of basal levels in healthy volunteer controls (Hansel et al. 2003). Subsequently, the drug appears to have been discontinued amid rumors of animal toxicity observed with the parent compound. Overall it seems that selective inhibition of *i*NOS is unlikely to provide benefit in asthma.

A preclinical case was also made for selective inhibition of *i*NOS in migraine, including efficacy in models of pain, but clinical studies with GW274150 (for migraine prophylaxis) and GW273629 (for acute migraine treatment) (Van der Schueren et al. 2009; Høivik et al. 2010; Hoffmann and Goadsby 2012; Barbanti et al. 2014) were convincingly negative.

The experience of testing selective *i*NOS inhibition in rheumatoid arthritis is somewhat similar; again, a preclinical case was made for this, resulting in progression into a clinical study with GW274150 (Cuzzocrea et al. 2002; Seymour et al. 2012). Although there were some beneficial trends in the GW274150 arm after 28 days dosing, they did not achieve statistical significance, in contrast to the positive control arm on prednisolone.

Similarly, a substantial body of work with *i*NOS inhibitors in vitro and in vivo supported the hypothesis that *i*NOS inhibition could be therapeutic in osteoarthritis (OA). Cindunistat (SD6010) was tested in a 2-year, multinational, double-blind, placebo-controlled trial, which enrolled 1,457 patients with symptomatic knee OA randomly assigned to cindunistat (50 or 200 mg/day) or placebo (Hellio le Graverand et al. 2013). Cindunistat did not slow the rate of Joint Space Narrowing (JSN) versus placebo overall. After 48 weeks, a subset of patients showed less JSN; however, the improvement was not sustained at 96 weeks. Thus, the loss of efficacy over time and lack of effect in more advanced OA patients suggest that alternative

biochemical catabolic pathways overcame the effects of NO inhibition and/or that the consequences of the increased intra-articular stress may not have been amenable to *i*NOS inhibition alone. It was not reported as to what degree of *i*NOS inhibition was achieved with 50 or 200 mg/day, either initially or on long-term dosing, so it could be that this was not sufficient or long-lasting enough to achieve efficacy. No further development of cindunistat has been reported.

AR-C102222, exhibited excellent efficacy in animal models of inflammation and arthritis following oral administration. AR-C102222 reduced plasma nitrate in LPS-treated rats and in adjuvant induced arthritis it was shown to be effective at reducing the onset and severity of symptoms and prevented the development of structural changes in the joints of these animals (Tinker et al. 2003). This is not an ideal model of human joint disease, not least because indomethacin is effective whereas it has little benefit in human arthritic disease. However, the results were very encouraging that *i*NOS inhibitors would be of great potential for therapeutic benefit in rheumatoid or osteoarthritis. Particularly when, in addition, AR-C102222 was found to abrogate a cytokine induced decrease in aggrecan production by human chondrocytes (Johnston et al. 2004). Apart from being effective in rodent models of joint disease this *i*NOS selective inhibitor also alleviated neuropathic and inflammatory pain in independent studies conducted by Adolor Corporation (LaBuda et al. 2006) In other work AR-C102222 has also been shown to ameliorate experimental pancreatitis and modulate gallbladder sphincter function (Sandstrom et al. 2004, 2005; Woods et al. 2007). In the studies from Adolor (LaBuda et al. 2006) it is noted that the inhibitor led to some reduction in motor activity in the experimental animals, and ultimately further concerns about reactivity with glutathione (Cheshire et al. 2011) prevented inhibitors from this series proceeding into clinical studies.

4.3 Cancer

The role of NO in tumor biology is complex (Vamvakas and Schmidt 1997). The immune system is normally the body's first defense against threats like cancer. However, sometimes cancer cells produce signals like programmed death-1 (PD-1) that prevent the immune system from detecting and killing them. Pembrolizumab blocks PD-1 so the immune system can detect and attack cancer cells. Most patients do not, or only incompletely, respond to PD-1 inhibitors due to cancer-related immunosuppression. The presence of nitric oxide synthase in the area around the cancer cells blocks the cancer-fighting ability of the immune system. In cancer, *i*NOS product, nitric oxide, is associated with the establishment of an immunosuppressive environment and poor survival due to increased tumor aggressiveness (Davila-Gonzalez et al. 2018). To help further boost the cancer-fighting ability of the immune system, L-NMMA is tested along with pembrolizumab. Thus, the use of L-NMMA and Pembrolizumab together may augment the immune response against cancer cells. Recently, L-arginine-derived NOS inhibitors, such as tilarginine, have been employed as potentially synergistic adjuncts to other candidate compounds to treat cancers when combined with the anti-PD-1 monoclonal antibody

pembrolizumab (Merck & Co.'s Keytruda), again as a blood flow disruptor (NCT03236935; Phase Ib). The purpose of this Phase Ib study is to test the safety of L-NMMA and pembrolizumab when used together in participants with melanoma, non-small cell lung cancer (NSCLC), head and neck squamous cell carcinoma (HNSCC), classical Hodgkin lymphoma (cHL), urothelial carcinoma, or microsatellite instability-high (MSI-H)/mismatch repair deficient (dMMR) cancer (Tables 1 and 2).

In an interesting work from the group of Weinberg at Duke University, following up a previous observation that non-selective NOS inhibitors induced apoptosis in cultured CLL (chronic lymphocytic leukemia) cells a positive correlation was observed between potency of *n*NOS inhibition (but not NOS2 inhibition) and the ability to induce cell death and apoptosis in these cells (Levesque et al. 2008). AR-R 17477 was identified as the most potent *n*NOS inhibitor in the study and the most effective at inducing cell death. The compound increased Caspase 3 expression and Annexin V binding suggesting the inhibitor was, indeed, inducing apoptosis. At the time of this study the incidence of CLL in the USA was not considered high enough to pursue the effect as a treatment solely for a sub-set of leukemias. However, in more recent times the incidence (though not the morbidity) of CLL has risen considerably. In light of this and the move toward more personalized and targeted therapy this unique application of *n*NOS inhibitors could, perhaps, be re-addressed.

4.4 Neuroprotection

In traumatic brain injury, ronopterin is the only and first NOS inhibitor to be clinically investigated. In a phase IIa study (NOSTRA, NCT02012582), ronopterin showed a significant improvement in clinical outcomes, however, induced acute renal failure that was dose-related (Stover et al. 2014). The effect of this compound on renal function was then examined in healthy volunteers (NCT02992236), showing a pharmacodynamic inhibitory effect on renal perfusion that was reversible (Ott et al. 2019). Now, a Phase III clinical trial to test the efficacy of this drug in patients with moderate and severe traumatic brain injury (NOSTRA-III, NCT02794168) (Tegtmeier et al. 2020b) is ongoing.

In ischemic stroke, inhibition of *n*NOS seems a potential neuroprotective therapy (Casas et al. 2019), however, has not been tested clinically so far. Preclinical studies with *n*NOS knockout animals or selective *n*NOS inhibitors show promising results in stroke (Huang et al. 1994; Willmot et al. 2005; Kleinschnitz et al. 2016). Examples of these compounds include 7-NI (Nanri et al. 1998), TRIM (Haga et al. 2003), BN 80933 (Chabrier et al. 1999), and ARL17477 (O'Neill et al. 2000). Also, Reif et al. provide a general overview of this class of inhibitors showing some of their potency as *n*NOS/NOS1 inhibitors, selectivity toward *e*NOS/NOS3 and *i*NOS/NOS2 and basic pharmacology and pharmacokinetics (Reif et al. 2000). The heterocyclic, thiene-substituted amidine compound, ARL17477, demonstrated neuroprotection when reducing infarct volume in a transient ischemia model in rats (Zhang et al. 1996) and toward hypothermic circulatory arrest in dogs (Tseng

et al. 1999). The ischemic model showed an inverted dose curve that Reif et al. suggest may be due to inhibition of *e*NOS (NOS3) at the higher doses.

The indirect inhibition of *n*NOS-induced NO production by using PSD-95 inhibitors has reached clinical trial stage. However, it is not known yet whether the efficacy of PSD-95 inhibition is superior to direct *n*NOS inhibition (Kleinschnitz et al. 2016). Indeed, infusion of Tat-NR2B9c (NA-1 or nerinetide), a PSD-95 inhibitor, resulted in fewer ischemic infarcts in patients with iatrogenic stroke compared to placebo in a small trial (NCT00728182) (Bruder 2012; Hill et al. 2012; Matsumoto 2013). Another trial for nerinetide (NCT02930018) has been completed with no better clinical outcomes in acute ischemic stroke patients. However, a subgroup analysis of patients not treated with alteplase showed that nerinetide was associated with improved outcomes compared to placebo (Hill et al. 2020). A third trial for nerinetide is ongoing (NCT02315443) and a fourth one has been withdrawn (NCT02056574).

The *n*NOS and *i*NOS inhibitor, 2-iminobiotin, has also reached the clinical trial stage for hypoxic brain injury. Neurophyxia B.V. has been recruiting patients for a Phase II study to prevent hypoxic brain injury in patients with out-of-hospital cardiac arrest (NCT02836340) using an intravenous administration within 6 h after the event. The current status of the trial is unknown (Zitta et al. 2017; van Hoogdalem et al. 2019; Biselele et al. 2020; Favié et al. 2020).

5 Conclusions

One major conclusion from studying *i*NOS/NOS2 inhibition preclinically and clinically is that the animal models have performed poorly in predicting outcomes in human clinical trials despite diligent attempts to match the preclinical and clinical characteristics and endpoints. One reason for this might be due to the difference in inducibility and the relevance of NOS2 between rodents and humans (Rico et al. 2007). In some cases, this is being addressed by evaluating more chronic, complex models; in other instances, it may be feasible to develop human/animal hybrid models, or to make greater use of more sophisticated in vitro human models with multiple cell types and/or matrices (Mercer et al. 2015). This makes the question of which NOS to target in which clinical indication particularly challenging. Four persistent questions remain after this massive international industry effort: (1) Were the previously chosen indications the best opportunities for *i*NOS/NOS2 inhibition? (2) Was the degree of inhibition sufficient to test the hypothesis fully? (3) Should *i*NOS/NOS2 inhibition be combined with other therapeutics/targets, i.e. for network pharmacology (Casas et al. 2019)? (4) Are there ways of selecting sub-populations of "responders to *i*NOS inhibition," i.e., mechanistic endophenotyping? With respect to *n*NOS/NOS1 inhibition the situation is probably more optimistic. Here acute indications were chosen, such as traumatic brain injury and stroke, with a dramatic phenotype and presumably uniform pathomechanism preserved in different species (Casas et al. 2017, 2019) so that preclinical animal

models are likely to be more predictive. With respect to *e*NOS inhibition, the advice is clear: leave it alone or else stimulate it!

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Conflict of Interest HHHWS is a minor shareholder in Vasopharm GmbH.
RGK is a former employee of GSK.
AW is a former employee of AstraZeneca.

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