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Pharmacokinetics and pharmacodynamics of nitric oxide mimetic agents

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ABSTRACT

Drug discovery focusing on NO mimetics has been hamstrung due to its unconventional nature. Central to these challenges is the fact that direct measurement of molecular NO in biological systems is exceedingly difficulty. Hence, drug development of NO mimetics must rely upon measurement of the NO donating specie (i.e., a prodrug) and a downstream marker of efficacy without directly measuring the molecule, NO, that is responsible for biological effect. The focus of this review is to catalog *in vivo* attempts to monitor the pharmacokinetics (PK) of the NO donating specie and the pharmacodynamic (PD) readout of NO bioactivity.

1. Introduction

Nitric oxide has received much interest in biomedical research since the discovery that endothelium derived relaxing factor (EDRF) is molecular NO· [1]. NO has emerged as a central regulator of cell function, survival, and death. NO is considered by many as a double-edged sword. Low concentrations of NO provide prosurvival effects and high concentrations of NO are proapoptotic [2–4]. Transient production of low nanomolar (nM) NO, approximately 1–50 nM, by the nitric oxide synthases (NOS), neuronal NOS (nNOS) and endothelial NOS (eNOS), is essential for numerous biological processes, including physiological control of vascular tone and neurotransmission [5–7]. These functions are largely attributed to nitrosylation of the metalloenzyme soluble guanylyl cyclase (sGC) which catalyzes the conversion of guanosine-5′-triphosphate (GTP) to cyclic guanosine monophosphate (cGMP) [8]. The direct addition of molecular NO· to sGC is the quintessential nitrosylation reaction in biological chemistry [9]. In addition to the canonical NO/sGC/cGMP kinase signaling cascade, NO also reacts directly with oxygen species to produce various nitrogen oxides, such as N_2O_3, which can react with nucleophiles (i.e., nitrosation) resulting in post-translational regulation of protein structure and function. Overproduction of NO results in nitrosative stress, nitration, and/or oxidative stress, leading to antipathogenic, bactericidal, and tumoricidal activity. Based on the broad range of effects, targeting NO related biological processes is of interest for a wide range of therapeutic indications, from Alzheimer's disease to anti-cancer therapy [10–14].

Exogenous administration of NO via molecules that release NO (e.g., NO donors) or agents that produce a related or undefined NO specie (such as NO, NO^+, NO^-, $ONOO^-$, HNO, NO_2^-, NO_3^-, N_2O_3), termed NO mimetics, has been important for understanding the biological chemistry of NO. Harnessing NO mimetic effects in therapeutic agents has faced several difficulties due to the “two faced” nature of NO and the relatively poor drug-like properties of NO mimetics. Designing agents that give off a desired amount of NO within a specific therapeutic target tissue has proven difficult. An appreciation for the reported pharmacokinetic (PK) properties of NO mimetics, as prodrugs, may be useful in the design of NO mimetics as therapeutic agents in the future.

This review analyzes reports of the PK and pharmacodynamics (PD) of NO mimetic agents. Measuring NO· directly is logistically challenging, especially in live animals. Therefore, drug development involving NO mimetics is forced to measure levels of an NO donating specie (i.e., prodrug) and a downstream marker of efficacy (see Fig. 1). NO mimetics have been the focus of numerous reviews in the past [15,16]. Here, we focus specifically on *in vivo* attempts to monitor and correlate levels of NO mimetic prodrug and a biochemical measure of efficacy in preclinical drug development. For the most part, only agents which have been studied in live animals will be considered, unless otherwise required to provide context of the current art. Furthermore, this article focuses on the last 10 years of literature, since plentiful reviews are available for NO mimetic agents in the preceding time period. In some instances we will review older accounts in the literature as appropriate, such as analysis archetypal molecules for a given class of NO mimetics. Within discussion for each class we define how NO release was measured (if available) and the PK/metabolic stability of the NO mimetic followed by consideration of indirect measurements related to NO mimetic activity (i.e., biochemical readouts) which may be considered as PD measures of efficacy.

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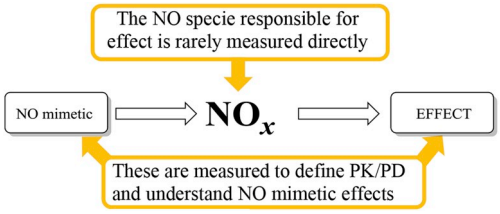


Fig. 1. Simplistic description of NO mimetic breakdown to produce NO and subsequent biological effects.

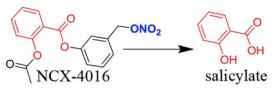
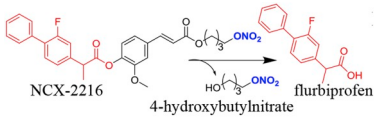
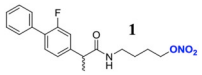
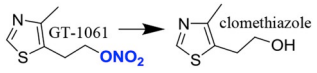
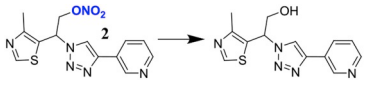
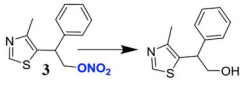
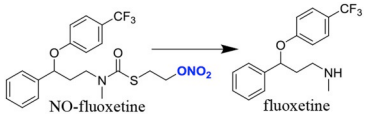
2. Nitrates

Therapeutic use of nitrates has a long history and pre-dates creation of the periodic table of elements. Indeed, nitrates have been used as safe and effective treatments for angina for over a century [17,18]. More recently, chimeric NO mimetic nitrates have been studied extensively as hybrids which possess NO mimetic activity and auxiliary pharmacophores for desirable complementary pharmacological benefit. A summary of some notable studies is provided in Table 1. It should be noted that nitrates are associated with development of tolerance (i.e., attenuation or loss of efficacy upon repeated administration) which results in diminished clinical effectiveness when used as long-term therapy. The mechanism of nitrate tolerance has been the subject of much debate and appears to be related specifically to bioactivation of nitrates rather than as a result of enhanced NO signaling, since other NO mimetic classes described in this review do not induce tolerance, including sydnonimines, furoxans, and NONOates.

Direct *in vivo* measurement of molecular NO˙ is not reported in any instance and the intact prodrug nitrate is rarely observed/reported, presumably because of poor metabolic stability and limited stability in plasma. Glyceryl trinitrate (GTN) is the archetypal NO mimetic and a potent nitrovasodilator. The mechanism of NO production from GTN has received much focus and debate. Nitrate groups are generally stable in acid and mild alkaline solution. In the presence of excess (> 5 mM) cysteine or ascorbate GTN activates sGC [19], however, there is much evidence that vascular bioactivation of GTN requires enzymatic turnover by mitochondrial aldehyde dehydrogenase (ALDH2) [20]. Several studies report nitrite (NO_2^-) levels in plasma as a *de facto* NO surrogate (measured via Griess assay). Others focus completely on biochemical readouts related to canonical NO signaling (such as sGC activity), anti-inflammatory activity (such as prostaglandin synthesis or cytokine production), or effects on learning and memory when studying for CNS activity. Specifically, carrageenan-induced paw edema was often used to screen anti-inflammatory effects of hybrid non-steroidal anti-inflammatory drugs (NSAID) containing an NO mimetic functionality. Inflammation induced by carrageenan produces acute edema, hyperalgesia, and erythema (superficial reddening of the skin) immediately following subcutaneous injection [21].

Prominent amongst the beneficial effects of NO are gastroprotective properties. NO is proposed to increase the blood flow in gastric mucosa,

Table 1
Summary of studies describing evaluation of stability and NO mimetic efficacy in preclinical animal models.

Prodrug and Main Metabolite	Administration Route, Dose	How was NO measured?[a]	Measured Specie (prodrug/metabolite)	PD readout (efficacy)[b]	References
 NCX-4016 → salicylate	p.o., 65 mg/kg	Griess assay on plasma from treated SD rats	Intact NCX-4016 not observed. Salicylate observed after 2 h T_{max} = 6 h.	Inhibition of platelet TXA_2 and increased levels of NO_2^- and NO_3^- in periphery [104]	Cuzzolin et al. [105] Tagliaro et al. [104]
 NCX-2216 → 4-hydroxybutylnitrate + flurbiprofen	p.o., 10 mg/kg	Brain dialysate from Wistar rats analyzed by Griess assay [30], or chemiluminescence detector [29]	Intact NCX-2216 not observed, flurbiprofen observed in brain and plasma (< 100 ng/mL) at all time points.	Decreased PGE_2, COX-1, IL-1β, and iNOS in the brain relative to treated with only flurbiprofen	Wallace et al. [106] Prosperi et al. [29,30]
 HCT-1026 → 4-hydroxybutylnitrate + flurbiprofen	p.o./i.p., 10 mg/kg	Brain dialysate from Wistar rats analyzed by Griess assay [30], *in vitro* [107], *in vivo* [108]	Intact drug not observed	Inhibition of osteoclast formation, cytokine inhibition, decreased PGE_2 expression, decreased NO_2^- (iNOS inhibition)	Armour et al. [107] Prosperi et al. [29,30] Idris et al. [108]
 1	i.p., 10 mg/kg	Griess assay; *in vitro* only	Intact nitrate observed in brain and plasma at 30 and 120 min	*In vitro* only. Protection against $Aβ_{42}$ and γ-secretase modulation	Schiefer et al. [109]
 GT-1061 → clomethiazole	i.v/i.p./p.o., 50 μmol/kg	Not reported	Intact GT-1061, 0.52 nmoL/ml in brain, 5 min after admin oral administration. B:P = 1.3	Increased sGC activity in hippocampus of C57Bl6 mice [34] or Long-Evans rats [110]	Luo et al. [34] Bennett et al. [110]
 2	i.p., 4.5 μmol/kg	Not reported	In brain 20 min after admin, intact **2** (2.1 ng/mL), metabolite (76.6 ng/mL)	Reversal of scopolamine induced deficits in contextual fear memory	Qin et al. [111]
 3	i.p., 4.5 μmol/kg	Not reported	In brain 20 min after admin, intact **3** (9.5 ng/mL), metabolite (9.5 ng/mL)	Reversal of scopolamine induced deficits in contextual fear memory	Qin et al. [111]
 NO-fluoxetine → fluoxetine	i.p., 15 mg/kg	Not reported	NO-fluoxetine not observed. Fluoxetine is main metabolite in plasma and brain (B:P ~ 1). 100 ng/mL 20 min after admin and 400 ng/mL at 120 min	Reversal of scopolamine induced deficits in contextual fear memory, anti-depressant activity in forced swim test.	Abdul-Hay et al. [37]

[a] Method used to measure NO production in animals treated with specified NO mimetic; technique used to evaluate NO mimetic efficacy in animals. Abbreviations: p.o., oral administration; PD, pharmacodynamics; T_{max}; admin, administration; TXA_2, thromboxane; Sprague Dawley, SD.

promoting repair and inducing the secretion of protective gastric mucus. Hence, many attempts have been made to combine NO functionalities with therapeutics that carry a risk of gastrointestinal damage. The classic example of this is the NO-aspirin, NCX-4016. NCX-4016 possesses multiple functional groups that are susceptible to saponification in aqueous environments, including an acetyl, a benzyl ester, and a nitrate. Hence, it is not surprising that no NCX-4016 was found in plasma of Sprague Dawley (SD) rats 0–24 h after oral (*per os* [p.o.]) administration. Oral administration of NCX-4016 results in the appearance of salicylate (salicylic acid), an NSAID, in plasma within 2 h. The amount of salicylate generated by NCX-4016 over a 6 h period was about 45% of that produced by the equivalent dose of aspirin.

The theory that a subset of NSAIDs, including flurbiprofen analogs, might hold potential for the treatment of Alzheimer's disease [22–26] resulted in the development of several NSAIDs containing nitrates (NO-NSAIDs) which potentiated the anti-inflammatory effects of the NSAID pharmacophore [27]. NO-flurbiprofens, such as NCX-2216 and HCT-1026 (Table 1), are neuroprotective and lower Aβ in AD mouse models; however, neither NCX-2216 nor HCT-1026 was detected in plasma or brain [28]. Studies by Prosperi et al. utilized transversal probes implanted in the cerebral cortex to perform microdialysis and measure nitrite levels in the brain following oral administration of NCX-2216 and HCT-1026 [29,30]. Both agents elicited anti-inflammatory effects based on decreased expression of IL-1β and iNOS. Dialysate was analyzed using either the Griess assay or a chemoluminescent NO analyzer (Seivers model 280 NOA). HCT-1026 doubled cortical nitrite levels at a relatively low dose (~15 mg/kg), reaching a maximum level ~60 min after oral administration. By contrast, NCX-2216 required a larger dose (100 mg/kg) to observe changes in cortical nitrite. The authors attributed differences in nitrite levels to differential absorption and distribution for HCT-1026 compared to NCX 2216. Comparison of the molecular weight of each of these molecules (HCT-1026 [361 g/mol] versus NCX-2216 [551 g/mol]) supports the hypothesis of the authors, based on the contemporary understanding that molecular weight is a key predictor of brain penetration (i.e., increases in molecular weight correlates with decreased brain penetration) [31]. In other studies, NCX-2216 decreases PGE_2 (prostaglandin E2) synthesis and COX-1 expression in the brain 48 h after administration, an activity not observed in flurbiprofen treated control animals (Wistar rats) [32]. Hydrolysis of HCT-1026 yields 4-hydroxybutyl nitrate, which could also be responsible for NO mimetic activity in the brain [33]. A separate report identified the intact nitrate for the NO-NSAID **1** containing an amide linkage, suggesting that ester hydrolysis, rather than bioactivation of the nitrate, is largely responsible for the inability to detect HCT-1026 immediately following administration. HCT-1026 also inhibits bone resorption in ovariectomized C57BL/6 mice with concurrent inhibition of PGE_2 synthesis and decreased nitrite levels compared to mice treated with flurbiprofen and a control NO mimetic (NONOate or SIN-1 [see below]), suggesting iNOS inhibition by the NSAID.

The Thatcher group has studied several NO chimeric nitrates, including GT-1061, a hybrid nitrate analog of clomethiazole, which is a potent neuroprotective agent and improves learning and memory upon chronic administration in several transgenic Alzheimer's disease mice [34]. GT-1061 is rapidly absorbed after i.p. or p.o. administration with peak plasma concentrations 3 min after dosing [35]. GT-1061 has a systemic half-life of approximately 5–10 min, with negligible remaining 60 min after administration, regardless of route. Direct effect of GT-1061 on NO signaling was demonstrated by significant increases in hippocampal sGC activity after depletion of cholinergic innervation of the hippocampus and cerebral cortex by intraventricular microinjection of IgG-saporin. Interestingly, GT-1061 did not affect aortic sGC activity, suggesting selective effects in the CNS. Structural related methythiazoles **2** and **3** (Table 1) were both observed in the brain 20 min after administration (i.p.). Biochemical efficacy measurement for **2** and **3** were limited to *in vitro* settings. In animals, NO mimetic efficacy was hypothesized based on ability to reverse scopolamine induced deficits in contextual fear memory using step-trough passive avoidance (STPA). The ability of agents which enhance NO/cGMP signaling to reverse cholinergic memory deficits in STPA is well characterized and has been benchmarked for nitrates, sGC activators, protein kinase G (PKG, a downstream kinase of cGMP) activators, and several phosphodiesterase inhibitors (PDEi; PDE is responsible for breaking down cGMP) [36–42]. In fact, reversal of scopolamine induced deficits in STPA is a reliable and resource efficient *in vivo* screen when developing NO mimetic agents for brain disorders.

Nitrate hybrids of selective serotonin reuptake inhibitors (NO-SSRI) have also been studied utilizing a unique thiocarbamate linker to create NO-fluoxetine which undergoes base catalyzed hydrolysis to yield fluoxetine (Table 1). The intact nitrate of NO-fluoxetine is not observed in the plasma or brain following i.p. administration. Biochemical efficacy measurement for NO-fluoxetine were limited to *in vitro* settings. In a similar manner to the nitrates described above, NO-fluoxetine reverses scopolamine induced deficits in contextual fear memory, an activity which was not observed in animals treated with fluoxetine alone. Side-by-side comparison demonstrated anti-depressant effects of NO-fluoxetine which were similar for those observed for GT-1061, based on performance in the forced swim test (FST).

3. Diazeniumdiolates (NONOates)

Perhaps no other NO mimetic class has provided as much insight into NO biochemistry as diazeniumdiolates (commonly referred to as NONOates). NONOates are stable in solid form but decompose in aqueous environments to release 2 M equivalents NO (Fig. 2). In contrast to other NO mimetics which require bioactivation (such as nitrates and furoxans), NONOates produce NO at a predictable rate in aqueous environments and since they do not require enzymatic activation, these NONOates do not develop tolerance [43]. Importantly, rate of NO release from NONOates and can range from minutes (for DEA/NO [Fig. 2]) to 24 h (DETA/NO). Comparison of gastroprotective and anti-inflammatory properties of the nitrate NCX-4016 with an analogous aspirin related NONOate (NONO-ASA [also known as CVM-01]) indicated comparable activity for NCX-4016 and NONO-ASA, with both compounds being devoid of gastric damage seen in Wistar rats treated with aspirin alone [44,45]. Direct measurement of NONO-ASA in the plasma was not reported, but level of nitrite in the plasma (160 ± 3 μM) peaked 4 h after oral administration, suggesting a reasonable physiological half-life. The best potential for NONOates to

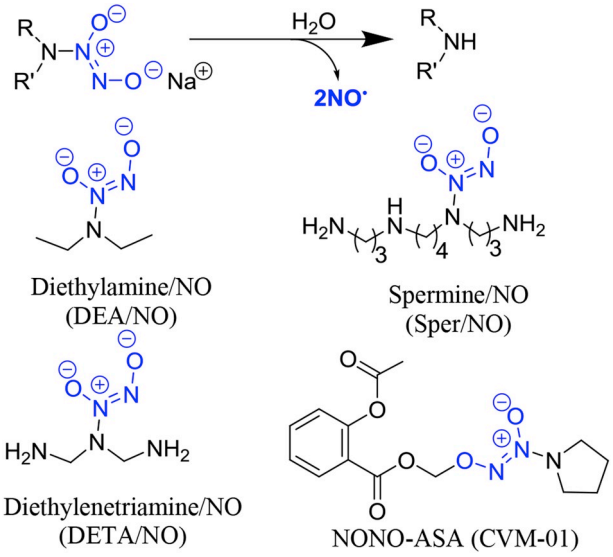


Fig. 2. NO release from and examples of NONOates.

benefit human populations is for the treatment of acute respiratory distress, since inhaled DETA/NO reduces pulmonary vascular resistance without affecting the systemic blood pressure [46,47]. At present NONOates are not approved for use in humans. Limited metabolic stability and corresponding large fluxes of NO from these agents may be associated with adverse effects which could explain the lack of use in the clinic. Regardless, NONOates continue to be an essential class of NO mimetics for unraveling the intricacies of NO related biological chemistry and therapeutic potential of NO mimetics.

4. Sydnonimines

Sydnonimines are mesoionic heterocycles (containing both negative and positive charges), which are an important class of NO mimetics that have been used clinically for over 25 years. Hundreds of sydnonimines have been explored, with the most well studied being molsidomine (Fig. 3), which will be the focus of this section. Molsidomine undergoes sequential degradation, initially via enzymatic conversion to SIN 1 (also known as linsidomine) followed by rapid decomposition at physiological pH to SIN 1A. SIN 1A is stable in anaerobic environments [48,49]. However, under biological conditions, oxidation of SIN 1A results in production of molecular $NO^{\cdot}$, requiring a SIN 1A cation intermediate to stabilize homolytic bond cleavage. The majority of NO produced is via oxidation in the presence of O_2, resulting in production of a stoichiometric equivalent of superoxide anion ($^{\cdot}O_2^-$). Since reaction rate between $NO^{\cdot}$ and $^{\cdot}O_2^-$ is a diffusion-rate limited process [3], peroxynitrite ($OONO^-$) is produced, resulting in lipid peroxidation and deleterious effects.

Although release of NO from molsidomine requires enzymatic bioactivation, the development of tolerance is not a clinically relevant problem because tolerance is much less pronounced than observed for nitrates [50,51]. Oral administration of molsidomine (2 or 4 mg) to healthy volunteers studied the relationship between plasma concentrations of molsidomine and SIN 1 with peripheral arterial resistance (i.e., in the form of finger plethysmography), venous distensibility (i.e., impedance plethysmography), heart rate, and blood pressure [52]. SIN 1 was confirmed to be the pharmacodynamically active form of molsidomine and the rate limiting step for prodrug breakdown was enzymatic hydrolysis and decarboxylation (i.e., conversion of molsidomine to SIN 1). The presence of SIN 1 in the plasma correlated strongly with increases in finger plethysmography and venous distensibility, without affecting blood pressure or heart rate. The mean peak plasma concentration was 76.1 ng/mL, which occurred 1.1 h after administration. Elimination t1/2 averaged 2.1 h. Appropriate formulation of molsidomine results in exceptional physiological stability, with approximately 5–10% remaining in the plasma 10 h after oral administration of a slow release preparation.

5. Furoxans (oxadiazole-N-oxides)

Furoxans are a class of thiol-dependent NO mimetics that have been studied for a variety of indications, which have been reviewed recently elsewhere [53]. Furoxans are unique amongst NO mimetics because they must react with a thiol to produce NO mimetic effects. Contrast this requirement with that of nitrates, *vide supra*, which are similarly bioactivated by thiols but are also activated by other means, including cytosolic and mitochondrial aldehyde dehydrogenases. Preference for thiophilic reactivity is associated with the electronic character of the furoxan ring system [54]. Manipulation of electron density within the furoxan ring via ring substitution pattern, allows modulation or 'tuning' NO release, potentially yielding attenuated slow onset NO mimetics [55]. Over the past 30 years, furoxan research has been largely centralized within the laboratory of Alberto Gasco at the University of Torino.

Furoxans are bioactivated by sufficiently reactive cellular thiols to produce reversible or irreversible (i.e., covalently modified) thiol adducts. Thorough analysis of the reaction products from incubations containing furoxans and generic biological thiols (such as cysteine or glutathione) is surprisingly sparse in the literature. Most reports examine thiol dependent NO production, measured via Griess assay, followed by progression to *in vitro* efficacy studies rather than analysis of the reaction with thiols and resulting reaction products (i.e., metabolites). Since furoxan metabolites are rarely characterized, none have been studied in animals. Hence, Table 2 only indicates structure of each parent furoxan without specifying metabolites.

There are two important studies which describe products of NO release from furoxans. The first of which characterized 4-phenyl-3-furoxancarbonitrile, referred to here as RVC-589 (Fig. 4). RVC-589 is the prototypical highly reactive furoxan which forms an irreversible thiol adduct [56]. RVC-589 has been studied for retinal recovery via increased ocular blood flow and was found to effectively release NO to a lesser extent than S-nitrosothiols [56]. Ultimately, RVC-589 possesses effects associated with large transient fluxes of NO, including potent antiparasitic efficacy against schistosomiasis [57,58]. Reaction of RVC-589 with cysteine produces a covalent adduct, based on LC-MS/MS, and a putative mechanism has been proposed involving the α-cyano group cyclizing with the resulting oxime group following NO release (Fig. 4).

To our knowledge, there are no reports of covalent thiol modification by a furoxan lacking the α-cyano group. This means either 1) covalent modifications of thiols by α-cyano furoxans is outlier-like behavior, or 2) covalent modification by other furoxans is underappreciated because it has not been studied. Indeed, analysis by Schiefer et al. described the reaction of several peptidomimetic furoxans, including 9a (Fig. 5), resulting in production of a keto-oxime product that is indicative of NO release. In this study the thiol dependent reaction products were characterized for several furoxans and a

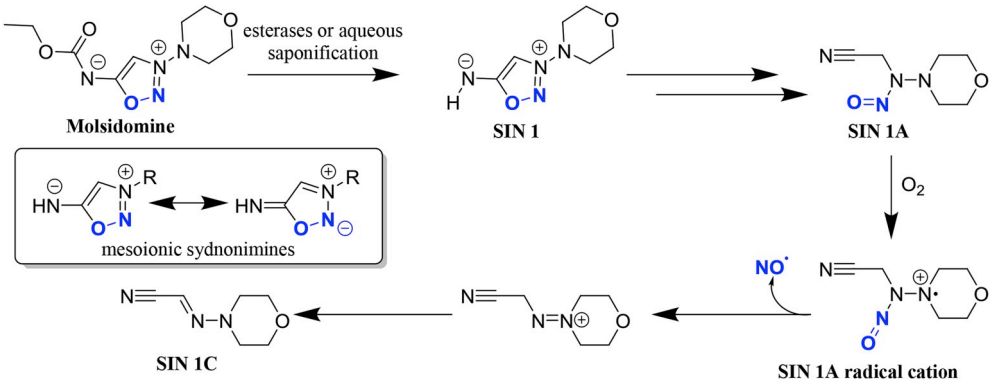
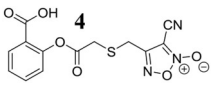
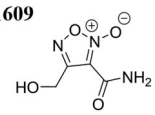
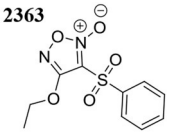
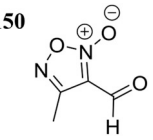
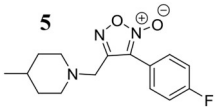


Fig. 3. Molsidomine breakdown to release NO.

Table 2
Summary of studies describing evaluation of stability and NO mimetic efficacy of furoxans in preclinical animal models.

Prodrug	Administration Route, Dose	How was NO measured?[a]	Measured Specie (prodrug)	PD readout (efficacy)[b]	References
 4	i.g., 120 mg/kg	Not reported	In vitro only. salicylate measured via HPLC-UV/Vis (incubated in human serum)	Inhibition of carrageenan-induced paw edema, decreased gastric lesion size	Lazzarato et al. [65] Cena et al. [63]
CAS 1609 	p.o., 0.5 mg/kg	Not reported	Intact drug not observed	Hemodynamic activity; conscious canine	Bohn et al. [112]
CHF 2363 	jugular infusion, 50 µl/min	Not reported	Intact drug not observed	Hemodynamic activity via NO-induced formation of methemoglobin in unconscious rats	Civelli et al. [113]
PRG150 	i.v., [^{13}N] = 23 ± 1 mCi; [^{11}C] = 25 ± 12 mCi	Not reported (Griess assay; *in vitro*)	^{13}N (NO) and^{11}C (prodrug) tracked via PET-CT	Analgesia; PWT in STZ-diabetic rats	Huang et al. [114] Pippin et al. [115]
5 	i.p. and p.o., 20 mg/kg (PK) i.p., 0.1, 1, 10, 20 mg/kg (behavioral)	Not reported	5 measured in brain and plasma via LC-MS/MS 1 h, 2 h, and 12 h post administration	Reversal of scopolamine induced deficits in contextual fear memory; co-incubation with ODQ results in loss of neuroprotection against oxidative stress	Horton et al. [55]

[a] Method used to measure NO production in animals treated with specified NO mimetic; technique used to evaluate NO mimetic efficacy in animals. Abbreviations: i.g., intragastric; p.o., oral administration;: i.v., intravenous; PD, pharmacodynamics; PWT, paw withdrawal threshold; STZ, streptozotocin induced; STPA.

covalent thiol adduct was observed in low abundance (< 1% of reaction products), indicating reversible modification of thiols. After 30 min in the presence of excess cysteine approximately 70% of 9a remains, versus RVC-589 which is completely consumed in 30 min. Side-by-side comparison with other peptidomimetic 3-arylfuroxan and 4-arylfuroxans indicates a similar reaction product profile, and the possibility that there are distinct NO release mechanisms for 3-arylfuroxan versus 4-4-arylfuroxans (Fig. 6). Neither furoxan reacts to produce NO in the absence of thiol. Importantly, conversion of the 3-arylfuroxan to only the keto-oxime product suggests it may be the most efficient NO mimtic furoxan, since 1 mol of 3-arylfuroxan yields 1 mol of keto-oxime metabolite resulting from NO release. Presumably, monitoring this keto-oxime as a measure of *in vivo* NO mimetic effects could be more useful than measuring nitrite as an NO surrogate, since nitrite is present in high concentrations in biological media but the keto-oxime is non-native.

It has been shown that NO donors, including a furoxan, can induce Nrf2 translocation to the nucleus [59–61], leading to the possible misconception that covalent modification of the furoxan ring is responsible for the dissociation of the Keap1-Nrf2 complex. However, it was shown by Um et al. that S-nitrosation can lead to the translocation, independent of covalent modifications of the furoxan. Furthermore, the lone instance in which a furoxan induced Nrf2 translocation was upon incorporation into a chalcone hybrid, which contains an additional Michael acceptor, thus convoluting whether or not it was the furoxan itself providing thiol modification. Experiments conducted with the NO donor S-nitroso-N-acetylpenicillamine (SNAP) showed translocation of Nrf2 to the nucleus after SNAP transnitrosation, not covalent bioactivation [62].

Similar to efforts with NONOates and nitrates, furoxan-aspirin hybrids have been studied to ameliorate gastric toxicity associated with NSAIDs. Efforts from Lazzarato et al. screened a library of furoxan

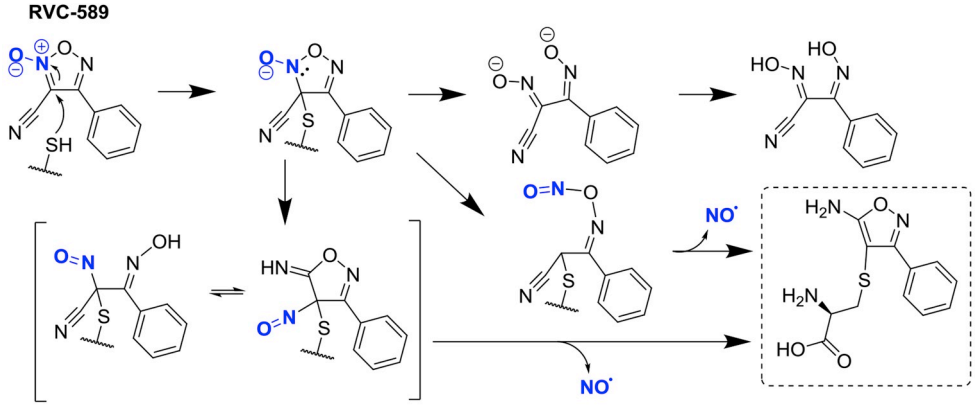
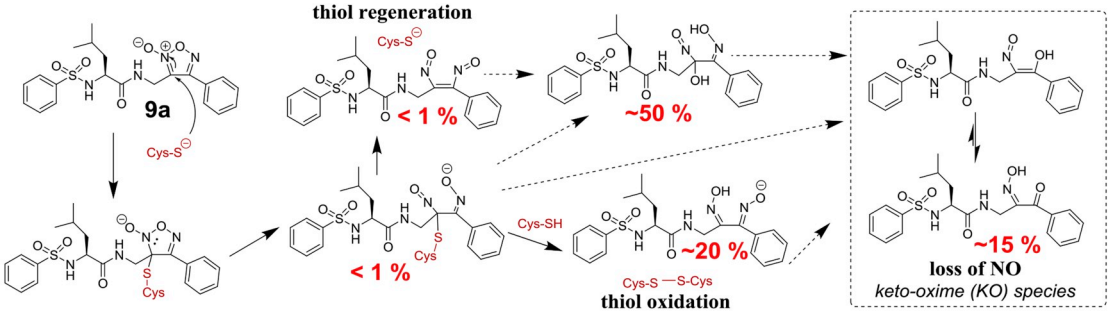


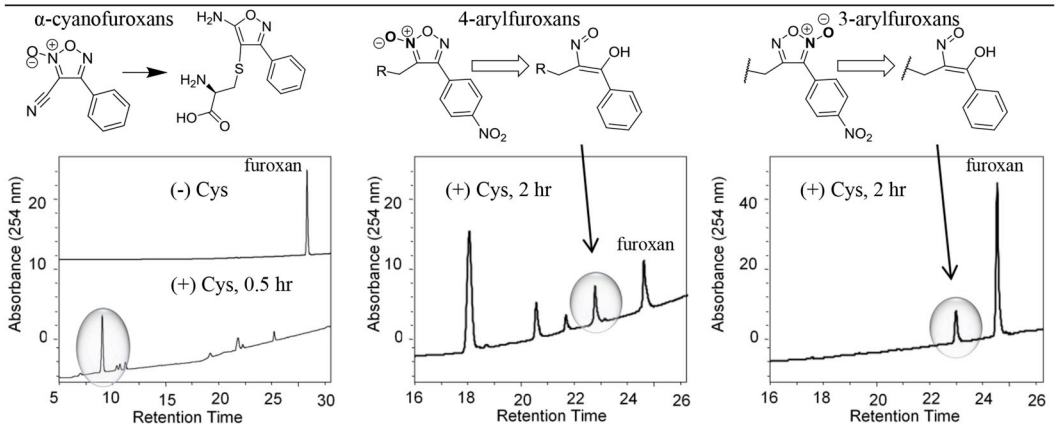
Fig. 4. Irreversible thiol modification by a furoxan.[a].
[a] Incubation of RVC-589 with excess thiol (5 mM) in PBS (50 mM, pH 7.4) results in a covalent thiol adduct based on LC-MS/MS analysis. RVC-589 is highly reactive based on complete consumption of RVC-589 within 30 min of thiol addition. Mechanism adapted from Medana, C.; et al., *J. Med. Chem.*, 1994, 37 (25), 4412-16.

[a] Incubation of RVC-589 with excess thiol (5 mM) in PBS (50 mM, pH 7.4) results in a covalent thiol adduct based on LC-MS/MS analysis. RVC-589 is highly reactive based on complete consumption of RVC-589 within 30 min of thiol addition. Mechanism adapted from Medana, C.; et. al., *J. Med. Chem.*, 1994, 37 (25), 4412-16.



[a] Incubation of **9a** with excess thiol (5 mM) in PBS (50 mM, pH 7.4) results in formation of several products. Relative % of each product is listed in red below each structure. The thiol adduct accounted for a negligible % of the reaction mixture based on UV-vis analysis. Notably, the NO release likely results in formation of the keto-oxime (or its enol tautomer). Mechanism adapted from Schiefer, I. T.; et. al., *J. Med. Chem.*, 2012, 55 (7), 3076-87.

Fig. 5. Reversible thiol modification by a furoxan.[a].
[a] Incubation of **9a** with excess thiol (5 mM) in PBS (50 mM, pH 7.4) results in formation of several products. Relative % of each product is listed in red below each structure. The thiol adduct accounted for a negligible % of the reaction mixture based on UV–vis analysis. Notably, the NO release likely results in formation of the keto-oxime (or its enol tautomer). Mechanism adapted from Schiefer, I. T.; et al., *J. Med. Chem.*, 2012, 55 (7), 3076-87.



[a] Incubations of indicated furoxans with excess cysteine (5 mM) in PBS (50 mM, pH 7.4) analyzed by tandem HPLC-PDA-LC-MS/MS results. These classes of furoxans produce distinct reaction product profiles. RVC-589 produces a covalent adduct as a product of NO release and is highly reactive, whereas the studied 4-aryl furoxans and 3-arylfuroxans are less reactive (based on % remaining starting material) and produce a keto-oxime as the product of NO release.

Fig. 6. Comparison of reaction product profiles for three types of furoxans.[a].
[a] Incubations of indicated furoxans with excess cysteine (5 mM) in PBS (50 mM, pH 7.4) analyzed by tandem HPLC-PDA-LC-MS/MS results. These classes of furoxans produce distinct reaction product profiles. RVC-589 produces a covalent adduct as a product of NO release and is highly reactive, whereas the studied 4-aryl furoxans and 3-arylfuroxans are less reactive (based on % remaining starting material) and produce a keto-oxime as the product of NO release.

incorporating aspirin analogs. A representative compound from this library, **4**, is shown in Table 2. Anti-inflammatory effects similar to those seen for NO-ASA (a nitrate) and NONO-ASA (a NONOate) were observed for **4**, including reduced paw volume in the carrageenan induced edema model in Wistar rats. **4** showed a significant reduction (54.9 ± 8.1%) over the furazan (incapable of NO donation, Fig. 7) control **4a** (38.9 ± 3.8%) and salicylic acid (36.8 ± 4.2%), and importantly, exhibited COX-independent platelet aggregation inhibition [63]. Furazans are often used as control compounds when studying furoxans due to the near identical physiochemical properties; however furazans are not capable of releasing NO and are not subject to nucleophilic conjugation. Decreased expression PGE_2 and decreased production of IL-1β was also observed for **4** [63,65]. The presence of **4** or/salicylate was not reported in the test animals, Wistar rats. A $t_{1/2}$ of 13 min was reported in stability studies using human serum spiked with **4**, indicating relatively high reactivity, in agreement with the reactivity of other α-cyano furoxans, such as RVC-589.

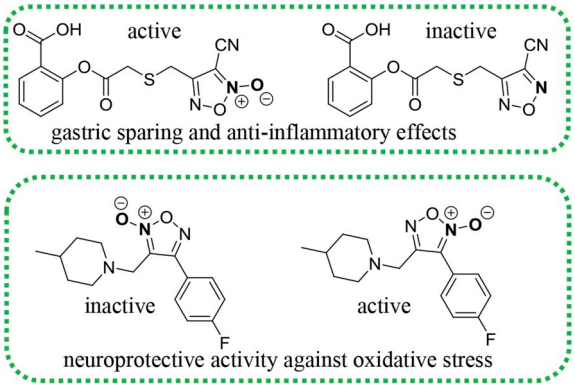


Fig. 7. Comparison of activity for noteworthy furoxan pairs in different models of NO mimetic efficacy.

Seminal work on the development of orally, bioavailable furoxans was completed by Bohn et al. at Cassella AG in the study of CAS 1609 [66,67]. Efforts were initially focused on producing an NO donating moiety that did not possess tolerance. Limited studies have been pursued on the PK properties of CAS 1609, instead relying on inference of biological half-life from duration of action studies. Of course, the structure of CAS 1609 creates inherent challenges for its measurement. Measuring CAS 1609 appears logistically challenging due to 1) the lack of a sufficiently delocalized π-electron system to allow measurement by spectrophotometry and 2) the small molecular weight (159 g/mol) is difficult to monitor by LC-MS/MS due to high background in this low molecule weight mass range. CAS 1609 was found to be a potent long lasting vasodilator, capable of lowering systolic blood pressure (10 mmHg, 0.5 mg/kg, 12 h), compared to glyceryl trinitrate (GTN) (15 mmHg, 0.3 mg, 3 min post-administration) [68]. Importantly, no tolerance was observed during a 5 day treatment in conscious dogs. This may be explained by the fact that, in contrast to nitrates, furoxans do not depend on the presence of any specific enzyme, such as mitochondrial aldehyde dehydrogenase. CHF 2363 was also under development concurrently by Civelli et al. at Chiesi Pharma as a potent vasodilator lacking tolerance drawbacks associated with GTN. Importantly, studies conducted on rat aortic strips demonstrated that endothelial relaxation could be produced after tolerance towards GTN developed, distinguishing the furoxan bioactivation from nitrates and nitrate tolerance [69].

Development of CAS 1609 and CHF 2363 was halted due to observations of genotoxicity. Follow-up studies exploring the genotoxicity of both compounds were performed based on the theory of NO genotoxicity at high concentrations [70,71]. No significant DNA damage was observed at therapeutic doses of CAS 1609, and genotoxicity was only observed at 100 fold above the maximum therapeutic dose where cytotoxicity was also observed. However, CHF 2363 was observed to possess significant genotoxic properties at 5 μM, much closer to the therapeutic dose of 1 μM. Since furoxans require thiol bioactivation in order to release NO, covalent modification of endogenous nucleophiles was hypothesized as potentially playing a role in genotoxicity. Computational studies by Boiani et al. [72], identified that the presence of leaving groups adjacent to the furoxan ring creates the potential for covalent adducts which may be associated with the genotoxicity observed by Balbo et al. [72]. This mirrors the results of RVC-589, which contains an α-cyano group adjacent to the furoxan ring, emphasizing the importance of the chemical moieties adjacent to the furoxan ring. In recent years, appropriately designed furoxans containing functional groups that are not energetically favored leaving groups are reliably devoid of genotoxicity seen with CHF 2363.

A noteworthy study of a structurally similar furoxan profiled the pharmacokinetics of PRG150 (Table 2 and Fig. 8), which has been studied for the treatment of painful diabetic neuropathy (PDN) [74,75].

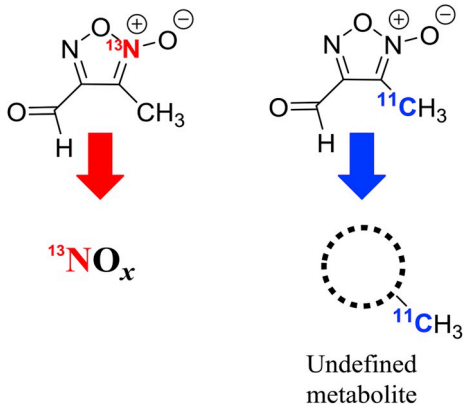


Fig. 8. Radiolabeled PRG150 PK experimental design.

To study the metabolic stability and biodistribution of PRG150, Pippin et al. employed radio-isotopic labeling and positron emission tomography (PET) combined with a computerized tomography (CT) scan. In order to study both the release of NO and the parent furoxan, ^{13}N and ^{11}C based probes were synthesized and administered intravenously (IV) to SD rats [76]. Their findings suggested that PRG150 is metabolized quickly ($t_{1/2}$ = 3 min in the distribution phase and 120 min in elimination phase). Localization of ^{13}N to the spinal cord remained outside the 60 min PET scan window, correlating with analgesia observed in the rat model of PDN. In order to explore PD properties of PRG150, experiments were conducted with streptozotocin induced diabetic rats. Analgesia was measured as paw withdrawal threshold. Interestingly, when PRG150 was administered with ODQ, a selective sGC inhibitor which blocks the canonical NO/sGC/cGMP signaling pathway, analgesic activity was not impacted until 1.5 h after ODQ administration, at which point analgesic effects were abolished.

The furoxan moiety has also been harnessed for potential treatment of Alzheimer's disease. The NO/sGC pathway is inhibited by oligomeric amyloid beta (Aβ42) [38,78], making the NO/sGC pathway an attractive drug target. NO/cGMP signaling is required for long-term potentiation (LTP)- i.e., sustained strengthening of synaptic signal [79,80]. The ability of NO to enhance synaptic plasticity is chiefly attributed to post-synaptic activation of NO sensitive sGC, resulting in kinase signal transduction via cGMP/PKG leading to phosphorylation of cAMP response element binding protein (CREB) at Ser-133 [79–84]. CREB phosphorylation is recognized as being a crucial regulator of synaptic plasticity, resulting in the production of neurogenic gene products, such as BDNF and Bcl-2, and the formation of new dendritic spines- ensuring the capacity for morphological flexibility of synaptic networks [85–92]. This pathway, cumulatively the NO/cGMP/PKG/CREB system, is disrupted in AD, mainly via Aβ-mediated inhibition of NO-induced CREB phosphorylation and synaptic plasticity [83,93,94]. Inhibition of any component of this system suppresses pCREB and weakens synaptic signal [95,96]. Reversal of Aβ induced deficits in LTP via agents which activate NO/cGMP signaling results in improved cognitive function- as demonstrated for nitrates [35,97,98], sGC activators [26,27], and phosphodiesterase inhibitors (PDEi) [99–103]. Recently, Horton et al. reported the first series of furoxans designed specifically to be 'slow onset' NO mimetics with attenuated reactivity targeted at the brain. PK studies in C57BL/6 mice via oral administration of 5 (Table 2), showed a brain to plasma ratio of 1.49 ± 0.52 as measured via LC-MS/MS. Importantly, the systemic t1/2 was measured at 50–110 min, with the intact furoxan present in the brain 12 h after oral administration. A reversal of a scopolamine-induced deficits in contextual fear was observed using an analogous behavioral model as described for nitrates in Table 1. Protection against oxygen glucose deprivation elicited by 5 in PC12 cells is abolished by co-incubation with ODQ. Despite evidence of NO/sGC pathway engagement and NO mimetic efficacy, 5 does not readily react with generic cellular thiols at physiological pH and no measurable changes in nitrite are observed, indicating an unusually stable furoxan.38 Interestingly, the tautomer represented in 6 (Fig. 8), lacks NO mimetic efficacy. Further exploration comparing and contrasting the reactivity and efficacy of 5 and 6 is ongoing.

6. Conclusions

NO plays a diverse set of biological roles, and to harness any specific effect requires a multi-faceted approach, including an intricate understanding of the mechanism of NO release and the pathological mechanisms involved in the therapeutic indication. There is a wealth of literature indicating the potential for increased utilization of NO mimetics clinically. However, appropriate utilization requires an understanding of the type of NO mimetic which is most appropriate for a given therapeutic indications and an appreciation for the distinct behavior of NO mimetics in different model systems. A better

understanding of NO release mechanism can facilitate an easier transition to tracking biological efficacy in complex systems via PK and PD readouts. With a few exceptions, the historic thrust of NO mimetic medicinal chemistry tends to overlook the importance of NO release mechanism in favor of progressing compounds into complex biological systems. Future studies should carefully benchmark NO mimetics effects of novel compounds versus established NO mimetics from a variety of classes, thus creating a profile of NO mimetic utility depending on assay and/or therapeutic indication.

Acknowledgements

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