An Introduction to NO-related Therapeutic Agents

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Abstract: Investigational NO-related therapeutic agents span the range from prodrugs that elevate NO levels, to scavengers of NO, and inhibitors of endogenous NO synthesis. Related agents that influence nitrogen oxides, in addition to NO itself, are also to be considered. Organic nitrates have been used for over 130 years in cardiovascular therapy and hybrid nitrates continue to lead the way in clinical development for an increasing range of disease states. Selectivity for inhibition of NO synthase (NOS) isoforms is a continuing goal. Conversely, N-hydroxyarginine derivatives are substrates for NOS and represent a new NO donor class. Diazeneimdiolate (NONOate) NO donors have been essential for understanding of NO biology and are being developed as NO donor and HNO donor prodrugs including as photolabile sources. N-Hydroxyurea has been used as a cancer drug for decades and is approved for sickle cell disease treatment, but also provides a lead compound for design of NO and HNO donors. Nitroaliphatics and nitrosoaliphatics also represent chemical classes that include NO donors and exhibit biological activity that mimics that of NO. Nitroprusside has been in use since the 1920’s and metal ion complexes continue to be explored, including as caged NO donors. Clear and exciting opportunities exist for new therapies based upon NO-related drugs. Despite the extensive clinical use of NO donor drugs and the role of NOs activation in the action of several prescription medications, challenges remain to the development of new NO-related therapeutics, but these are surmountable and are outweighed by the opportunities.

1. PURPOSE

NO-related therapeutic agents include prodrugs that elevate NO levels, scavengers of NO, and inhibitors of endogenous NO synthesis. The wider field also includes compounds that modulate the concentration and actions of nitrogen oxides derived from or related to NO, in particular peroxynitrite and HNO (commonly termed nitroxyl). To open the field even wider, one might incorporate those drugs with mechanisms of action associated with elevation of NOS activity and of NO levels, for example, the statins [1-3]. However, for reasons of space, a focus has been chosen. In this issue, reviews in the area of NO-related therapeutic agents have been selected primarily to provide a very broad range of chemical classes (Fig. 1), and also to incorporate a range of topics from preclinical discovery to clinical drug candidates. Several omnibus reviews have appeared, but this issue includes only reviews by experts who are leaders in each individual area of NO-related therapeutics. Hopefully, this selection emphasizes the exciting progress towards NO-related therapeutics, together with providing detailed information on the biological and medicinal chemistry.

2. APPROACHES

The importance of NO in biology was recognized in 1998 by the award of the Nobel Prize to R. Furchgott, L. Ignarro, and F. Murad. In the first decade after the discovery of the identity of endothelium derived relaxing factor and the expanding roles of NO in biology, medicinal chemists focused upon the task of developing selective inhibitors of the three NOS isoforms, especially constitutive neuronal nNOS, and inducible iNOS. The rationale for these targets was the cellular cytotoxicity and tissue damage thought to contribute to the pathophysiology of human disease states, deriving from (a) iNOS induction by endotoxins and inflammatory cytokines yielding high concentrations of NO, and (b) the excessive production of NO produced by sustained activation of nNOS. NOS inhibitors were expected to block the synthesis of “the major injurious agent, nitric oxide”, and to attenuate tissue damage resulting from chronic inflammation and the prolonged elevation of cytosolic Ca²⁺, as encountered in CNS excitotoxicity [4]. These approaches have not borne fruit [5], in part because of the emerging complexity of NO biology, but also because of the challenge in selective inhibition of isozymes with very similar active sites [6, 7]. A thorough review of progress towards selective nNOS inhibitors is presented in the first article in this issue [8]. Arguably, most research activity has been directed at iNOS inhibitors, studied in preclinical models including sepsis, asthma, diabetes, amyotrophic lateral sclerosis, meningitis, and several chronic inflammatory conditions. In a similar vein, a drug candidate proposed to act as an NO scavenger (N-methyl-D-glucamine dithiocarbamate-Fe²⁺) is in early clinical trials for sepsis and has been examined for other indications [9].

Progress over the past 15 years in understanding the chemical biology and physiology of NO has been made possible by the availability and the extensive use of the NONOates (diazeneimdiolate NO donors) in preclinical studies. These compounds, unlike the majority of all other NO donors, cleanly release NO at predictable rates. The second article in this issue spotlights the preclinical studies on NONOates that suggest that this family of NO donors represent drug candidates with significant potential [10]. Various derivatized NONOates have been reported which are pro-prodrugs, designed to confer some cell or tissue selectivity by using metabolic bioactivation. In the third...
article, derivatized photolabile NONOates are discussed amongst other approaches to caged NO photochemical sources and potential photodynamic therapy [11]. This latter article includes some discussion of organometallic NO donors, the most venerable of which is sodium nitroprusside, in use as a clinical nitrovasodilator for 80 years. Organo-metallic transition metal complexes have been studied for a number of years alternately as NO donors and NO scavengers. Metalloporphyrins, such as FP15 (FeCl-tetrakis-2-(triethylene glycol monomethyl ether) pyridyl porphyrin [12], are in preclinical studies as scavengers of peroxynitrite (although scavenging of hydrogen peroxide and nitroxyl is also claimed) [13].

Interest in nitroxyl has increased in recent years, on the basis of the interesting cardiovascular activity of Angeli’s salt and confirmation of its HNO donor capacity. The fourth contribution to this issue discusses HNO donors including inhibitors of aldehyde dehydrogenase that have been subsequently shown to be donors of the thiophilic HNO [14]. Indeed, N-hydroxyurea, a therapeutic agent in use for decades in cancer chemotherapy and approved for sickle cell disease treatment, can behave as a mixed NO/HNO donor. The biological chemistry of N-hydroxyurea and its role as a lead compound for design of NO and HNO donors is discussed in the fifth article in this issue [15]. The Nef reaction is a base-catalyzed reaction of nitroalkanes via a nitronic acid intermediate that is reported to hydrolyze to yield HNO. Nitroaliphatic lipids have been reported as endogenous species with NO mimetic biological activity [10]. Interestingly, the hydroxyimine NO donors developed by Fujisawa (now commercially available as experimental probes; NOR-1 to NOR-5) are nitroaliphatic hydroxyamines that may decompose to NO via a nitronic acid intermediate (Fig. 1). The sixth article reviews the Fujisawa program from genesis to commercial termination [17].

S-Nitrosothiols are endogenous NO donors, whereas O-nitroso compounds, such as amyl nitrite, are nitrovasodilators and recreational drugs. Amyl nitrite is also used “routinely” in cyanide poisoning antidote kits, in combination with sodium nitrite. Ethyl nitrite has also been the focus of recent clinical studies [18]. C-Nitroso compounds include dimeric species such as diazetine dioxides and furoxans that show NO donor activity in preclinical studies. These and other
nitrosoaliphatics are reviewed in the seventh contribution to this issue [19].

Organic nitrates have been used for over 130 years in cardiovascular therapy and continue to lead the way in clinical development in the NO donor field. Classical nitrates continue to be of clinical use for angina pectoris, despite the apparent hindrance of clinical tolerance to nitroglycerin (GTN), and controversy surrounding the phenomenon [20]. A recent Phase III clinical trial of a drug combination including the classical nitrate, isosorbide dinitrate (ISDN), was halted early because of very positive outcomes in heart failure prevention [21] Hybrid nitrates that conjugate a nitrate group to an established drug via a labile linker have been the subject of numerous preclinical and clinical studies and have attracted the interest of Merck, Astra, and Pfizer. The biological chemistry of classical nitrates, hybrid nitrates, and NO chimeras (novel nitrates that incorporate a pharmacophore in addition to the NO mimetic nitrate group) has recently been reviewed [20]. The most well described examples of hybrid nitrates are NO-NSAIDs that along with other hybrid nitrate approaches are discussed in the eighth article in this issue [22].

Finally, in this issue, we come full circle back to NOS, in this case using the catalytic activity of the enzyme to produce NO, not from arginine supplementation, but from N-hydroxyarginine derivatives that are substrates and show isoform selectivity; representing a novel approach to NO donors [23].

3. OBSTACLES

Obstacles to development of the undoubted potential of NO-related therapeutics are often of perception and sometimes of substance. (#1) NO donors do not fit the traditional receptor-targeted drug design paradigm; NO donors are by definition prodrugs. NO is a ubiquitous biological messenger molecule and therefore NO donor drugs are expected to have multimeric actions and pleiotropic effects; though in most disease states this should be seen as a benefit rather than disadvantage. Selectivity may be engineered, by using the localization of the cellular components required for bioactivation to provide tissue selectivity. As prodrugs, NO donors are not readily amenable to in vitro high throughput drug screening.

(#2) The biological chemistry of NO is complex and confusing even to those researchers specializing in NO biology. The biological activity of NO may be exerted simply by NO coordination to the Fe(II)-heme of soluble guanylyl cyclase (sGC) and elevation of intracellular cGMP, but additionally through cGMP-independent mechanisms via a variety of other possible reactive nitrogen oxide species (RNOS) (Fig. 2). The oxidative metabolism of NO and RNOS is intimately linked with oxygen and thiol concentrations, cellular redox state (and therefore is intrinsic to redox signaling), and is profoundly influenced by transition metal catalysis, and microenvironment [24]. The diverse range of unstable and metastable chemical species cause difficulties in detection and quantitation, which has resulted in measurement of the readily detected, stable end products of NO metabolism as surrogates for NO itself: that is NO$^\cdot$, NO$_2^-$, N$_2$O, NO-Hb. Inorganic nitrite and hydroxylamine are well known to be biologically active at higher concentrations, mimicking aspects of NO and HNO, respectively. Under hypoxic conditions, NO$_2^-$ has been shown to function as an NO donor at lower concentrations: nitrite infusions are in clinical trials and inhaled NO$_2^-$ is in preclinical studies [26, 27]. It has even been suggested that HNO donors act as hydroxylamine donors and that some NO donors are functionally donors of NO$_2^-$.

Difficulties with direct detection of NO and HNO at pharmacological concentrations of donors, and the reversible conversion of NO, RNOS and detected end products often provide for ambiguous evidence that NO donors function only by direct release NO in vivo. Thus, we have preferred the term NO mimetic for drug classes such as nitrates.

(#3) To avoid explaining complex NO biological chemistry, the term NO donor is used almost universally for NO-related drugs manifesting biological activity that mimics endogenous NO. Thus, the very different classes of “NO donors” tend to be lumped in one basket. Numerous pharmacological observations undermine this premise. Furthermore, examination of the “NO donor” drugs and the RNOS that mediate the biological activity of NO reveals a wide span of oxidation state (Fig. 3); often the redox interconversion of these species is not trivial: endogenous formation of NO from arginine is an oxygen-dependent 5e$^-$ oxidation requiring multiple cofactors; whereas, bioactivation of a nitrate to NO is a 3e$^-$ reduction requiring oxygen atom transfer. Thus, it is reasonable that a given “NO donor” might provide NO mimetic biological activity that does not require generation of NO. As a hypothetical example, nitrate 2e$^-$ reduction to organic nitrite [20], is reasonably accompanied by nitrosation of a metal centre or thiol, and the organic nitrite, like its putative endogenous equivalent N$_2$O$_3$, is an excellent nitrosating agent:

$$\text{RONO}_2 + R'SH \rightarrow \text{RONO} + R'SOH$$
$$\text{RONO} + R'SH \rightarrow \text{ROH} + R'SNO$$

Thus, nitrosation and nitrosation, two NO mimetic actions, have been accomplished without the intermediacy of NO. In order for NO itself to have accomplished these actions via the endogenous nitrosating agent, N$_2$O$_3$, the following reactions would be described:

$$2\text{NO} + O_2 \rightarrow N_2O_4$$
$$\text{N}_2\text{O}_4 \rightarrow 2\text{NO}_2$$
$$\text{NO} + \text{NO}_2 \rightarrow 	ext{N}_2\text{O}_3$$
$$\text{N}_2\text{O}_3 + R'SH \rightarrow R'SNO + \text{NO}_2^- + \text{H}^+$$
$$\text{NO}_2^- + R'SH \rightarrow R'S^0 + \text{NO}_2^- + \text{H}^+$$
$$R'S^0 + \text{NO} \rightarrow R'S\text{NO}$$
$$R'S^0 + O_2 \rightarrow R'S\text{OO}^- \rightarrow R'S\text{OH}$$

This sequence requires several NO equivalents and O$_2$; high concentrations of NO are often associated with formation of the potentially cytotoxic oxidants NO$_2$ and peroxynitrite, ONOO$^-$:

$$\text{NO} + O_2^- \rightarrow \text{ONOO}^-$$
$$\text{ONOO}^- + \frac{1}{2}\text{H}^+ \rightarrow \frac{1}{2}\text{NO}_2 + \frac{1}{2}\text{HO}^- + \frac{3}{2}\text{NO}_3^-$$
Fig. (2). A variety of NO-related species can result from oxidative or reductive metabolism of NO, often involving more than a single reaction step rather than direct conversion. Several short-lived free radicals not depicted are also implicated in these reactions, which are strongly dependent on $O_2$ concentration and on transition metals. Reactions of the RNOS can be classified as nitroxidation (oxidation by RNOS [25]), nitration, nitrosation and amination. Reactions of RNOS with S-functional groups, in particular low molecular weight and protein thiols (RSH), yields a variety of further species ranging in lability, some of which may interconvert, or serve as intermediates in thiol oxidation to disulfides and S-oxides, and in “glutathiolation”; processes central to cellular redox signaling. This cornucopia of chemical species may react with proteins yielding biological effects originating from the initial modulation of NO levels; however, of such known reactions the major ones are: (a) nitrosylation/displacement/redox reactions at protein-transition metal centres; (b) nitration of aromatic, olefinic, and thiol groups; (c) nitrosation at N, S, and O, to form nitrosoamines, nitrosothiols, and nitrates, respectively; and (d), oxidation at S.

Formula: $\text{NO} + \frac{1}{2} \text{O}_2 \rightarrow \text{NO}_2^+$

Fig. (3). NO donor drugs may decompose directly to NO, but NO mimetic biological activity will also result from direct nitrosation, nitration, and nitroxidation reactions, or direct formation of RNOS that mediate these. NO mimetic drugs contain N in oxidation states (N Ox#) from -1 to +5, which mirrors the oxidation numbers (Ox#) of RNOS and also of the biomolecule adducts formed on nitration, nitroxidation, or nitrosation. NO itself has oxidation number +2 and mediates its biological activity via binding to the regulatory Fe(II)-heme site of soluble guanylyl cyclase (sGC).

Formula: $\text{ONO}^- + \frac{1}{2} \text{O}_2 \rightarrow \text{NO}_2^+$

ONOO$^- + \frac{1}{2} \text{CO}_2 \rightarrow \frac{1}{2} \text{NO}_2 + \frac{1}{2} \text{CO}_3^{2+} + \frac{1}{2} \text{NO}_3^-$

There is a mistaken perception that cytotoxicity is inherently linked with NO, which partly derives from the association of NO with atmospheric pollutants, before its renaissance as an essential component of human health. The recent progress of new hybrid nitrates to Phase II/III clinical trials shows that toxicity does not represent a major obstacle to drug development. There is also 130 years of drug therapy with classical nitrates to support the safety of NO mimetic medications and nitrates in particular. Under some
pathophysiological conditions, where cellular NO/thiol homeostasis is perturbed, removal of NO by use of NOS inhibitors will be of benefit. However, this is evidence for the benefits of therapeutic modulation of NO, not for the general toxicity of NO. Numerous reports on NOS inhibitors showing neuroprotective and chemo-preventive effects are counterbalanced by a large body of data showing neuroprotection and chemoprevention by NO donors. The complexity of NO physiology is amply demonstrated by animal studies on NOS knockouts (reviewed in [28]), which can only partly be explained by adaptive responses of remaining isozymes to the absence of the one NOS knockout.

As with any drug class, there are considerations with toxicological assessment. At higher concentrations in vitro, NO and nitrosating agents (such as acidified NO⁻) can lead to DNA-modification [29]. There is no evidence for carcinogenicity of GTN or other clinical nitrates, but GTN is reported as mutagenic in several bacterial strains in the reverse mutation assay (AMES test), as are diethylamine-
NONOate, NO gas, and sodium nitrite [30, 31]. Positive results were also reported for GTN in at least one other cell culture mutagenesis assay, but ISDN and other nitrates have had reported negative Ames tests. The relevance of several results in cell culture is unclear, since in vivo measurements have been made of normal, endogenous concentrations of NO⁻ and nitrosamines at micromolar and nanomolar levels, respectively [32]. The recent progress of new hybrid nitrates through clinical development clearly shows that any perceived obstacles are not insurmountable.

(#5) NO (and HNO) donors are readily accepted as systemic, cardiovascular drug candidates, but applications in other indications can be less warmly received. This situation is changing, in part due to the quality of data generated on anti-inflammatory and other actions of hybrid nitrates [22]. Supported by animal model data demonstrating cognition enhancement and neuroprotection, an NO mimetic novel nitrate recently entered clinical trials directed at Alzheimer’s disease [33]; other exciting applications of NO mimetics as neuroprotective agents have been described [34]. Given the crucial role of NO in cell signaling in the CNS, this development is projected to continue. It is to be expected that NO mimetics will manifest some cardiovascular effects, including vasodilation, however, appropriate drug design and delivery can elevate the doses at which any undesired vascular effects are observed to well above the therapeutic dose, providing functional selectivity.

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