Part 1 Chemistry of NO Donors

1
NO and NO Donors

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Nitric oxide (NO), a magic free radical gas molecule, has been shown to be involved in numerous physiological and pathophysiological processes. Among its diverse functions, NO has been implicated in the relaxation of vascular smooth muscle, the inhibition of platelet aggregation, neurotransmission (Viagra reverses impotence by enhancing an NO-stimulated pathway), and immune regulation [1]. It was named the molecule of the year in 1992 by *Science* and was the subject of the Nobel Prize in 1998. NO has limited solubility in water (2–3 mM), and it is unstable in the presence of various oxidants. This makes it difficult to introduce as such into biological systems in a controlled or specific fashion. Consequently, the development of chemical agents that release NO is important if we are to target its bioeffector roles to specific cell types for biological and pharmacological applications. Based on our comprehensive review of NO donors [2], this chapter focuses on recent progress and current trends in NO donor development and novel applications which are not covered by the following chapters.

1.1
Introduction to NO Biosynthesis and NO donors

1.1.1
Nitric Oxide Synthases

Endogenous NO is produced almost exclusively by L-arginine catabolism to L-citrulline in a reaction catalyzed by a family of nitric oxide synthases (NOSs) [3]. In the first step, Arg is hydroxylated to an enzyme-bound intermediate N^{ω}-hydroxy-L-arginine (NHA), and 1 mol of NADPH (nicotinamide adenine dinucleotide phosphate, reduced form) and O_2 are consumed. In the second step, NHA is oxidized to citrulline and NO, with consumption of 0.5 mol of NADPH and 1 mol of O_2 (Scheme 1.1). Oxygen activation in both steps is carried out by the enzyme-bound heme, which derives electrons from NADPH. Mammalian NOS consists of an N-terminal oxy-

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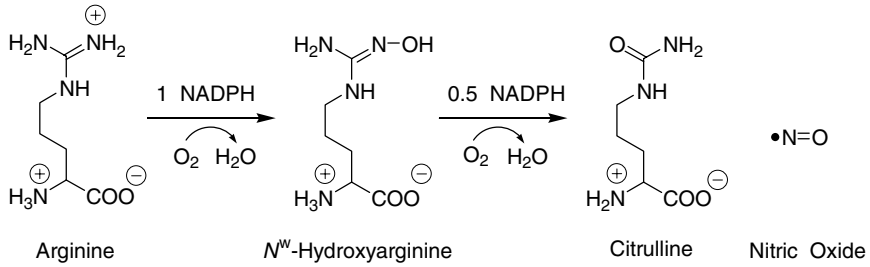


Scheme 1.1 Endogenous synthesis of nitric oxide.

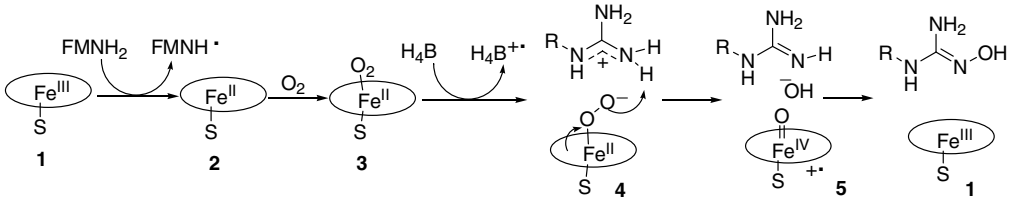
genase domain that binds iron protoporphyrin IX (heme), 6-(*R*)-tetrahydrobiopterin (H_4B) and Arg, and a C-terminal reductase domain that binds FMN (flavin mononucleotide), FAD (flavin adenine dinucleotide), and NADPH, with a calmodulin binding motif located between the two domains. To be active, two NOS polypeptides must form a homodimer. The reductase domains each transfer NADPH-derived electrons, through FAD and FMN, to the heme located in the adjacent subunit. Three distinct isoforms of NOS have been identified – neuronal, macrophage and endothelial types, and each is associated with a particular physiological process (Table 1.1). Constitutive endothelial NOS (eNOS or NOS III) regulates smooth muscle relaxation and blood pressure; constitutive neuronal NOS (nNOS or NOS I) is involved in neurotransmission and long-term potentiation; the NO produced from inducible NOS (iNOS or NOS II) in activated macrophage cells acts as a cytotoxic agent in normal immune defense against microorganisms and tumor cells. The constitutive isoforms (nNOS and eNOS) require added Ca^{2+} and calmidulin for activity and produce a relatively small amount of NO, while the inducible isoform (iNOS) has tightly bound Ca^{2+} and calmodulin, and produces a relatively large amount of NO.

Tab. 1.1: Properties of NOS isoforms.

NOS	Locations	Characteristics	Major Biological Functions
nNOS (NOS-I)	Brain, spinal cord, peripheral	Constitutive, Ca^{2+} dependent	Neuromediator
iNOS (NOS-II)	Macrophages, other tissues	Inducible, Ca^{2+} independent	Host defender, cytotoxic
eNOS (NOS-III)	Endothelium	Constitutive, Ca^{2+} dependent	Vasodilator tone modulator

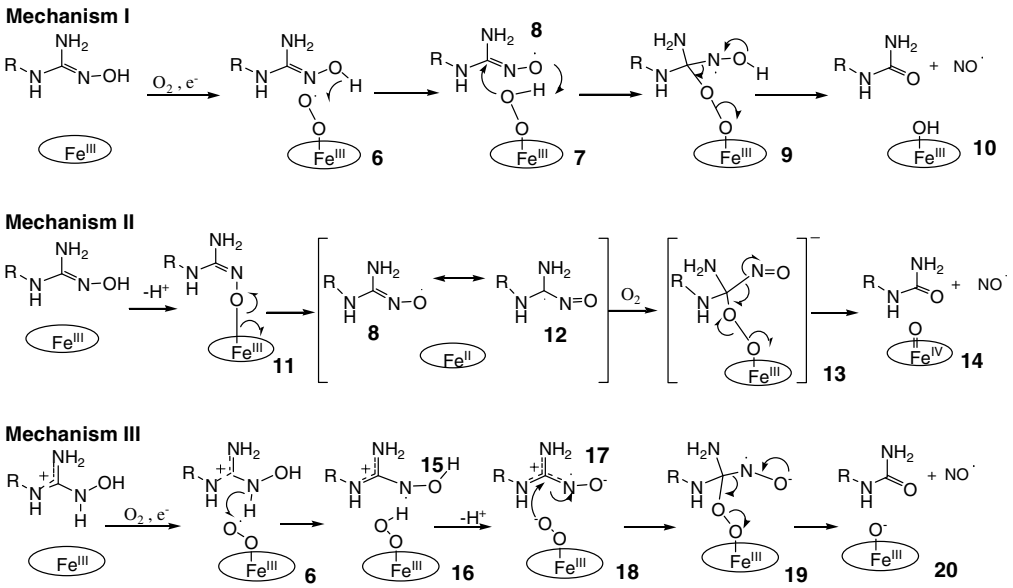
The first step of an NOS catalyzed reaction is a "classical" P450-dependent *N*-hydroxylation of a guanidine, except for the involvement of H_4B. As shown in Scheme 1.2, Fe(III)heme **1** first accepts one electron to give Fe(II)heme **2**, which binds O_2 to produce ferrous-dioxy heme **3**. The second electron from H_4B reduces **3** to peroxy-iron **4**. Arg donates a proton to **4** to facilitate O–O bond cleavage to generate an oxo-iron (IV) cation radical species **5**, which then rapidly hydroxylates the neutral guanidinium to NHA [4].

The second step of NOS oxidation is a greater challenge to enzymologists since there is no direct analogy in other systems. A variety of proposed reaction steps can be



Scheme 1.2 The first step of NOS reaction.

roughly summarized in three mechanisms (Scheme 1.3). The popular Mechanism I was proposed by Marletta and modified by Ingold and others [5, 6], a superoxo-iron(III)heme intermediate **6** abstracts the hydrogen atom of the NHA to furnish an iminoxy radical **8**, which upon nucleophilic attack by the hydroperoxoiron(III)heme **7** on its carbon generates NO and citrulline. This mechanism, however, appears not to be supported by the crystal structure analysis of the NOS-NHA complex [7–9] or by a recent spectral study [10]. The second mechanism was proposed by Groves (Mechanism II), where the NOS-catalyzed aerobic oxidation of NHA occurs via a radical-type auto-oxidation process [11, 12], i.e., NHA is oxidized by the Fe(III) heme to generate an iminoxyl radical **8**, which tautomerizes to the α-nitroso radical **12**. Insertion of a dioxygen molecule between **12** and Fe(II) heme forms an energetic α-nitrosoperoxy Fe(III) heme intermediate that decomposes to generate NO [13, 14]. However, direct ligation of NHA to heme iron has been precluded by the X-ray crystallographic data [7–9]. The third mechanism, proposed by Silverman and others [15–18], mainly in-

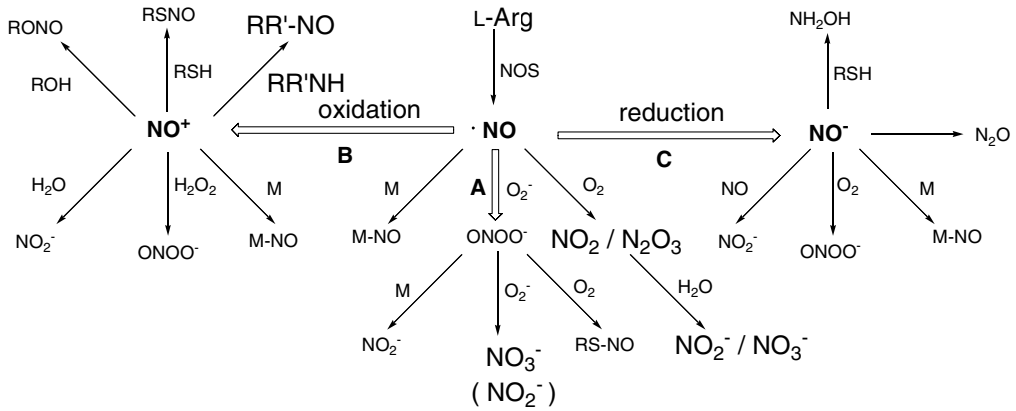


Scheme 1.3 The second step of NOS reaction.

volves the oxidation of the nitrogen on the protonated *N*-hydroxyguanidino moiety (Mechanism III). It was suggested that the initial N–H bond cleavage by superoxo-iron(III)heme **6** generates a radical cation intermediate **15**, which, upon heterolysis of the O–H bond, gives the iminoxy radical **17**. The nucleophilic attack of peroxo-iron(III)heme **18** on **17** gives an intermediate similar to **13**, which decomposes to NO and citrulline. More recently, Stuehr has emphasized the involvement of H_4B in the second step of the NOS reaction [19–21].

1.1.2 Chemistry of Reactive Nitrogen Species

One of NO's major biological actions is to activate guanylate cyclase directly to generate cyclic guanosine monophosphate (cGMP) as an intracellular second messenger, followed by kinase-mediated signal transduction. In another pathway, NO undergoes oxidation or reduction in biological systems to convert to many different reactive nitrogen species (RNS). It can react with molecular oxygen (O_2), superoxide anion ($O_2^{-\bullet}$) or transition metals (M) to produce RNS such as N_2O_3, NO_2, NO_2^-, NO_3^-, peroxynitrite ($OONO^-$), and metal-nitrosyl adducts (Route A, Scheme 1.4) [22, 23]. Among these RNS, peroxynitrite stands out as an important species [24, 25]. The reaction between NO and $O_2^{-\bullet}$ produces peroxynitrite at a diffusion controlled rate [26–28]. Peroxynitrite is a strong oxidizing and nitrating species that causes molecular damage leading to disease-causing cellular dysfunction [29, 30]. NO can also be rapidly oxidized by oxygen, superoxide or transition metals to nitrosonium (NO^+) which reacts with nucleophilic centers such as ROH, RSH and RR′NH to produce RO–NO, RS–NO or RR′N–NO, respectively (Route B, Scheme 1.4) [31, 32]. These products subsequently undergo other reactions to exhibit their biological effects. In addition, NO also undergoes a one-electron reduction to produce nitroxyl (NO^-) (Route C, Scheme 1.4). The reducing potential of this reduction is approximately +0.25 V [33]. Nitroxyl converts rapidly to N_2O under physiological conditions. Other competing reactions



Scheme 1.4 Oxidation and reduction of reactive nitrogen species.

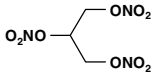
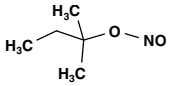
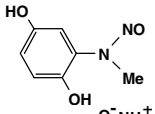
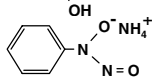
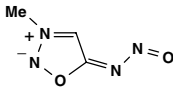
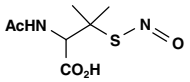
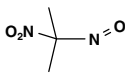
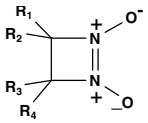
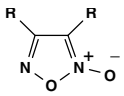
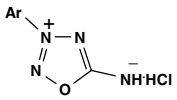
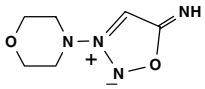
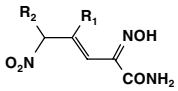
of nitroxyl include addition to thiol groups (singlet NO^-) to generate NH_2OH, and reaction with oxygen (triplet NO^-) to form peroxynitrite ($OONO^-$). Nitroxyl has also been proven to exhibit many biological functions [34], such as vasodilatation [35–37] and cytotoxicity [38–40].

1.2 Classification of NO Donors

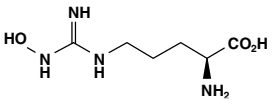
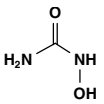
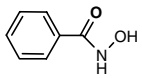
Intensive research on the biological functions of NO and other reactive nitrogen oxide species demands exogenous sources of NO donors as research tools and pharmaceuticals. Since the mid-1980s, the development of new NO donors has offered several advantages over the previous NO donors, such as spontaneous release of NO, donation of NO under controlled rates, and even the targeting of NO to certain tissues. The structural dissimilarities of the diverse NO donors have led to remarkably varied chemical reactivities and NO-release mechanisms. Generally NO donors release NO through three kinds of mechanisms. The first route is that donating NO spontaneously, which releases NO through thermal or photochemical self-decomposition of e.g. S-nitrosothiols, diazeniumdiolates, oximes. The second route is that releasing NO by chemical reactions with acid, alkali, metal and thiol. Organic nitrates, nitrites and syndnonimines give NO though this mechanism. The third route is enzymatic oxidation where NO donors, for example, *N*-hydroxyguanidines, need metabolic activation by NO synthases or oxidases for NO release. Some NO donors release NO by more than one route, e.g. organic nitrates can also generate NO by enzymatic catalysis. Classification of all NO donors could be confusing, since all nitrogen–oxygen-bonded compounds have the potential to decompose, be oxidized, or be reduced to produce reactive nitrogen species. However, similar chemical structures usually have a similar NO-releasing mechanism, so all current NO donors and their pathways of NO generation are summarized in Table 1.2 according to their chemical classification.

Many medicines may work by an NO-dependent mechanism. Recent studies have shown that angiotensin-converting enzyme (ACE) inhibitors (i.e. Enalapril, Captropril, Cilazapril) improve endothelium-dependent vasodilator responsiveness [41–43]. ACE inhibitors inhibit the degradation of bradykinin, thereby augmenting NO production. Another calcium channel blocker, amlodipine, also releases NO from blood vessels, and kinins mediate the generation of NO [44]. These new findings give a good explanation for the cardioprotective effects of these drugs. Furthermore, estrogen, statins (HMG-CoA reductase inhibitor) and essential fatty acids have the ability to augment NO synthesis [45, 46]. All of the above molecules do not have structural moieties which can release NO directly, so they can be called NO stimulators, and they are not discussed in this book. Currently used NO donors will be introduced in the following chapters.

Tab. 1.2: Current major classes of NO donors.

Chemical Class	Representative Compounds	Pathway of NO Generation	
		Non-enzymatic	Enzymatic
Organic nitrate		Thiols	Cyt-P450, GST, etc
Organic nitrite		Hydrolysis, trans-nitrosation, thiols, light, heat	Xanthine oxidase, etc
Metal-NO complex	$Na_2[Fe(CN)_5(NO)]\bullet 2H_2O$	Light, thiols, reductants, nucleophiles	A membrane-bound Enzyme
N-Nitrosamine		OH^-, light	Cyt-P450 related enzyme
N-Hydroxyl nitrosamine		Light, heat	Peroxidases
Nitrosimine		Thiols, light	?
Nitrosothiol		Spontaneous, enhanced by thiols, light, metal ions	Unknown enzymes
C-Nitroso compound		Light, heat	?
Diazetine dioxide		Spontaneous, thiols	?
Furoxan & benzofuroxan		Thiols	Unknown enzyme
Oxatriazole-5-imine		Thiols	?
Syndonimine		Spontaneous, enhanced by light, oxidants, pH>5	Prodrugs require enzymatic hydrolysis
Oxime		Spontaneous, O_2/Fe^{III}-porphyrin	Cyt-P450

Tab. 1.2 (continued)

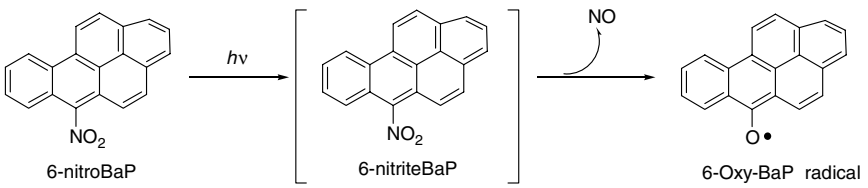
Chemical Class	Representative Compounds	Pathway of NO Generation	
		Non-enzymatic	Enzymatic
Hydroxy-amine		Anto-oxidation enhanced by metal ions	Catalase/H_2O_2
N-Hydroxy-guanidine & guanidine		Oxidants	NOS, Cyt-P450
Hydroxy-urea		H_2O_2/CuZn-SOD or ceruloplasimin, H_2O_2/Cu^{2+}, heme-containing proteins	Peroxidase
Hydroxamic acid		?	Guanylate cyclase

1.3
New Classes of NO Donors under Development

Different types of NO donors will be discussed in the other chapters except for the following two classes.

1.3.1
Nitroarene

6-Nitrobenzo[a]pyrene (6-nitroBaP) was found to release NO under visible-light irradiation, while no such photodegradation was observed with other nitrated BaPs, such as 1- and 3-nitroBaPs [47]. It can induce DNA strand breaks upon photoirradiation. NO is generated from 6-nitroBaP via 6-nitriteBaP, which is produced from 6-nitroBaP by an intramolecular rearrangement mechanism (Scheme 1.5). This finding may be useful for the development of a new type of photochemically triggered NO donors.



Scheme 1.5 Photochemical reaction of 6-nitroBap.

1.3.2
Hydroxamic Acids

Hydroxamic acids [general formula RC(O)N(R′)OH] have been used as inhibitors of peroxidases [48], ureases [49] and matrix metalloproteinases, and as anti-hypertensive, anti-cancer, anti-tuberculous and antifungal agents [50, 51]. Although some of these bioactivities are attributed to the chelating ability of the hydroxamate group, the hypotensive effects are due to their ability to release NO [52]. Experiments have shown that hydroxamic acids can transfer NO to ruthenium(III) and cause vascular relaxation in rat aorta by activation of the iron-containing guanylate cyclase enzyme. Of the hydroxamic acids investigated, benzohydroxamic acid (Fig. 1.1) showed higher NO releasing ability than aceto-, salicyl-, and anthranilic hydroxamic acids.

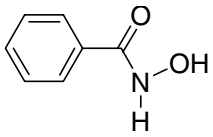


Fig. 1.1 Benzohydroxamic acid.

1.4
Development of NO-Drug Hybrid Molecules

An innovative approach to harnessing the beneficial properties of NO is to attach an NO-releasing moiety to an existing drug (Fig. 1.2). Different hybrid compounds can offer various drug actions with synergistic effects, with reduced toxicity and side effects. Several pharmaceutical companies are actively engaged in this research area. A series of compounds are currently in the Phase-I or Phase-II clinical study. For example, Nicox in France (www.nicox.com) has developed the NO-releasing derivative of acetylsalicyclic acid, NCX-4016, which is claimed to be able to overcome the major drawback associated with the use of aspirin as a pain reliever [53]. NCX-4016 also shows a broader mechanism than aspirin and can inhibit additional inflammatory mediators [54]. NitroMed in Boston (www.nitromed.com) has reported that nitrosylated α-adrenoreceptor antagonists moxisylate (S-NO-moxisylate) had lower toxicity and fewer adverse side effects in the treatment of erectile dysfunction [55].

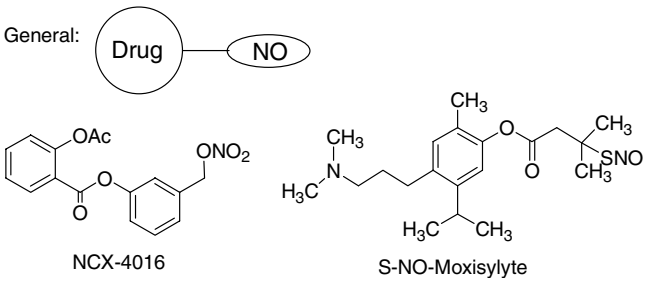
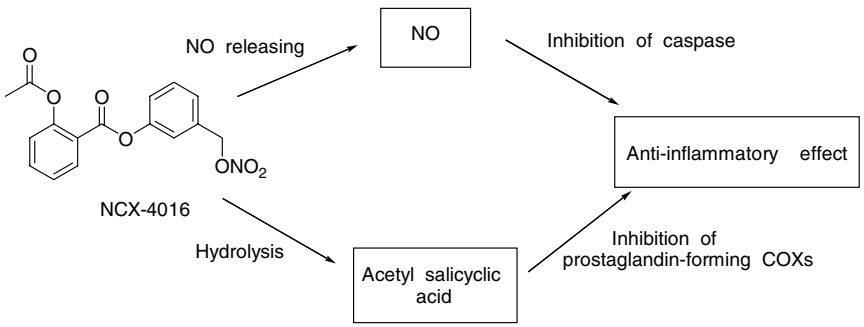


Fig. 1.2 NO-drug conjugates.

1.4.1 Nitrate Hybrid Molecules

Recently, the combination of a nitrate moiety with another bioactive substructure in a single molecule has received particular attention. Nitric-oxide-releasing nonsteroidal anti-inflammatory drugs (NO-NSAIDs) are chemical entities obtained by adding a nitroxyalkyl moiety via an ether linkage to a conventional NSAID, such as NO-aspirin (NCX-4016), a prototype NO-NSAID. Because the use of NSAIDs is associated with significant gastro-intestinal toxicity, the development of safer NSAIDs, NO-NSAIDs, has been demonstrated to be rational and successful [56]. NO-NSAIDs inhibit inflammation via cyclo-oxygenase (COX)-dependent and independent NO-related effects. The mechanism can be explained by a general feature of NO, its capability to modify proteins that contain cysteine residues by causing the S-nitrosation of thiol groups in the enzyme catalytic center [57]. It has been proved that NCX-4016 causes the S-nitrosation and inhibition of interleukin(IL)-1β converting enzyme(ICE)-like cysteine proteases (caspases) involved in pro-IL-1β and pro-IL-18 processing, which are pivotal in the pro-inflammatory cytokine hierarchy. The NO-NSAIDs are not only devoid of gastro-intestinal toxicity, but are also more effective anti-inflammatory drugs than their parent compounds (Scheme 1.6). A similar compound, B-NOD, showed a similar activity, and does not affect blood pressure [58]. NCX-4215 and NCX-456 are designed according to the same principle. NO-NSAIDs are currently undergoing clinical Phase II trials.



Scheme 1.6 Anti-inflammatory mechanism of NO-NSAIDs.

NCX-1000 (Fig. 1.3) is another prodrug obtained by adding a nitroxybutyl moiety to ursodeoxycholic acid (UDCA), a steroid that is selectively metabolised by hepatocytes [59]. Because NCX-1000 is almost exclusively metabolised in the liver, NO is delivered directly to hepatic cells. It has been demonstrated that NCX-1000 protects against liver damage induced by Con A in mice by modulating the live-resident immune system, and reduces portal pressure in cirrhotic rats. This drug may provide a novel therapy for the treatment of patients with portal hypertension or immunomediated liver injury [60]. Using a similar approach, NCX-1015, a nitro-prednisolone, was designed and showed NO-releasing ability in biological fluids [61]. It was more

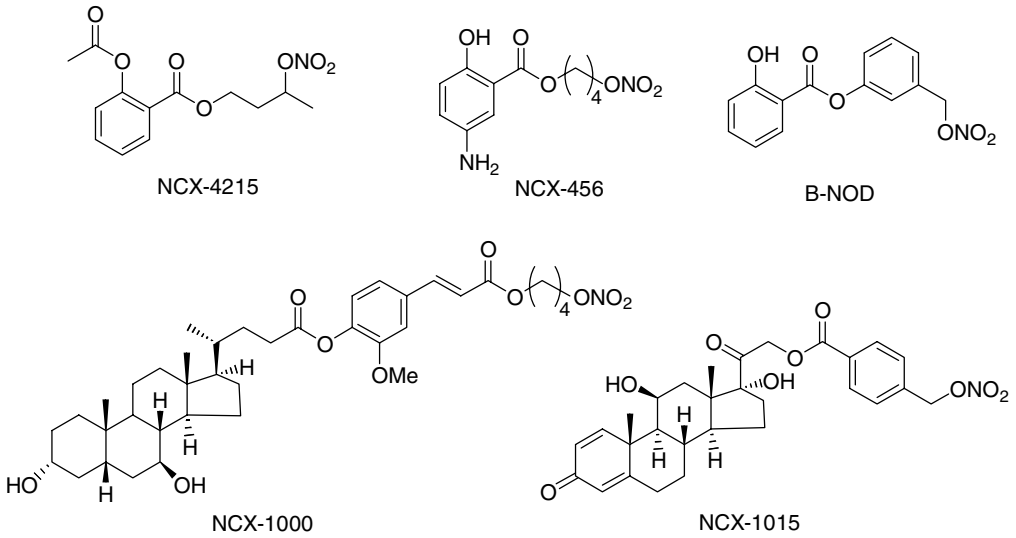


Fig. 1.3 Structures of NO-NSAIDs.

potent than prednisolone in acute and chronic inflammation. NO not only synergizes with the glucocorticoid moiety to produce the anti-inflammatory effect, but also counteracts the osteoclast activity of prednisolone, which causes a major side-effect of glucocorticoid drugs. Moreover, NCX-1015 showed significant bronchodilating activity [62]. As inhaled NO has been suggested as a useful therapy to induce airway dilation [63–65], NO-linked steroids may be a very effective therapy for airway diseases such as asthma and chronic obstructive pulmonary disease (COPD). The potential synergism between NO and steroids locally released in a damaged tissue, may reduce the therapeutically effective doses of steroids and prevent the side-effects of steroids.

The combination of beta-blockers with nitrovasodilators is an efficient therapeutic approach in coronary heart disease. Therefore, nitration of beta-blockers could produce an NO donor, while keeping its beta adrenergic receptor blocking effects. The *S-S* enantiomer of a metoprolol derivative named PF9404C (Fig. 1.4) was synthesized accordingly [66]. Pharmacological and biochemical experiments showed that when in contact with living cells, PF9404C can generate substantial amounts of NO, leading to cyclic GMP formation and vasorelaxation. Unlike rapid NO donors, PF9404C produces a slowly developing and sustained relaxation of the vessel. Its beta-blocking potency is close to that of *S*(-)-propranolol, four-fold higher than metoprolol. When beta-blockers alone are administered to hypertensive patients, the total peripheral resistance remains higher than in normotensives. If PF9404C is used in hypertensive patients, the NO actions, including potent vasodilating actions, inhibition of leukocyte–endothelial cell interaction, as well as platelet adherence and aggregation and vascular smooth muscle cell proliferation, may exert beneficial effects in hypercholesterolemia and ischaemic heart injury. Thus, PF9404C exhibits antihypertensive

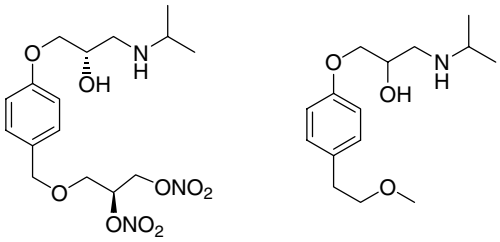
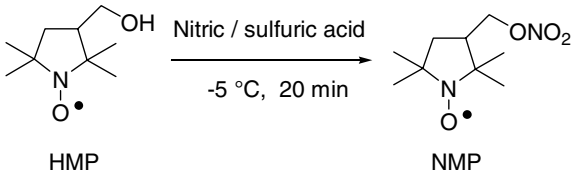


Fig. 1.4 Structures of β-blocker NO hybrid.

and cardioprotective actions through a double mechanism, NO donation and beta-blockade.

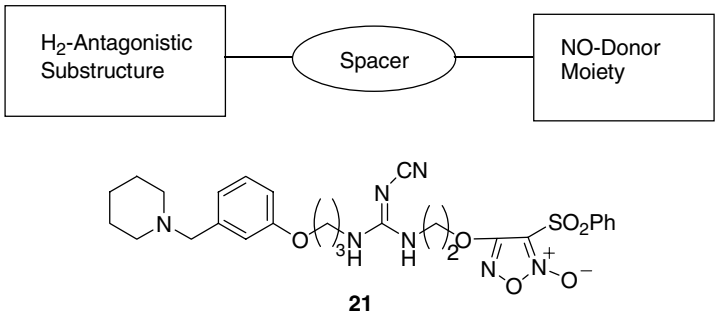
Several reports indicate the involvement of superoxide in the mediation of tolerance [67–69]. Based on these reports, a bifunctional superoxide dismutase-mimic NO donor was designed by Haj-Yehia's group [70]. The nitrate ester was incorporated into a nitroxide such as 3-hydroxymethyl-2,2,5,5-tetramethyl-1-pyrrolidinyloxy (HMP) by its conversion into 3-nitratomethyl-PROXYL (NMP) (Scheme 1.7). HMP is a stable, metal-independent, low-molecular weight SOD-mimic with excellent cell-permeability. So NMP is the first compound that can simultaneously generate NO and destroy superoxide. This may lead to novel nontolerance-inducing nitrovasodilators.



Scheme 1.7 Preparation of NMP.

1.4.2
Furoxan Hybrid Molecules

H_2-antagonists are a class of drugs used in the management of peptic ulcer and gastric-acid-related disorders. Since they fail to trigger protection against gastric damage induced by NSAIDs, it would be useful to combine the antisecretory activity of H_2-receptor antagonists with the NO-dependent gastroprotective effect in the same molecule. Some new H_2-antagonist-containing NO-donor moieties have been synthesized [71]. The H_2-antagonistic substructures were derived from lamtidine and tiotidine, respectively. NO-releasing moieties were chosen from phenysulfonyl furoxan, nitrates and nitrosothio functions. The experimental results showed that only the hybrid compounds were able both to antagonize histamine effects on guinea pig papillary muscle and to display in vivo antisecretory and gastroprotective action. The best results were obtained with the lamtidine/furoxan hybrid structure, such as

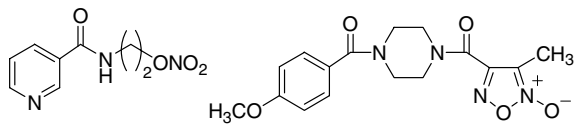


21

Fig. 1.5 General structure of H_2-antagonist/NO hybrids and example compound.

21 (Fig. 1.5), while others hybrids showed ambiguous results. These compounds could be the prototypes of a new class of drugs, which may be useful in the therapy of gastric hypersecretion combined with inflammatory disorders.

Nicorandil is an antianginal drug, which has the properties of both K^+ channel openers and NO donors [72]. Structurally, it is a nicotinamide derivative with a nitrate group in its chemical structure (Fig. 1.6). The hybrid molecules of furoxan and nicorandil derivatives may achieve an ideal cardiovascular drug with good selectivity, efficiency and low toxicity [73]. A series of hybrid drugs designed by linking the furoxan ring to nicorandil analogues was investigated. Several of these compounds had good vasodilatory activity [74]. Compound **22** was further tested for its hypotensive effects in anaesthesized rats, and was able to significantly reduce blood pressure 3 h after administration. Its hypotensive effects could prevail for a further 3 h. These preliminary results indicate that the furoxan-nicorandil derivatives are a useful lead in the design of NO-donor compounds for hypertension.



Nicorandil

22

Fig. 1.6 Structures of Nicorandil and its hybrid.

1.5
New Therapeutic Applications of NO Donors

Due to the numerous possible reactions and related biological consequences, inappropriate overproduction of NO can cause a series of disease states such as a variety of neurodegeneration diseases including inflammation, rheumatic disease, septic shock, diabetes mellitus, and cerebral ischemia. Therefore development of isoform-specific NOS inhibitors to regulate NO synthesis has been an active research area.

On the other hand, insufficient NO production also causes serious medical problems. Many diseases such as hypertension, atherosclerosis and restenosis involve a deficiency of NO production. Therefore, a compound that can release NO under specific conditions can be used therapeutically to palliate NO underproduction. In fact, the best known NO donor, glyceryl trinitrate, has been used for over a century to relieve acute attacks of angina pectoris.

In 1998, Carl Djerassi published a book titled "NO", where he plotted the success of a biotech company producing NO donor compounds to treat male impotence [75]. Currently, NO donor compounds have a variety of biomedical applications. Though current understanding of NO physiology and pathology seems incomplete, the largely indirect, correlative information suggests that both NO excess and insufficiency can elicit tissue injury and diseases. So far the most purported NO-insufficiency diseases are cardiovascular. The oldest NO donor drug, glyceryl trinitrate, has been used as a vasodilator since 1879. Besides supplementation of NO in a situation where an NO insufficiency may underlie the pathology, NO donors can also regulate an NO-based physiological pathway, i.e., male erectile dysfunction, and improve drug safety and efficacy, i.e., gastrointestinal toxicity of nonsteroidal anti-inflammatory drugs. In addition to cardiovascular disorders [76], nerve system diseases [77] and inflammation [78], the benefit of NO in many other diseases has now been recognized.

1.5.1 NO Donors against Cancer

NO produced by activated macrophages plays an important role in modulating the host defense mechanism against tumor cells [79, 80]. Several in vitro studies have also shown that NO donors are cytotoxic to tumor cells leading to apoptosis, mainly involving changes in mitochondrial permeability transition and release of cytochrome *c* from the mitochondria [81]. NO released from inducible NO synthase inhibits metastasis at a higher level, but at a lower concentration, NO may cause induction of NO resistance and permit the growth of tumor cells [82]. Although there are reports indicating the genotoxicity of NO, exposure of whole cells to NO donors resulted in no appreciable mutations as compared to alkylating agents [83]. The reason is that large amounts of NO are required to generate DNA alterations and the formation of other reactive species and nitrosated DNA from NO is limited. There are also numerous defense mechanisms, such as ascorbate and glutathione, as well as intra- and intercellular consumption of NO, which limit the mutation of DNA [84]. The dual role of NO in carcinogenesis is very confusing, so further studies are still needed to clarify it [85].

1.5.1.1 Diazeniumdiolates (NONOates) as Promising Anticancer Drugs

Diazeniumdiolate compounds have already shown anti-leukemia activity [86]. However, these NO donors release NO systemically and cause severe side effects on the vascular system, so their therapeutical use has been limited. Upon modulation at the

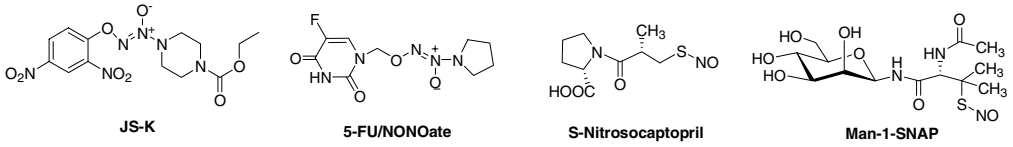
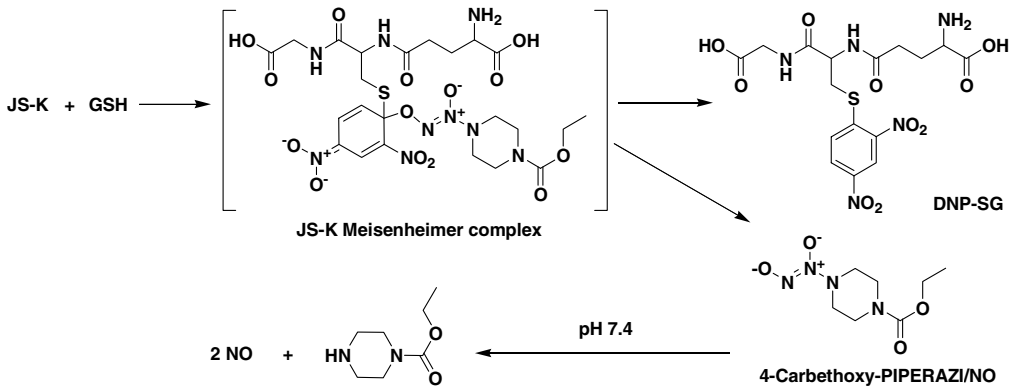


Fig. 1.7 Anticancer NO donors.

oxygen with nitro-aromatic substituents, these derivatives can release NO in target cells after a hydrolytic or enzymatic action. JS-K (Fig. 1.7), an example of O-protected diazeniumdiolate developed by the US National Cancer Institute (NCI), has attracted much attention [87]. JS-K can be attacked by the nucleophilic thiol group of glutathione (GSH), with the formation of the Meisenheimer complex; then the NONOate moiety leaves, and at physiological condition, the NONOate decomposes to release NO (Scheme 1.8). The aryl moiety of JS-K is bonded to the thiol group of GSH to give *S*-(2,4-dinitrophenyl)glutathione (DNP-SG). The drug kills, or slows the growth, of cancer cells without harming healthy cells [88]. For example, acute myeloid leukemia (AML) is the most common and deadly form of leukemia. Tests with the drug showed it triggered the destruction of AML cells grown in vitro. In the HL-60 human myeloid leukemia assay system, the IC-50 of JS-K is 0.5 μM, while the IC-50 of a chemotherapeutic agent, daunorubicin, is 0.01 μM. It also slowed the growth of AML cells in mice. In other tests on cell cultures, JS-K did the same, but to a lesser extent, in prostate, colon, and breast cancer cells. It also inhibited the growth of prostate cancer cells in mice. JS-K is found to react with glutathione S-transferases (GST), which help pump foreign substances out of certain cells. GSTs help the liver get rid of toxic substances in blood, but they also help cancer cells resist chemotherapy drugs. When GSTs in cancer cells interact with JS-K, there are two anticancer effects: GST activity is inhibited, making the cells less resistant to chemotherapy drugs, and NO is released.



Scheme 1.8 Mechanism of JS-K.

It has been shown that JS-K inhibited cell growth with concomitant activation of mitogen-activated protein kinase (MAPK) members, ERK, JNK, p38 and their downstream effectors, c-Jun and AP-1 [89]. Inhibitors of these MAPK pathways abrogated the growth inhibitory actions of JS-K. In addition to the actions of JNK as a kinase for c-Jun, it was shown that c-Jun is also an ERK target. Furthermore, JS-K generated NO in culture and NO inhibitors antagonized both MAPK induction and the growth inhibitory effects of JS-K. These results suggest two possible mechanisms for the mediation of JS-K growth inhibitory actions, namely, NO-induction of MAPK pathway constituents as well as possible arylation reactions. The data support the idea that prolonged MAPK activation by JS-K action is important in mediating its growth-inhibitory actions. In 2003, NCI accepted JS-K into its Rapid Access to Intervention Development (RAID) program, which tries to speed the development of new cancer therapies. Work done so far by the University of Utah within the RAID program has shown that JS-K is active against a broad spectrum of cancer cells. JS-K thus represents a promising platform for novel growth inhibitory analog synthesis.

1.5.1.2
The Synergistic Effect of NO and Anticancer Drugs

The 5-fluorouracil (5-FU) and NONOate conjugates (Fig. 1.7) were prepared and their cytotoxicity was tested [90]. The median effect doses of the conjugates for DU145 and HeLa cancer cell lines were 2–4-fold lower than that of 5-FU. In another study by Wink et al., the cytotoxicity of cisplatin was enhanced about 60-fold after NONOate pretreatment for 30 min [91]. The enhancement of cytotoxicity of 5-FU/NONOate conjugates and cisplatin-NONOate combination has shown that there is a synergistic effect between anticancer drugs and NO. Another study by Jia et al. demonstrated that the cytotoxicity of Taxol was enhanced by *S*-nitrosocaptopril (Fig. 1.7) [92]. This effect is primarily mediated via the increased influx of Taxol by NO into intracellular compartments, while NO-induced cytotoxicity cannot be excluded.

In another separate study, researchers found organic nitrates can also increase the efficiency of cytostatic therapy and retard the development of drug resistance [93]. The combined therapy results in significant increase in life span and number of survivors among mice bearing leukemia P388 and L-1210. Comparative studies of organic nitrates and a similar compound in which ONO_2 moieties were replaced by OH groups demonstrated that the presence of NO_2 is required for adjuvant activity of compounds and confirmed that NO modifies the antitumor effects of cytostatics. It was also shown that the NO donor retards the development of drug resistance to cyclophosphamide.

1.5.1.3
NO-NSAIDs as a New Generation of Anti-tumoral Agents

Non-steroidal anti-inflammatory drugs (NSAIDs), such as aspirin, were utilized primarily to protect from inflammation. Other biological effects were also found gradually, for example, induction of apoptosis [94, 95], stimulation of immune activity

[96], and inhibition of angiogenesis [97]. Studies of patients with familial adenomatous polyposis (FAP) have demonstrated a reduction by approximately 50% of colonic adenomas or colorectal cancer among patients while using aspirin [98]. The growing evidence suggests that tumor inhibition may be mediated by at least two major cellular events. These involve the ability of NSAIDs to maintain the equilibrium between proliferation and apoptosis rates in colonocyte, and their inhibitory effect on angiogenesis [99, 100]. The main problem with the regular use of NSAIDs is the occurrence of side effects such as the increased risk of gastrointestinal bleeding and the developments of ulcers [101]. In recent years, NO-NSAIDs, such as NO-aspirin (NCX 4016) (Fig. 1.2), have been developed as "safe" NSAIDs [102]. NO-aspirin is 2500–5000-fold more potent than traditional aspirin in inhibiting the growth of colon cancer cells in vitro [103]. The corresponding test in mice also confirmed the strong inhibitory effect of NO-aspirin in intestinal carcinogenesis and suggests that NO-NSAIDs merit further evaluation as chemopreventive agents against colon cancer [104]. A detailed study showed that NO-aspirin inhibits β-catenin/T cell factor (TCF) signaling in colon cancer cells by disrupting the nuclear β-catenin/TCF association, whereas aspirin has no affect [105].

Two clinical trials of aspirin for the prevention of cancer were published in March 2003 [106, 107]. In one study, 635 patients with a recent history of adenomas received either the placebo or 325 mg of aspirin per day. A colonoscopy was performed on 81% of the patients after at least one year. One or more adenomas were found in 17% of patients in the aspirin group compared to 27% in the placebo group. Another study gave a similar result. These two studies indicate that daily use of aspirin is associated with a significant reduction in the incidence of colorectal adenomas in patients with previous colorectal cancer. If aspirin therapy is stopped, the reduction in the risk of adenomas dissipates. Also, NSAIDs may decrease the incidence of carcinomas of the esophagus, stomach, breast, lung, prostate, urinary bladder and ovary [108]. The clinical use of these agents is limited to patients with FAP. Due to the clear protective effect of aspirin, NO-NSAIDs can be a good alternative, which can give the beneficial effects of both NO and NSAIDs. In March 2003, NCI awarded a grant to the University of Michigan to conduct a clinical trail of NO-releasing aspirin (NCX-4016). This placebo-controlled study will assess the pharmacokinetics of different doses of NCX 4016 in patients at risk of colon cancer.

1.5.1.4 Other NO Donors with Anticancer Activity

As well as NONOates, other NO donors also showed anticancer activity independently. Sodium nitroprusside (SNP), a metal-NO complex, showed cytotoxic effects on the cells of some patients with malignant lymphoma (ML), acute myelocytic leukemia (AML) or chronic myelomonocytic leukemia (CMMoL), but not with multiple myeloma [109]. SNP and cytosine arabinoside (Ara-C) did not share the drug resistance. Interestingly, SNP had no effect on lymphocytes of healthy volunteers. These results suggest that SNP has an anti-tumor effect on human hematological malignant cells.

A series of sugar-*S*-nitrosothiols (sugar-SNAPs), for example, glucose-1-SNAP, have shown promising pharmacokinetic properties [110]. These compounds were designed based on the observation that facilitated transport of monosacharrides in mammalian cells was accomplished by the glucose transporter family of transmembrane properties. They were constructed from an aglycone unit conjugated with a mono- or oligosaccharide. Compared to SNAP, sugar-SNAPs had higher stability and slower NO-releasing properties in aqueous solution. Glucose-SNAPs were more cytotoxic than SNAP. The enhanced cytotoxicity of glucose-1-SNAP and glucose-2-SNAP may be related to their affinity for glucose transporters present on plasma membranes, but relative experiments have not yet been done. Another possible explanation is that glucose-SNAP binds to glucose transporters and decomposes to release NO, then NO causes the apoptosis of cancer cells. Recently, mannose-SNAPs were also developed, for example Man-1-SNAP (Fig. 1.7). The cytotoxicity of Man-1-SNAP was just as potent as that of glucose-SNAP [111].

Hydroxamic acid derivatives, which belong to a new class of NO donors, have been shown to inhibit the matrix metalloproteinases (MMPs) [112]. MMPs are a family of zinc-dependent endopeptidases, which play a critical role in multiple steps in the metastatic cascade and facilitate neoangiogenesis. Numerous hydroxamic acids, such as marimastat, have been developed, that bind the zinc atom in the active catalytic domain of MMPs. During a randomized Phase III trial, comparing marimastat with placebo in patients with metastatic breast cancer, marimastat was not associated with an improvement in progression-free survival or overall survival. Other studies also indicated no benefit for MMP inhibitors when used either in combination with chemotherapy or sequentially after first-line chemotherapy in a variety of cancers [113]. Currently, many pharmaceutical companies have suspended clinical development of this kind of agent.

1.5.2 NO against Virus

1.5.2.1 HIV-1 Induces NO Production

It was found that the HIV envelope glycoprotein in vitro increases the production of NO by human monocyte-derived macrophages [114]. NO production is increased in patients who have AIDS [115], and the increased concentrations of nitrite in AIDS patients with opportunistic infections is caused by *T gondii*, *Pneumocystis carinii*, *Mycobacterium tuberculosis*, and *Mycobacterium avium*, whereas nitrite concentrations are normal in symptom-free patients. It was also confirmed that there was increased production of NO in the sera of children with HIV-1 infection, and of circulating cytokines, such as interleukin 1β, tumor necrosis factor α, and interferon γ. It is postulated that rises in the concentrations of these cytokines may represent a substantial stimulation of NO production [116]. In contrast, it has been shown that there was no altered endogenous nitrate formation in eight patients with AIDS, most of whom had opportunistic infections [117]. It has also been noted that there were high

nitrite and nitrate concentrations in 39 patients with AIDS, especially in those with lower CD4 cell counts, whereas in symptom-free patients no such increase was seen [118]. However, AIDS patients with opportunistic infections were not selected for assessment of NO production.

Groeneveld and colleagues [119] have shown that serum nitrate concentrations are higher in symptom-free HIV-1-infected patients than in healthy individuals. NO production was measured in vitro from peripheral blood leucocytes of HIV-1-infected patients after measuring nitrite concentrations from peripheral blood mononuclear cells and polymorphonuclear leucocytes [120, 121]. An increase in nitrite production in AIDS patients, especially in those with opportunistic infections, was also seen.

There is substantial induction of the iNOS gene in primary cultures of human monocyte-derived macrophages, concomitant with the peak of virus replication, and exposure to low concentrations of NO donors results in a significant increase in HIV-1 replication [122]. Acute infection of macaques with a pathogenic strain of the simian immunodeficiency virus increased gene expression of iNOS in mononuclear cells obtained from bronchoalveolar lavage [123]. At the time of systemic viral load peak, NO production was greatly raised in the monkeys [123]. The activated lung macrophages of neonatal rats produced significantly more NO than did those of infant and adult rats [124]. Since HIV-1 infection in neonates progresses to AIDS more rapidly than does infection in later life in human beings, these investigators speculate that excessive NO may explain the rapid progression of HIV-1 infection to AIDS during infancy.

The concentrations of nitrite or nitrate in the sera of patients infected with HIV-1 are substantially raised, especially in those with low CD4 cell counts [118]. However, during HIV-1 infection, it is difficult to find out whether the NO production is attributable to virus replication or to opportunistic infections, or both. In vitro there is a substantial rise in nitrite concentrations from blood mononuclear cells and polymorphonuclear leucocytes from patients with AIDS, especially in those with neurological disorders and pulmonary disease caused by intracellular opportunistic pathogens [121]. Interestingly, the serum concentrations of nitrate are positively correlated with plasma and cell-associated viral loads, which suggests that HIV-1 may induce NO synthesis in vivo [119]. However, the results clearly show that there is a close relation between viral replication and iNOS expression or peaks of plasma nitrate in the absence of any opportunistic infections, in either in macaques or infected patients [119, 122, 123].

NO acts as an autocrine factor that mediates HIV-1 replication; as at the molecular level, NO seems to stimulate long-terminal repeat-mediated transcription [125]. It was noted that exogenous NO increases replication of HIV-1 T-tropic isolates in primary T cells or T-cell lines, and inhibitors of iNOS partly block HIV-1 replication, especially that induced by tumor necrosis factor α [125]. The contrasting effects of exogenous NO, particularly NO donors, may depend on the type of NO donors, their releasing kinetics, and the dose used in the study design.

1.5.2.2
Antiviral and Proviral Activity of NO

Antiviral effects of NO are known for several viruses, including murine poxvirus, herpes simplex virus, Epstein-Barr virus, coxsackievirus, and influenza virus [126, 127]. Virus infection induces directly or indirectly (through interferon γ production) overproduction of NO because of localized iNOS expression in the area of infection [128]. Many pathological effects of NO are thought to be produced via its interaction with oxygen radicals, producing peroxynitrite [129]. Since the antiviral effects of NO do not require immune recognition of infected cells, and since NO can pass readily into cells, it provides a useful early defence against viral infections before the development of a specific immune response; thus NO may be a host response modulator rather than a simple antiviral agent.

Viral infections against which NO and its derivatives are thought to have inhibitory effects include DNA and RNA viruses, such as poliovirus, Japanese encephalitis virus, mouse hepatitis virus, vesicular stomatitis virus, herpes simplex virus type 1, vaccinia virus, and Epstein-Barr virus [126]. NO may inhibit an early stage in viral replication, and thus prevent viral spread, promoting viral clearance and recovery of the host. The earliest host response to viral infections is non-specific and involves induction of cytokines, especially tumor necrosis factor α and interferonγ. These cytokines are potent inducers of iNOS, which generates large amounts of endogenous NO [130]. Thus, NO could be a vital factor in inducing the host's innate immunity to control the initial stages of viral infections.

Despite the protective effect of NO against various viral infections, workers in several studies have shown a harmful role of NO in many systems. NO seems to play a part in the development of pneumonia caused by influenza virus [128], in the pathogenesis in mice of tick-borne encephalitis flavivirus infection [131], and in worsening the course of the murine myocarditis caused by coxsackievirus B3 [132]. In addition, pneumonia in mice induced by herpes simplex virus type 1 could be suppressed by the inhibitor of iNOS [133]. The issue of whether NO acts as an inhibitor of viral replication or as a harmful agent, therefore, remains unanswered. This issue is particularly evident in HIV-1 infection, since NO seems to act as a double-edged sword in the pathogenesis of HIV-1.

The antiretrovirus properties of NO were shown in mice infected with Friend leukemia virus, a murine retrovirus. NO produced by NO-generating compounds or activated macrophages inhibits viral replication in fibroblast cultures, and is involved in defens against this murine retrovirus in vivo [134]. It was also reported that NO donors can inhibit HIV-1 replication in human monocytes through induction of iNOS [135].

The life cycle of many viruses, including retroviruses, depends on viral proteases that cleave viral glycoproteins into individual polypeptides, and these enzymes are necessary for viral replication. NO can inactivate coxsackievirus [136]. Since cysteine proteases are critical for the virulence and replication of many viruses, nitrosation of viral cysteine proteases may be a mechanism of antiviral host defense. NO mediates nitrosation of cysteine and aspartyl proteases of HIV-1, and it was suggested that this

mechanism may have a role in the inhibition of HIV-1 replication [137]. Later it was shown that NO donors inhibit the HIV-1 reverse transcriptase activity, through the modulation of the catalytic activity of cysteine enzymes [138]. The modulation mechanism of the HIV-1 reverse transcriptase activity may be relevant for the development of new strategies for inhibition of HIV-1 replication.

NO has complex roles in immunological host responses against viruses, and especially against HIV-1 infection. In HIV-1 infection, NO cannot be rigidly classified as an anti-inflammatory or proinflammatory molecule, but it can be deemed a true inflammatory mediator. Many studies support a proviral effect of NO in HIV-1 infection, mainly based on stimulation of viral replication, and on toxic effects on various cells, including central nervous system cells, via oxidative injury that may cause cellular and organ dysfunction, and immunosuppression and immunopathology, especially in the central nervous system.

In several studies on the antiviral effects of NO on HIV-1 infection, the proviral or antiviral effects of NO seem to be strictly related to the active production of NO during HIV-1 infection. The universal speculative interpretation of the dichotomous effect of NO is that overproduction of this substance, especially in the primary infection and in late stages of the disease, leads to active viral replication with consequent harmful effects on the course of the disease. Conversely, low production of NO may cause a reduction in or inhibition of HIV-1 replication, especially during the symptomless stage of the disease, or during treatment with highly active combined antiretroviral drugs.

Recently, it has been found that NO donors inhibit HIV-1 replication in acutely infected human peripheral blood mononuclear cells (PBMCs), and have an additive inhibitory effect on HIV-1 replication in combination with 3′-azido-3′-deoxythymisylate (AZT) [139, 140]. S-nitrosothiols (RSNOs) inhibit HIV-1 replication at a step in the viral replicative cycle after reverse transcription, but before or during viral protein expression through a cGMP-independent mechanism. In the latently infected U1 cell line, NO donors and intracellular NO production stimulate HIV-1 reactivation. These studies suggest that NO both inhibits HIV-1 replication in acutely infected cells and stimulates HIV-1 reactivation in chronically infected cells. Thus, NO donors may be useful in the treatment of HIV-1 disease by inhibiting acute infection, or reactivating a latent virus.

1.5.3
Inhibition of Bone Resorption

NO is recognized as a mediator of bone cell metabolism, where it regulates osteoblast and osteoclast activity [141–143]. Osteoporosis, which frequently occurs in postmenopausal women, is a systemic skeletal disease associated with abnormal bone resorption. Addition of NO or NO donors to osteoclasts in vitro results in a reduction in bone resorption, whereas NO synthase inhibitors increase bone resorption, both in vitro and in vivo. Further research has shown that NO reduces bone resorption, via inhibition of the cysteine protease cathepsin K, which is believed to be a key protease in bone resorption. Most of the NO donors, i.e., nitroglycerin, 3-

morpholinosydnonimine (SIN-1), *S*-nitrosothiols, sodium nitroprusside (SNP), have an IC50 value for cathepsin K from 0.01 μM to about 1000 μM. So NO donors may be a new generation of therapeutic agents for inhibiting the bone resorption activity of osteoclasts.

There is also evidence suggesting that prostaglandin plays an important role as a regulator of bone remodelling in response to various stimuli, such as cytokines, sex hormones, and mechanical loading [144, 145]. Moreover, both cytokine-induced activation of NO and prostaglandin E_2 (PGE_2) pathways may act in a concerted manner to influence bone cell activity and bone turnover [146, 147]. So the combination of cyclooxygenase (COX) inhibitors and NO donors would exert more potent modulatory effects on bone turnover and bone mass. A report from NiCox indicated that flurbiprofen nitroxybutylester (HCT1026) was significantly more efficacious than the parent compound, flurbiprofen, at inhibiting osteoclast formation and bone resorption in vitro and prevented ovariectomy-induced bone loss in vivo [148]. HCT1026 may be of clinical value in the prevention and treatment of inflammatory diseases such as rheumatoid arthritis, which are characterized by joint inflammation as well as periarticular and systemic bone loss.

1.5.4
Treatment of Diabetes

SIN-1, a non-enzymatic NO donor, has been reported to inhibit insulin release in isolated pancreatic islets [149]. However, another report showed that L-arginine, an NO donor, could stimulate glucose-induced insulin secretion from the pancreas of diabetic rats [150]. Further studies showed that non-enzymatic NO donors such as SIN-1, sodium nitrite, sodium nitropusside, and *S*-nitroso-*N*-acetyl-DL-penicillamine (SNAP), increased insulin sensitivity through stimulation of NO production in the liver [151]. It was found that, besides the known vascular effect, enzymatic NO donors, such as organic nitrates, also have a hypoglycaemic/antihyperglycaemic effect. A pharmaceutical combination for the treatment and prevention of diabetes mellitus was invented, comprising at least one enzymatic NO donor and optional antidiabetic active ingredients [152]. The basis of the invention is the recognition of a new insulin-sensitizing effect and synergism using NO donors and conventional antidiabetic drugs.

1.5.5
Thromboresistant Polymeric Films

Hydrophobic polymer materials that slowly release NO can be used on the surface of medical devices. Many medical devices suffer from the surface adhesion of blood platelets. To minimize this thrombogenic effect, blood thinners such as heparin, coumarin, and aspirin are often used. However, systemic administration of antiplatelet agents could increase the risk of uncontrolled bleeding elsewhere in the body. In contrast, biocompatible polymer films would solve this problem [153]. It is possible to create polymeric surfaces that mimic the inner surface of a blood vessel by

locally releasing NO. Such in situ NO generation would inhibit platelet adhesion and activation on the polymer surface. Because of its short-life in blood (<1 s), NO is potentially an ideal species for improving the thromboresistivity of polymeric materials for biomedical applications [154]. Various diazeniumdiolate NO donors have been incorporated into polymer matrices [155, 156]. These films showed good NO-releasing ability and improved blood compatibility.

1.5.6 Inhibition of Cysteine Proteases

S-nitrosylation reactions, which transfer NO from a NO donor to a protein sulfhydryl group, can affect protein functions in biological systems [157]. If *S*-nitrosylation occurs in the active sites of the enzymes it may inhibit the catalytic activity of the enzymes. Cysteine proteases comprise a large class of enzymes from plant, animal, and bacterial sources. They play important roles in various biological processes [158]. NO donors inhibit cysteine proteases by modification of the cysteine catalytic residue of the enzymes, including Coxsackievirus and Rhinovirus cysteine proteases, cruzain, Leishmania infantum cysteine protease, falcipain, papain, as well as mammalian caspases, cathepsins and calpain [159]. Since cysteine proteases are critical for the replication of many viruses, bacteria, fungi, and parasites, cysteine proteases appear as promising targets for anti-parasite chemotherapy [160]. NO-releasing drugs could have an enhancing role in the therapeutic treatment of parasitic diseases, such as malaria [161]. Caspases, a family of cysteine proteases activated during apoptosis, are also therapeutic targets for modulating inflammation, as ICE/caspase-1 activation is the limiting step in the process of maturation of the cytokines IL-1β and IL-18, which are pivotal in the pro-inflammatory cytokine hierarchy [162]. Specific inhibitors are being developed by various pharmaceutical companies [163]. NO-NSAIDs are promising anti-inflammatory drugs as caspase inhibitors.

1.6 Conclusion

The synthesis of compounds which can release NO is relatively easy but, for therapeutic uses, they must have tissue selectivity, evenly controlled manner, and remain in a subtoxic range. Notwithstanding the many new classes of NO donors that have been reported, organic nitrates, diazeniumdiolate and *S*-nitrosothiols are still the three most important NO donors. They have the obvious advantages of well-proven NO donors, decomposing rapidly in solution, and mimicking the endogenous nitrosothiols. In clinical use, only organic nitrates and sodium nitroprusside show on prescriptions. However, patients taking long-term nitrates often develop tolerance, and prolonged sodium nitroprusside administration can give rise to cyanide accumulation in the body. *S*-nitrosothiols do not share these drawbacks. Perhaps it will take several years for new NO donor drugs to be used extensively. For targeted therapy, protected diazeniumdiolates as prodrugs may be more suitable than other

classes. By selecting a protecting group that can be metabolically removed by enzymes unique to the target tissue, NO release should be concentrated at the target site. The liver selective NO donor, V-PYRRO/NO, is a successful example[164]. The development of hybrid NO donors, such as NO-NSAIDs, which are formed by linking an NO-releasing moiety to a well-established bioactive molecule, seems a promising trend. These hybrid compounds can either abolish detrimental side effects, or reduce toxicity, or produce synergistic effects. Upon understanding the complex chemistry, biochemistry, and molecular biology of NO, NO donors should have more therapeutic applications.

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